MICROMORPHOLOGY AND GENE EXPRESSION IN MUSCLE AND SHELL DEVELOPMENT OF THE MOLLUSCA

Dissertation

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Descent being on my view the hidden bond of connexion which naturalists have been seeking under the term of the natural system. On this view we can understand how it is that, in the eyes of most naturalists, the structure of the embryo is even more important for classification than that of the adult.

(Charles Darwin: *The Origin of Species*, 1859)

Diversity of opinion about a work ... shows that the work is new, complex, and vital.

(Oscar Wilde: *The Picture of Dorian Gray*, 1890)

For Martina

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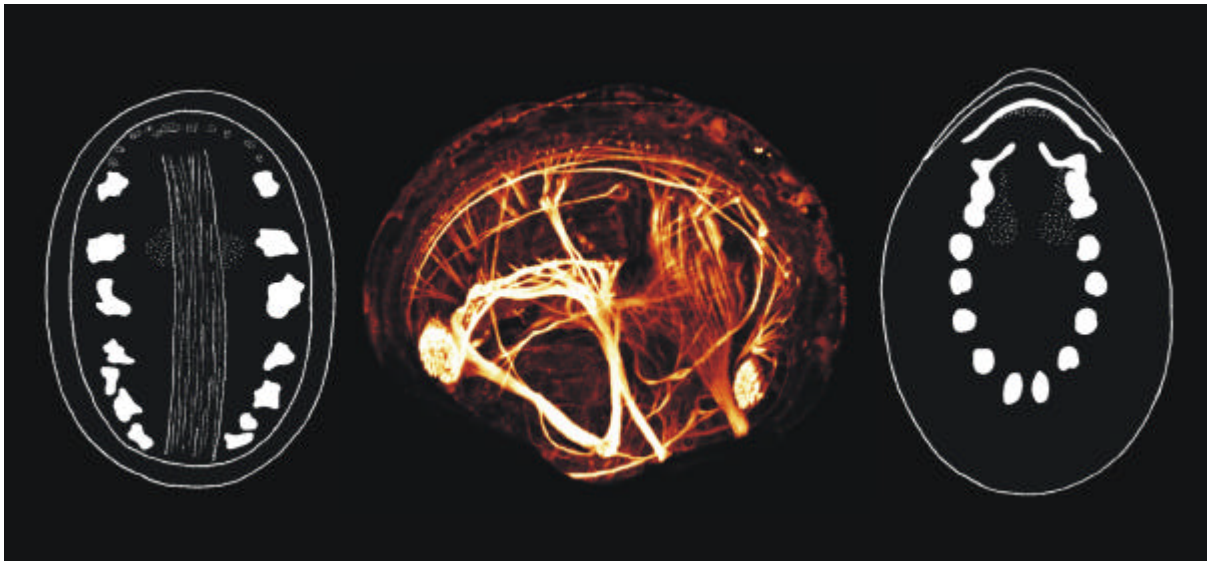
GENERAL REVIEW



SUMMARY. This work comprises detailed studies by scanning electron microscopy (SEM), transmission electron microscopy (TEM), fluorescence staining combined with confocal laser scanning microscopy (CLSM), as well as serial sectioning analyses and reconstruction techniques to elucidate the development of the larval and adult musculature of several basal representatives of the molluscan classes Polyplacophora, Bivalvia, Scaphopoda, and Gastropoda. Special reference is given to the shell musculature. In addition, aspects of the myo-anatomy of adult Solenogastres are reconsidered. A further part of this study deals with scaphopod shell morphogenesis and expression of the homeobox gene *engrailed* (*en*), in order to gain insights regarding the scaphopod-bivalve relationship. The results enable far reaching conclusions regarding the evolution and the phylogeny of the Mollusca.

Solenogastres

TEM analysis of adult Solenogastres revealed a mesenchymate body wall musculature which consists of outer ring, intermediate diagonal, and inner longitudinal muscles and resembles the condition of other worm-shaped taxa. The ventrally inter-crossing dorso-ventral musculature, which is diagnostic for the Mollusca, is arranged in multiple serial units along the anterior-posterior body axis.

Polyplacophora

During development, the chiton larva undergoes an intermediate stage in which the dorso-ventral musculature is serially arranged as in adult Solenogastres. The concentration into seven (and later eight) functional shell plate muscle units is a secondary condition which takes place after metamorphosis. Thus, assumptions of a primarily "segmented" (i.e. annelid-like) character of the polyplacophoran shell plate musculature are rejected. In addition, the anterior (i.e. pre-trochal) body region of chiton larvae shows a muscular grid which is lost at metamorphosis and resembles the body wall musculature of adult aplacophoran (Solenogastres + Caudofoveata) molluscs. Both, the multiple seriality of the dorso-ventral muscles and the apical muscle grid are regarded as ontogenetic recapitulation of the basal molluscan condition which is fully expressed in the adult body plan of Solenogastres. This infers a non-segmented, worm-shaped ancestor at the base of molluscan evolution.

The existence of a larval ring-shaped muscle that underlies the prototroch cells (prototroch muscle ring) is a shared feature of polyplacophoran, gastropod, and bivalve larvae (see below) and suprataxic homology of this organ is proposed.

Bivalvia

Besides a rather complicated set of larval retractor muscles, the veligers of autobranchs (i.e. all Bivalvia except the Protobranchia, the latter with a test-cell larva) exhibit a distinct prototroch muscle ring similar to chitons and gastropods. Both systems are entirely larval and are resorbed during metamorphosis.

Scaphopoda

The general ontogeny and especially myogenesis in the dentaliid scaphopod *Antalis entalis* proceeds much more direct than in polyplacophorans or gastropods. Accordingly, distinct larval muscle systems are lacking. However, the paired cephalic and pedal retractors both form additional fibers which project into the region of the prototroch and are lost at metamorphosis. The existence of a distinct, paired cephalic retractor system, which is also found in the basal gastropod and cephalopod bauplan but not in the Bivalvia, suggests a clade comprising the Scaphopoda and Gastropoda + Cephalopoda. This is strengthened by expression data of the homeobox gene *engrailed*, which plays a significant role in molluscan shell formation. While two dorso-lateral centers of *engrailed* expression, which correspond to the two centers of initial shell calcification, are found in early bivalve veligers, *engrailed* is exclusively found in mantle margin cells surrounding the single anlage of the embryonic scaphopod shell. In contrast to bivalves, the scaphopod shell is thus formed from a single center of calcification, and a scaphopod-bivalve sistergroup relationship is therefore rejected.

Gastropoda

Primitive gastropods, such as the patellogastropods *Patella vulgata* and *Patella caerulea*, show one pair of asymmetrically positioned larval retractor muscles which have distinct insertion sites at the embryonic shell. Another strict larval muscle system is the prototrochal muscle ring. All these muscle are lost before, during, or shortly after metamorphosis. Parts of the adult mantle musculature as well as the muscles of the cephalic tentacles are formed prior to metamorphosis, while the buccal musculature is of entire post-metamorphic origin.

The process of gastropod ontogenetic torsion is mainly caused by muscular activity of the larval retractors, while the adult shell musculature arises after the completion of torsion. Thus, ontogenetic torsion is regarded as an entirely larval process inferring that the arrangement of the adult shell musculature - which can often be reconstructed by muscle scars

on fossilized shells - is not indicative for the question whether paleozoic univalved molluscs were torted or not.

1. INTRODUCTION

Until this study, molluscan myogenesis has been widely neglected by zoologists and developmental biologists alike. The only detailed data hitherto available were recruited from bivalves (Hatschek 1880, Meisenheimer 1901, Cragg 1985, Cragg and Crisp 1991) and from the basal gastropods *Haliotis* (Crofts 1937, Degnan et al. 1997, Page 1997), *Polinices* (Page 1998), and *Patella* (Smith 1935, Crofts 1955). However, most of these works acquired data from very few developmental stages, leaving most aspects of larval and early juvenile muscle morphogenesis obscure. This together with often limited methodology led to contradicting results regarding muscle development in gastropods. Thus, Crofts (1937, 1955) and most followers (e.g., Bandel 1982) regarded at least parts of the larval shell musculature as direct ontogenetic precursors of the adult shell musculature in *Haliotis* and *Patella*, rendering both systems as homologous, while Smith (1935) argued in favor of independent larval retractor systems in *Patella*. In the late 90s, Page (1997) and Degnan et al. (1997) rejected Crofts' earlier findings for *Haliotis* by demonstrating independence of larval and adult muscle systems in this genus. The data of Smith (1935), however, have not been re-investigated since, but are crucial for inferring the basal gastropod (myo-) groundplan, because *Patella* is a member of the most basal gastropod taxon, the Patellogastropoda (see Haszprunar 1988, Ponder and Lindberg 1997). Accordingly, patellogastropod myogenesis is reinvestigated in order to elucidate whether genuine larval retractors are basal for the Gastropoda and, if so, maybe for all Conchifera (Monoplacophora + Bivalvia + Scaphopoda + Gastropoda + Cephalopoda) or even Testaria (Conchifera + Polyplacophora).

The Polyplacophora show numerous characters that are considered plesiomorphic for the Mollusca, e.g., a chitinous cuticle with calcareous spicules, lack of jaws, a cord-like tetraneuran nervous system, and bipectinate ctenidia (gills). In addition, several organ systems are serially repeated along their anterior-posterior axis, such as the shell plates, dorso-ventral shell plate muscles, ctenidia, the pedal commissures, and the latero-pedal connectives of the nervous system (see, e.g., Wingstrand 1985). This has led to the still popular hypothesis that chitons, and thus the entire Mollusca, may have a primary annelid-like segmented ancestor, proposing a direct sister-group relationship of annelids and molluscs (Götting 1980, Ghiselin

1988, Lake 1990, Nielsen 1995, Scheltema 1996; but see Russell-Hunter, 1988). Such an assumption, however, infers either a secondarily acquired worm shape (e.g., Edlinger 1989) or evolution by progeny of the aplacophoran taxa Solenogastres and Caudofoveata as proposed by Scheltema (1993). The bauplan of the Solenogastres, indeed, shows multiple seriality of the dorso-ventral musculature and the famous aplacophoran "larva of Pruvot" was described as bearing seven shell plate rudiments, similar to the anlagen of the first seven shell plates in late chiton larvae (Pruvot 1890, 1892). However, alternative theories suggest that aplacophorans have retained the original basal (i.e. non-segmented) molluscan bauplan (e.g., Boettger 1955, Salvini-Plawen 1969, 1980, 1981, 1991, Salvini-Plawen and Steiner 1996). This hypothesis assumes successive concentration of an ancestral aplacophoran-like serially arranged dorso-ventral musculature which consequently led to a final, single pair of (shell) retractors in gastropods and cephalopods. Accordingly, this theory regards the Polyplacophora with their eight sets of paired shell plate muscles as an intermediate stage of dorso-ventral muscle concentration, thus linking the aplacophoran clades Solenogastres and Caudofoveata to the Conchifera. However, due to the lack of recent ontogenetic data on aplacophoran development, this interpretation remains problematic. In order to provide new data for this discussion, which is directly associated with the question regarding basal features of the molluscan bauplan, investigation of the muscle morphogenesis in the Polyplacophora appeared crucial.

Aside from the aplacophorans and the tryblidians (i.e. extant Monoplacophora), the Scaphopoda are the least known molluscan class regarding any aspect of ontogeny. In fact, except for mere sketch drawings (Lacaze-Duthiers 1857, Kowalevsky 1883) and few (though detailed) experimental cell lineage analyses (Dongen and Geilenkirchen 1974, 1975; Dongen 1976), no recent data on their development (especially organogenesis) are available. Thus, their larval muscle anatomy remains unknown, and especially the question whether scaphopods bear a specific, independent larval musculature, as found in bivalves (Hatschek 1880, Meisenheimer 1901, Cragg 1985, Cragg and Crisp 1991; see also Fig. 1 herein), remains unclear. In addition, general scaphopod ontogeny is crucial for the traditionally proposed but recently questioned direct scaphopod-bivalve sistergroup relationship, which was based on the assumption that both taxa possess a primarily bivalved shell, which, during larval development, fuses ventrally in the Scaphopoda. This led to the current text book version of a scaphopod-bivalve clade, the Diasoma, which was first proposed by Runnegar and Pojeta (1974). While a bipartite early shell anlage has been clearly demonstrated for the Bivalvia (e.g., Waller 1981), this assumption remains purely hypothetical for the Scaphopoda.

Investigation of scaphopod shell morphogenesis should thus yield significant insights regarding the Diasoma concept. Recently, Moshel et al. (1998) and Jacobs et al. (2000) showed that the homeobox gene *engrailed*, which has been sequenced from all testarian classes except the Tryblidia (Wray et al. 1995), seems to play an important role in early shell development of gastropods, bivalves, and chitons. It seemed thus very likely that combined analyses of scaphopod muscle and shell organogenesis (together with *engrailed* expression data) would provide a new base for the discussion of molluscan phylogenetics as a whole.

2. MATERIALS AND METHODS

A brief overview of the species investigated and the methods applied is given in Table 1. For detailed descriptions, see the relevant appendices.

Table 1. List of species investigated and methods applied. CLSM - confocal laser scanning microscopy in combination with fluorescence staining of F-actin; *en* - staining of the *engrailed* transcript; LM - light microscopy analysis of serial semithin sections; SEM - scanning electron microscopy; TEM - transmission electron microscopy.

CLASS/ *Species*	stages investigated larval	juvenile	adult	Methods applied	Appendix
SOLENOGASTRES					
Dondersia sp.	-	-	+	TEM	I
CAUDOFOVEATA					
Chaetoderma nitidulum	+ (few)	-	-	CLSM	IV
POLYPLACOPHORA					
Mopalia muscosa,	+	+	-	CLSM, SEM, TEM	II
Chiton olivaceus	+	+	-	CLSM, SEM, TEM	II
TRYBLIDIA					
Laevipilina antarctica	-	-	+	TEM	I
BIVALVIA					
unidentified	+	-	-	CLSM	Review
SCAPHOPODA					
Antalis entalis	+	+	-	CLSM, LM, *en*, SEM, TEM	III, IV
GASTROPODA					
Patella caerulea,	+	+	-	CLSM, SEM	V, VI, VII
Patella vulgata	+	-	-	CLSM, SEM	V, VI

3. RESULTS

3.1. General remarks and terminology (see also Appendix I)

An overview on all larval and adult muscle systems which are found in the Mollusca is given in Appendix I. In this review, emphasis is lain on the main focus of this thesis, namely the development of the individual muscle systems of selected species of the molluscan classes Polyplacophora, Bivalvia, Scaphopoda, and Gastropoda. In addition, the first detailed SEM analysis of scaphopod larval and early juvenile development is provided along with a gene expression pattern analysis of the homeobox gene *engrailed* in relation to scaphopod shell development. Ages of specimens are given in hours post fertilization (hpf), hours post metamorphosis (hpm), or days post metamorphosis (dpm).

The prototroch of basal, lecithotrophic molluscan larvae corresponds ontogenetically and phylogenetically to the velum of more derived planktotrophic forms (higher Bivalvia and Gastropoda). Thus, the terms "prototroch (muscle) ring" and "velar (muscle) ring" should be regarded as structures belonging to the same, homologous organ.

Shell terminology follows Haszprunar et al. (1995). Accordingly, tryblidians, basal bivalves, scaphopods, and basal gastropods show a distinct embryonic shell (protoconch I) which is directly followed by the adult shell (teleoconch) with different shell sculpture. Certain gastropod taxa (e.g., Caenogastropoda and Heterobranchia) as well as autobranch Bivalvia show an additional, intermediate shell stage, the larval shell (protoconch II). In planktotrophic species, this shell type is often ornamented and thus easily distinguishable from the embryonic and the adult shell. For a more detailed definition and discussion on the subject, see Appendix III and V.

This review provides a summary of the results given in Appendix I-VII. These data are cited according to the following example: IV: 2B, p. 103 = Appendix IV, Fig. 2B, page 103.

3.2. Myogenesis in Polyplacophora (Appendix II)

Myogenesis was investigated in the two chiton species *Chiton olivaceus* Spengler, 1797 and *Mopalia muscosa* Gould, 1846, and followed the same developmental patterns and chronology. Myogenesis in *Mopalia* starts at 74 hpf (II: 1A, 2A, p. 61-62) with the dorsal anlagen of the prototroch muscle ring and the first two myocytes of the putative rectus muscle, which are situated more ventrally. Slightly later, the delicate, paired longitudinal

muscle appears ventro-laterally on both sides of the larva and starts to extend post-trochally (II: 2A right, p. 62). On the ventral side, the anlage of the dorso-ventral musculature becomes visible. At this stage, the first ring muscles of the pre-trochal muscle grid become visible on the dorsal and ventral side (II: 2B, p. 62).

Subsequently, new myocytes of the rectus muscle differentiate laterally on both sides, which results in a bilaterally symmetrical muscular system. The newly formed fibrils diverge towards the anterior pole of the larva and only the two earliest formed fibers mark a strict anterior-posterior axis through the animal. In addition, ring muscles are formed in the pre-trochal region, which form a muscular meshwork around the fibers of the rectus muscle (II: 2C-F, p. 62; 3A, p. 64). This "apical grid" is engulfed laterally by a circular muscle that later becomes the ventral enrolling muscle. In the post-trochal body region, transversal muscle fibers are formed underneath each putative shell plate just dorsal of the fibers of the rectus muscle (II: 2C-F, p. 62; 3B, p. 64).

At around 129 hpf, the rectus muscle forms a predominant, dorsal, longitudinal unit which extends antero-laterally. The apical grid surrounds the pre-trochal body part as a three-dimensional muscular net which consists of outer ring and inner diagonal muscle fibers and encircles the rectus muscle. The prototroch ring is a solid band of muscle fibers located directly underneath the prototrochal epithelium. Laterally, the enrolling muscle encircles all other muscle systems and forms a border against the outer mantle. The ventro-lateral longitudinal muscle pair lies more ventral and medial to the latter muscle and consists of two distinct muscle strands that do not contact each other anteriorly. This ventro-lateral longitudinal muscle interconnects on both sides with the dorso-ventral musculature via numerous short muscle fibers (II: 2D-E, p. 62). The dorso-ventral musculature appears as a multiple repetition of minute myofibrils that intercross in the pedal region (II: 2D-F, p. 62).

During metamorphosis, the larval prototroch muscle ring and the apical muscle grid degenerate (II: 2G-H, p. 62). The buccal musculature arises immediately after metamorphosis and consists of numerous fibers that insert on the first shell plate. The former distinct, dorso-ventral shell plate muscle fibers start to concentrate (II: 2G, p. 62), and ten days after metamorphosis, the paired shell plate muscle bundles have differentiated under each shell plate. Additionally, the radular retractor muscles, which insert on the second shell plate, are formed on both sides of the rectus muscle (II: 2H, p. 62). The paired ventral longitudinal muscle persists through metamorphosis. The circular enrolling muscle is already functional in early juvenile animals (i.e. at one day after metamorphosis, see II: 2G, p. 62), enabling the animal to protect its soft body parts on the ventral side if separated from the substratum.

The myofibrils of the dorsal rectus muscle undergo considerable rearrangement during larval life and especially at metamorphosis: their strong anterior divergence ceases (II: 2C-F, p. 62), and after metamorphosis all fibers follow a strict longitudinal anterior-posterior orientation. (II: 2A-B, G, p. 62).

Fine structural analyses revealed the smooth character of most larval and adult chiton muscle systems (II: 3, p. 64), except for the obliquely striated buccal musculature.

3.3. Larval development and shell formation in Scaphopoda (Appendix III)

Herein, the first SEM study of the larval and early post-metamorphic development of a scaphopod - *Antalis entalis* (Jeffreys, 1869) - is presented. In addition, the expression pattern of the homeobox gene *engrailed* is analyzed.

Early larvae are poorly differentiated with the prototroch cilia being not yet fully developed and the prototroch cells not arranged in the three parallel rows as found in individuals aged 32 hpf or older (III: 1A-B, p. 81). The post-trochal area consists only of a small cluster of cells. From approximately 35 hpf onwards, dramatic morphological transformations occur (III: 1C-I, p. 81): The prototroch is subsequently reduced and is finally recognized as a narrow band of ciliated cells only (III: 1F-I, p. 81). The larval apical organ is completely lost prior to metamorphosis (III: 1C, E-G, p. 81). The post-trochal area starts to grow and at 39 hpf the calcified, single primordium of the embryonic shell is formed. Likewise, the anlagen of the foot and the mantle become visible (III: 1D, p. 81). The formation of the mantle and the embryonic shell starts dorsally and both structures grow in anterior and ventral direction. At 64 hpf the mantle and the embryonic shell are ventrally closed. The successive calcification of the protoconch in dorso-ventral direction and its anterior growth until metamorphic competence is indicated by the suture, the ventral fusion line of the embryonic shell (III: 1H-I, p. 81). The surface of the protoconch is completely smooth, without any distinct growth lines. The foot anlage appears as a symmetrical hump on the ventral side but remains non-functional until metamorphic competence (III: 1D-E, p. 81).

Larvae induced at 94 hpf or later performed metamorphosis within two hours. During metamorphosis, the prototroch is lost and the paired anlage of the first captacula (anterior tentacles) is formed dorso-laterally in the cephalic region of the juvenile (III: 2A-B, p. 82). The foot differentiates and develops its characteristic three-lobed morphology, which is retained through adulthood. Accordingly, the animal switches from a planktic

free-swimming to a benthic creeping-burrowing locomotion. Only the anterior tip of the predominant central lobe of the foot is ciliated (III: 2, p. 82). The formation of the protoconch stops at the onset of metamorphosis and the adult shell (teleoconch), which shows striking growth lines but lacks a suture, is generated. These differences enable easy distinction of the protoconch and the teleoconch in the post-metamorphic juvenile scaphopod (III: 2D, p. 82).

3.4. *Engrailed* expression in Scaphopoda (Appendix III)

The *engrailed* transcript is first localized in early trochophores at the age of 28.5 hpf, where it is expressed in two unequally sized cell clusters of the dorsal ectoderm. The much larger anterior cluster consists of approximately 15 cells which are arranged in a semi-circle just behind the prototroch around the putative anlage of the embryonic shell field. The second cluster of about three cells is situated more posteriorly (III: 3A, p. 85). Slightly later, *engrailed* is found in cells surrounding the anlage of the embryonic shell, which has started to differentiate between the two former *engrailed* clusters (III: 3B, p. 85). During subsequent development, *engrailed* expressing cells form the margin towards the outer mantle epithelium (III: 3C-E, 4, p. 85). The gross morphology of the shell field and the pattern of *engrailed* expression clearly reflect the primary univalved character of the protoconch (III: 4, p. 85). After ventral closure of the mantle and the embryonic shell field, the protoconch is fully established and the *engrailed* expressing cells are found around both the anterior and the posterior edges of the shell field (III: 5A-B, E, p. 86).

In 80.5 hpf old specimens *engrailed* is also found in a few cells of the body mass which probably belong to the adult nervous system (III: 5C-D, p. 86). At metamorphic competence, *engrailed* expression disappears at the margins of the protoconch. In contrast, expression is now found in several body regions, e.g., in the anterior region of the putative cephalic ganglia, in two centers of the mid-body, the foot, and in the visceral mass (III: 6, p. 87).

At 61 hours after metamorphosis *engrailed* expression is restricted to a few cells that contribute to the adult cerebral ganglion (III: 7, p. 87). All other former expression sites remain without signal. *Engrailed* is not expressed in cells that are involved in teleoconch formation (cf. III: 2D, p. 82; 7, p. 87). In individuals aged 13 days post metamorphosis no *engrailed* expressing cells were found.

3.5. Myogenesis in Scaphopoda (Appendix IV)

Muscle development in *Antalis* starts at around 50 hpf in two dorso-laterally positioned, bilaterally symmetrically arranged regions (cf. IV: 1A, p. 101; 2A, p. 103). Slightly later, these anlagen have differentiated into fibers of the putative paired cephalic and pedal retractors which run dorsally from their posterior shell attachment site into the mid-body region (IV: 2B, p. 103). After ventral closure of the protoconch, the pedal retractor fibers project ventrally into the foot hump and interconnect with the newly formed myocytes of the pedal plexus. In contrast, the cephalic retractors run in anterior direction towards the mantle fold. Both retractors show additional fibers which penetrate the prototroch area and serve as prototroch retractor muscles (IV: 2C, p. 103). These muscle portions, however, are not independent but are connected to either the cephalic or the pedal retractors. No prototroch muscle ring is present in *Antalis* larvae. At metamorphic competence, the buccal musculature, which is represented by a muscular ring encircling the region of the foregut, and the laterally situated mantle retracting fibers have started to form (IV: 2D, p. 103).

During metamorphosis the prototroch retracting muscle fibers are resorbed (IV: 2E, p. 103). The foot musculature (pedal plexus) starts to arrange. Its middle piece consists of circular, longitudinal, and diagonal fibers which form a three-dimensional muscular meshwork (IV: 2G-H, p. 103). From their posterior dorso-lateral shell attachment sites, the pedal retractors run slightly more dorsal and more lateral to the cephalic retractors into the mid-body of the juvenile until they reach the buccal muscle ring. (IV: 2H-I, p. 103). From the inner sides of this ring, both foot retractor muscles run in ventral direction into both sides of the foot and form the lateral longitudinal muscle bundles of the foot (IV: 2K-L, p. 103). The distinct cephalic retractors run from their postero-dorsal origin in anterior direction until they reach the buccal ring, which they cross dorsally, and insert in the buccal region of the animal (cf. IV: 1C, p. 101; 2H, p. 103).

Anatomically, the central (main) lobe of the anterior part of the foot mainly consists of outer circular and inner longitudinal muscle fibers with occasionally present diagonal myocytes (IV: 3B, D-E, p. 105). The two lateral lobes consist of few diagonal, several longitudinal, and additional circular muscle fibers (IV: 3B, E, p. 105), while the middle piece and the foot basis are formed by a muscular grid of longitudinal and intercrossing diagonal muscles (IV: 3B, F, p. 105). The musculature of the captacula starts to form several days after metamorphosis and develops rapidly, leaving little space for a distinct captacular cavity (IV: 2L, p. 103; 3, p. 105). Ultrastructural analyses show that all identified larval muscle systems

in *Antalis entalis*, including the prototroch retracting fibers and the juvenile buccal musculature, are smooth (IV: 4, p. 106).

3.6. Myogenesis in Bivalvia (previously unpublished data)

Numerous specimens of unidentified bivalve veliger larvae were obtained by plankton toes in order to determinate the complete myo-anatomy of autobranch larvae. These studies show that, aside from the previously identified complex larval velum retractor systems, a specific velum muscle ring is present in autobranchs (Fig. 1A-B). Both muscle systems are lost during metamorphosis and the anterior and posterior adductor muscles, which are already present and functional in the late veliger stages (Fig. 1B), form the major adult shell muscle system. The velum ring resembles those of polyplacophorans (see above) and gastropods (see below).

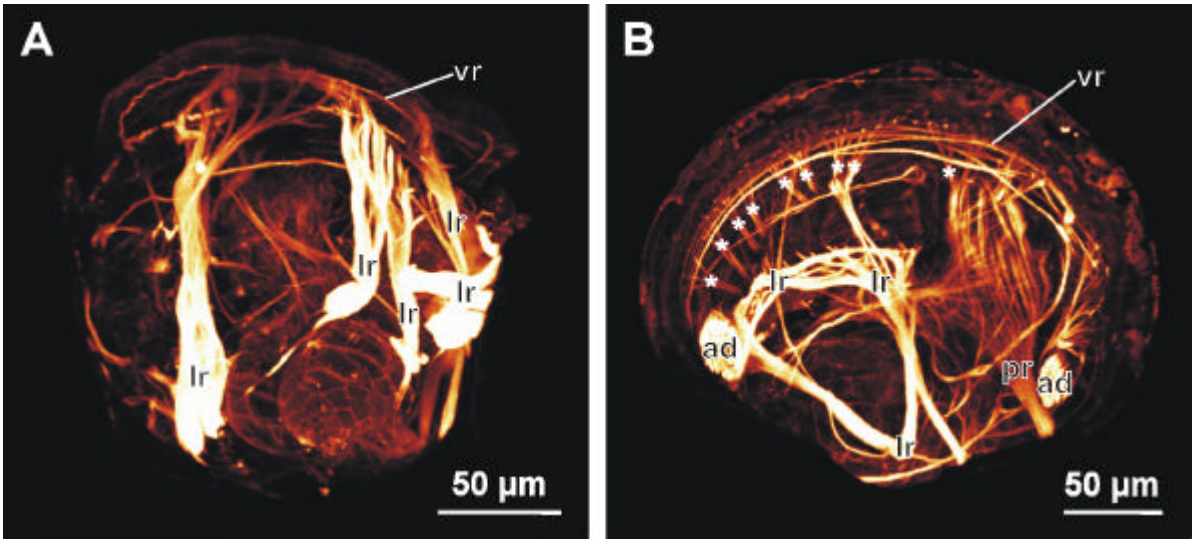


Fig. 1. Unidentified bivalve (autobranch) larvae showing the complicated larval retractor system (lr) with numerous anteriorly branching muscle fibers (marked with asterisks in B) which insert at the velum muscle ring (vr). The existence of such a velar ring (vr) in autobranchs, similar to those of polyplacophorans and gastropods, is new to science. The adult pedal retractor (pr) and adductor muscles (ad) are already well developed in late veligers. Anterior faces upwards. **A.** Relaxed specimen. **B.** Specimen with velum retracted into the mantle cavity.

3.7. Myogenesis in Gastropoda (Appendix V, VI)

Since the Patellogastropoda represent the most basal gastropod taxon, they are the ideal taxon for studying the larval gastropod myo-groundplan. Herein, two species, *Patella*

vulgata L. and *Patella caerulea* L., were investigated and showed only minor differences in their general ontogeny as well as regarding muscle development.

The anlage of the main larval retractor, which is characterized by several fine, dorsally situated muscle fibers, is the first recognizable muscle structure (V: 1A-B, 2A-B, p. 127-128). Slightly later, the velar muscle ring, which consists of several spindle-like muscle cells, forms (V: 1C, p. 127). In the next stage, the main larval retractor consists of two portions: the dorsal and more central portion runs as a dense bundle into the apical area, whereas the smaller ventral and lateral portions run into the pedal region (V: 1D, 2C, p. 127-128). Moreover, two additional muscle systems are visible for the first time, namely the accessory larval retractor, situated ventro-terminally, and the pedal muscle plexus, which consists of a yet weak and irregular muscular grid (V: 1D, p. 127). Prior to torsion the main larval retractor shows a distinct insertion area at the embryonic shell slightly left to the center of the visceral hump of the larva (V: 1E, 2D, p. 127-128). Seen from posterior, the accessory larval retractor is attached right of the main larval retractor to the embryonic shell. In *Patella vulgata*, three myocytes of the accessory larval retractor run into the mantle margin and one reaches the anterior pedal region, while in *Patella caerulea* several fibers project into the mantle and velar region but not into the foot.

After torsion is completed the insertion area of the main larval retractor is placed to the upper left of that of the accessory larval retractor (V: 3-5, p. 130-132; VI: 1, 3, p. 146) but most of the fibers of the main larval retractor are situated on the right side. The larval operculum is associated with two thin, symmetrical muscle fibers which curve upwards from the posterior end of the foot into the ventral region of the larva (V: 6A, p. 133). These fibers form the anlagen of the left and right adult shell muscles. Subsequently, these fibers form distinct, laterally placed insertion areas at the shell (V: 3B-D, p. 130; 4A-D, p. 131; 6B, p. 133). During late larval development two new, longitudinal muscle fibers become visible in the mantle region and are positioned dorsally of the accessory larval retractor. Towards metamorphosis the fibers of the accessory larval retractor degenerate and its insertion area at the protoconch is lost. Two additional, transversal muscle fibers occur at the apical mantle margin (V: 4D, 5A-D, 6B-C, p. 131-133). The musculature of the cephalic tentacles arises *de novo* and consists of two longitudinal, cross-bridged fibers.

Prior to metamorphosis the two adult shell muscles grow in size and volume and increasingly interconnect with the fibers of the pedal plexus (V: 4A, p. 131; 6A, p. 133). The main larval retractor remains prominent, whereas the accessory larval retractor degenerates (V: 5A-B, 6B-D, p. 132-133). Eventually, the accessory larval retractor loses its insertion area

at the protoconch. The adult shell muscles become predominant and the pedal plexus is continuously elaborated (V: 5C-D, p. 132). The velum ring disappears simultaneously with the reduction of the prototroch at metamorphosis, while the longitudinal and transversal mantle fibers are retained through metamorphosis (V: 5A-B, 6B-D, p. 132-133). This is also true for the main larval retractor in early postmetamorphic stages, whereas the accessory larval retractor is completely reduced immediately after metamorphosis. The first anlage of the buccal musculature becomes visible between the bases of the tentacular muscle systems (V: 5C, p. 132). Finally, the main larval retractor is lost and the buccal apparatus forms the most prominent anterior muscle system of the juvenile animal (V: 5D, 6D, p. 132-133; VI: 1, p. 146).

In *Patella,* the fibers of both the main and the accessory larval retractor are obliquely striated (Wanninger et al. 1999), whereas the velar and pedal system as well as the adult shell musculature are smooth. All larval and adult shell muscles insert at the shell via so-called tendon cells which are characterized by a high density of actin filaments within the cytoplasm.

3.8. Torsion in Patellogastropoda (Appendix VI, VII)

In *Patella caerulea*, ontogenetic torsion started between 32 and 39 hpf at a rearing temperature of 20-22°C. In the pretorsional larva the foot lies on the same side as the mantle fold (VI: 2, p. 146; VII: 1A1-A3, p. 161). At the onset of torsion the operculum, the embryonic shell, and both asymmetrically positioned, contractile larval shell muscles are already well developed (VII: 1A1, E1 insets, p. 161-162). Both muscles contract simultaneously every 30 seconds. Since the foot still lies between the mantle fold and the prototroch, retraction of the cephalopedal region of the larval body into the embryonic shell is not yet possible. Instead, the embryonic shell acts antagonistically against the activity of the larval shell muscles, which results in a clockwise movement of the head/foot region relative to the visceral portion. These muscular contractions are followed by slow, gradual pumping movements of the foot, which causes transportation of body fluid from the visceral part into the pedal region of the animal. After 30 seconds the next series of muscular contractions occurs, followed by hydraulic movements and so on. 30 minutes after the onset of torsion, 45° of the 180° twist are performed. (VII: 1A1-A4, p. 161). 30 minutes later, 90° of torsion is achieved (VII: 1C1-C4, p. 162) and a further half hour on, 135° of rotation is reached (VII: 1D1-D4, p. 162). About two hours after the onset of torsion all specimens have completed the 180° twist. The foot with its attached operculum is now situated on the opposite side of the

mantle fold (VI: 3, p. 146; VII: 1E1-E4, p. 162). Only from this stage onwards, retraction of the larval body into the embryonic shell is possible.

The formation and growth of the larval operculum starts in late pretorsional larval stages and thus occurs independently of the ontogenetic torsion process (VI: 2, p. 146; VII: 1A1-A3, p. 161).

4. DISCUSSION

4.1. General notes

The results presented herein are the first detailed comparative account of molluscan muscle development and were obtained by a combination of various methodologies such as fluorescence F-actin labeling, confocal microscopy, serial sectioning, and electron microscopy. This and the fact that muscle morphogenesis was followed from its onset through metamorphosis in basal Polyplacophora, Scaphopoda, Gastropoda, and, partly, in Bivalvia are the main innovations of this work and provide significant insights regarding molluscan ontogeny and phylogeny. In addition, results on scaphopod shell development may revive the discussion of scaphopod relationships in the Mollusca.

It is important to be aware of several serious handicaps when discussing the phylogenetic significance of the various features of molluscan ontogeny. First of all, data for the most basal molluscan taxa Solenogastres and Caudofoveata as well as for basal conchiferans (Tryblidia, i.e. extant monoplacophorans) are still either entirely missing or were obtained several decades ago (Pruvot 1890, 1892, Baba 1938, 1940, Thompson 1960). This makes conclusions about basal ontogenetic characters for the Mollusca or the Conchifera quite speculative. This uncertainty increases because the molluscan sister taxon still remains unknown (Haszprunar 1996, 2000, Waller 1998). In addition, basal Bivalvia (protobranchs) undergo larval development via a derived larval type, the so-called test cell or pericalymma larva. Only higher bivalves (autobranchs) show a typical, planktotrophic veliger-type larva. Thus, conclusions regarding basal bivalve development is likewise problematic. Cephalopod ontogeny is generally highly derived and thus provides little information for the discussion of conchiferan or even general molluscan development.

4.2. Myo-anatomy and muscle development

Since the main focus of this work is to deduce phylogenetic and evolutionary conclusions for the Mollusca and their possible outgroups, only muscle systems relevant for these questions are discussed here.

4.2.1. *Larval muscle systems*

Two distinct, genuine larval muscle systems, namely the larval shell retractors and the prototroch (= velum) muscle ring, have been identified in some of the molluscan taxa investigated.

Larval retractor muscles, which are characterized by distinct shell insertion areas and an oblique striation pattern, have been found in several planktotrophic bivalve taxa (Hatschek 1880, Meisenheimer 1901, Cragg 1985, Cragg and Crisp 1991). However, the presence of such retractors in the bivalve groundplan remains debatable. On the one hand, this is due to the phenomenon that basal bivalves (protobranchs) show a derived larval development (see above), the myo-anatomy of which is still unknown. The main bivalve larval type, a planktotrophic veliger-like larva, is only found in the otherwise more derived autobranch taxa such as mytilids, ostreids, or heterodonts. These larvae have a well defined velum and larval retractor muscles (see Fig. 1), but this might be apomorphic for the Autobranchia.

The existence of one, strongly asymmetric pair of larval retractor muscles has been demonstrated for all of the major basal gastropod taxa (patello-, veti-, and caenogastropods) (Degnan et al. 1997, Page 1997, 1998, and herein, V: p. 119-141; VI: p. 142-150) as well as heterobranchs (Page 1995). Muscular activity of these retractors has been shown to be a main cause of gastropod ontogenetic torsion, the major autapomorphy of the class Gastropoda (VII: p. 151-171). These findings clearly indicate that asymmetric larval retractors are a basal feature of the gastropod larval bauplan.

For inference of the basal condition within the entire Mollusca regarding the presence of larval retractors, only the polyplacophorans, scaphopods, and gastropods provide relevant data, because the conditions in the Tryblidia, Caudofoveata, and Solenogastres still remain obscure (see above). The Polyplacophora and Scaphopoda lack distinct larval retractors (see above and II: p. 56-75; IV: p. 75-95), while they are present in autobranch bivalves (Fig. 1) and gastropods (Degnan et al. 1997, Page 1997, 1998, and herein, V: p. 119-141; VI: p. 142-150). Applying the parsimony principle and proposing larval retractors as part of the ancestral bivalve bauplan, the evolutionary origin of larval retractor muscles at the interface of the

Bivalvia versus Scaphopoda, Gastropoda, and Cephalopoda (inferring secondary loss in the Scaphopoda and Cephalopoda) is equally parsimonious than their twice independent evolution in the Bivalvia and the Gastropoda (cf. IV: 5, p. 109). A correlation of the expression of larval retractors and planktotrophy can be ruled out, since the basal, larval retractor bearing gastropod taxa all show entirely lecithotrophic larval stages.

The second larval muscle system, the prototroch muscle ring, renders similar problems regarding its evolutionary origin. To date, it has been found in the Polyplacophora (II: p. 56-74), Bivalvia (Fig. 1), and basal Gastropoda (Degnan et al. 1997, Page 1997, 1998, V: p. 119-141; VI: p. 142-150), but not in the Scaphopoda (IV: p. 96-118). According to the parsimony principle, these data suggest its evolution at the polyplacophoran-conchiferan interface (secondary loss in the Scaphopoda), but due to missing data for the aplacophorans and the tryblidians, this issue requires further investigation.

4.2.2. *Adult muscle systems*

Body wall musculature

Many authors nowadays believe that the aplacophoran taxa Solenogastres and Caudofoveata form the most basal molluscan classes, with the Solenogastres being the earliest offshoot of the phylum (Haszprunar 2000 and IV: 5, p. 109; but see, e.g., Scheltema 1996, or Edlinger 1989, 1991 for recent contrary view). Both taxa have a three-layered body wall musculature which consists of outer ring, intermediate diagonal, and inner longitudinal muscles (Salvini-Plawen 1969, 1972, Scheltema et al. 1994, and herein, I: p. 40-55), similar to other worm shaped taxa such as annelids or nemertines. This worm-like gross morphology is regarded basal for the Mollusca (Salvini-Plawen 1991, Haszprunar 1992, 2000). Functionally, this body wall musculature is necessary in these taxa to maintain a stable body shape against the pressure of the inner body fluid. In polyplacophoran larvae, a similar worm grid is found in the pre-trochal region (II: p. 56-74). Anatomically, this meshwork resembles the aplacophoran body wall musculature. Together with large parts of the pre-trochal body region, this muscle grid is lost in the chiton larva during metamorphosis. It appears probable that the apical muscle grid of the chiton larva likewise serves to maintain the shape of the anterior body region until it is reduced at metamorphosis. Thus, structurally and functionally, the chiton worm grid can be regarded as a relic of the body wall musculature of a proposed worm shaped molluscan ancestor and homologous to the aplacophoran body wall musculature. Its reduction during chiton metamorphosis and its complete absence in the

Conchifera (note that the larval condition in tryblidians is still unknown) is probably due to the introduction of a body stabilizing "exoskeleton", the shell (plates) (see IV: p. 96-118).

Dorso-ventral (shell) musculature

Regarding the arrangement of the molluscan dorso-ventral musculature, the Polyplacophora represent a link between the aplacophoran and the conchiferan condition (IV: p. 96-118). While the Solenogastres (and the anterior part of certain Caudofoveata) show numerous serially repeated dorso-ventral muscle fibers along their anterior-posterior axis, the conchiferans are characterized by concentrated and numerically reduced shell muscles (see I: p. 40-55; II: p. 56-74; IV: p. 96-118). In chiton larvae, the development of the dorso-ventral musculature undergoes an initial stage of multiple seriality, which corresponds to the situation found in adult Solenogastres. Considerable time after metamorphosis, these fibers concentrate into seven (and later eight) paired muscle bundles which insert on both sides of each shell plate (II: p. 56-74). This demonstrates that the typical eight-seriality of the adult dorso-ventral shell musculature of chitons is a secondary condition. Thus, the serial organization of the adult shell plate musculature in the Polyplacophora is not indicative for deriving chitons or the entire Mollusca from a metameric, segmented, annelid-like ancestor.

Considering the data on the molluscan body wall and the dorso-ventral musculature, two evolutionary trends within the Mollusca become obvious: The evolution of protective epidermal structures from calcareous spicules (Solenogastres and Caudofoveata) via shell plates (Polyplacophora) to a uni- or bivalved shell (Conchifera) coincides with a subsequent concentration and numeric reduction of the dorso-ventral (shell) musculature and with a complete loss of the original body wall musculature (see above and IV: 5, p. 109).

Buccal musculature

Extant and fossil tryblidians and polyplacophorans show distinct buccal (= radula) retractor muscles (Wingstrand 1985, Fig. 2 herein, as well as I: p. 40-55; II: 2H, p. 62). This paired muscle - like other components of the buccal musculature - contains myoglobin, in contrast to all remaining muscles which bear hemoglobin or hemocyanine (Terwilliger and Read 1970, Graham 1973, Nisbet 1973, herein, I: p. 40-55). Both, the cephalic retractor of scaphopods, gastropods, and cephalopods and the buccal retractor of polyplacophorans and tryblidians are situated inwards of the dorso-ventral musculature (Fig. 2), but only the latter muscle inserts directly at the buccal cartilage, thus mainly serving as a radula retracting system. Accordingly, it is clearly distinct from the cephalic retractor which is not associated

with the buccal cartilages. Instead, the cephalic retractor projects into the dorsal part of the cephalic region and serves as a true "head" retracting system (Lang 1900, Wells 1988, herein, IV: p. 96-118). Unfortunately, the buccal retractors are often misinterpreted as cephalic retractors, especially in fossil monoplacophorans where they leave striking muscle scars (e.g., Harper and Rollins 2000: fig. 2). However, a comparison of the surface of the muscle scars of the buccal retractor and the cephalic retractor reveals a spotted scar-type for the tryblidian and polyplacophoran buccal retractor, while the patellogastropod cephalic retractor leaves a much more homogeneous imprint (Fig. 2).

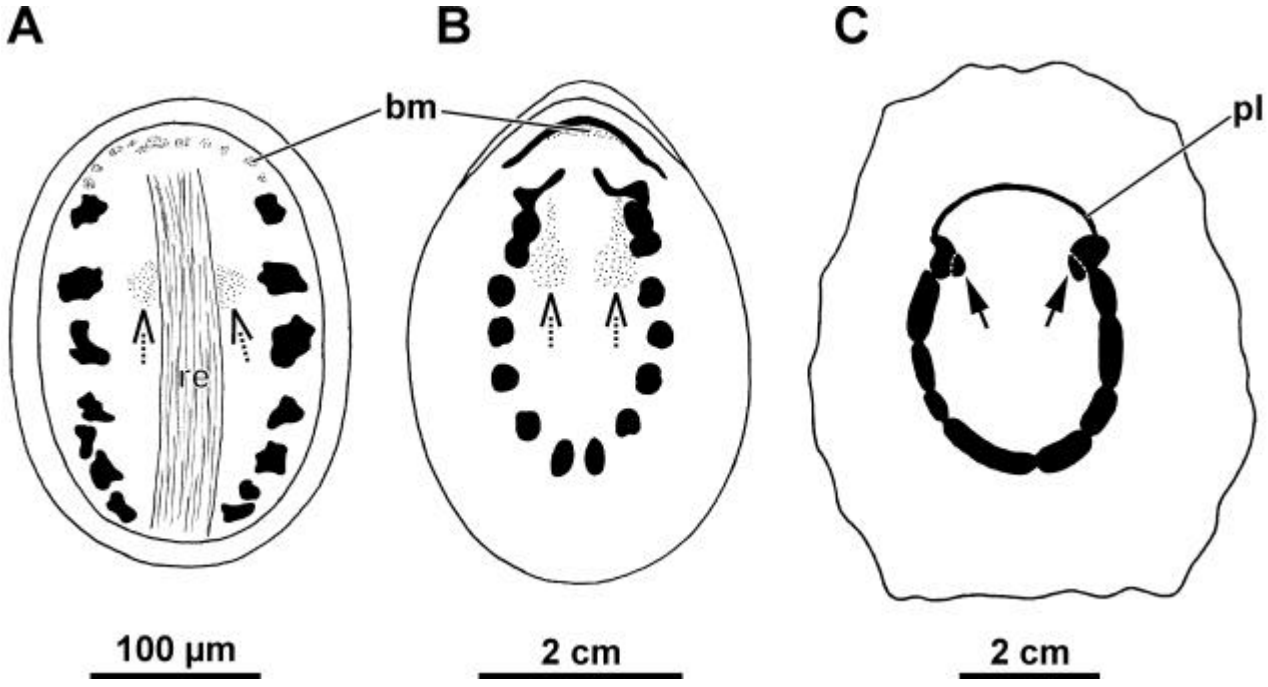


Fig. 2. Shell insertion sites of the dorso-ventral musculature (large, black filled areas), the radula retractors (dotted arrows), the buccal musculature (bm), and the cephalic retractors (full arrows) in early juvenile Polyplacophora (A), fossil Tryblidia (B), and Patellogastropoda (C); all dorsal view. In Polyplacophora and Tryblidia, the radula retractors (dotted arrows) and the buccal muscles (bm) show distinct, spotted shell insertion areas. These muscles insert within the cephalic epithelium in gastropods, thus leaving no shell scars. Gastropods, however, have genuine cephalic retractors (full arrows), which are situated adjacent to the anterior portion of the dorso-ventral muscles. **A.** *Chiton olivaceus*, juvenile specimen aged 13 days after metamorphosis with dorso-median rectus muscle (re) and seven pairs of differentiated dorso-ventral shell muscles. The eighth pair is formed later in development. After phalloidin-stained specimen in II: 2H, p. 62. **B.** Muscle scars of fossil *Pilina unguis*. After Wingstrand (1985). **C.** Adult *Patella caerulea* showing anterior pallial line (pl, i.e. insertion sites of mantle retractor muscles). After Stützel (1984).

Cephalic retractors

Within the Mollusca, gastropods and cephalopods alone have a free, movable head (Salvini-Plawen and Steiner 1996; but see Waller 1998, who interpreted the scaphopod buccal cone as a similar free movable head structure). All "head-less" classes, however, do have a

distinct "cephalic region" which is characterized by a buccal apparatus (which is secondarily lost in the Bivalvia) and distinct cerebral ganglia with a commissure. So far, adult (i.e., post-metamorphic) cephalic retractors were only reported for gastropods and cephalopods (Lang 1900, Wells 1988, Salvini-Plawen and Steiner 1996, Haszprunar 2000). However, Lacaze-Duthiers (1857) already stated that one of the two retractor pairs in juvenile *Dentalium* projects into the antero-dorsal region of the animal, close to the mouth, but he misleadingly interpreted this muscle as a mantle retractor. The analysis presented herein (IV: p. 96-118) shows that the fibers of the true mantle retractor insert much more anteriorly (i.e. closer to the buccal apparatus) and are loosely arranged rather than forming a solid muscle bundle (cf. IV: 2E, H, p. 103 and Lacaze-Duthiers 1857: pl. 9, fig. 2). It seems most likely that the "rétracteurs ... du manteau" described by Lacaze-Duthiers (1857) is indeed the distinct, independent scaphopod cephalic retractor as presented herein (cf. IV: 2, p. 103).

Because of positional, structural (smooth), and functional similarities, the cephalic retractor system of scaphopods, gastropods, and cephalopods is regarded as suprataxic homologous and synapomorphic for a clade comprising Scaphopoda and Gastropoda + Cephalopoda. This hypothesis is supported by the fact that unequivocal apomorphies for an earlier proposed scaphopod-bivalve clade (Diasoma; see Runnegar and Pojeta 1974) are entirely lacking (Waller 1998, Haszprunar 2000, herein, III: p. 75-95).

Enrolling muscles

The body plan of solenogastres, caudofoveates, and polyplacophorans includes a laterally positioned enrolling muscle system (Salvini-Plawen 1972, Wingstrand 1985, herein, I: p. 40-55; II: p. 56-74). Salvini-Plawen (1972) proposed homology of the aplacophoran and the polyplacophoran enrolling muscles and the tryblidian pedal ring muscle. However, fluorescence staining analyses of the musculature in chiton larvae and early juveniles diagnosed this muscle as a single, circular organ in chitons (II: p. 56-74), while it is paired in adult Caudofoveata and Solenogastres (Salvini-Plawen 1972). Thus, the aplacophoran enrolling muscles probably arose as strengthened parts of the longitudinal body-wall musculature (Salvini-Plawen 1972). In contrast, it is formed as an entirely independent system in the Polyplacophora (II: p. 56-74). These data argue against a proposed homology of aplacophoran and polyplacophoran enrolling muscles, but studies on aplacophoran muscle ontogeny are needed for final clarification.

Foot musculature and functionality

The foot musculature (pedal plexus) is formed in late larval stages of scaphopods and gastropods (IV: p. 96-118; V: p. 119-141), but could not be detected through polyplacophoran early juvenile development (II: p. 56-74).

Functionally, the foot of most basal gastropod (and nearly all polyplacophoran) taxa serves as a locomotory organ on mainly hard bottom substrates. Thus, a ciliary gliding sole is usually the major organ for gastropod movement. In contrast, many bivalves and scaphopods use the foot as a burrowing rather than a gliding organ. Consequently, specific mechanisms, which are reflected in the anatomy of these taxa, evolved (see Kier 1988 for details). One of the two different functional and micro-anatomical modes of the molluscan foot is the so-called muscular-hydrostat system, which is based on antagonistic muscle activity. Such a system is characterized by a complex three-dimensional muscular pattern, which usually leaves little or no space for a hemolymphic cavity and is generally found in body appendages that require fast movements such as the cephalopod arms (for the capture of prey) or the squid mantle (for producing the jet propulsion) (Trueman 1980, Kier 1988). The second type, a hydraulic system, is based on a combination of hemolymphatic pressure (for relaxation) and muscular activity (for contraction). This is usually found in body regions which produce a steady force, such as the burrowing foot of many bivalves or the body and tentacles of pulmonate gastropods (Trueman 1966, 1967). Thus, a distinct and often wide lumen is present in these organs. The scaphopod bauplan expresses both foot types in the two primary subtaxa (Steiner 1992a). While the elongation of the foot of Gadilida is caused by hydraulic pressure alone, its counterpart in the Dentaliida stretches by combined hydraulic and muscular-hydrostat activities (Steiner 1992a, but see Morton 1959 and Trueman 1968 for an alternative view and IV: p. 96-118 for extensive discussion). The present study on juvenile *Antalis entalis* (see IV: 2G-L, p. 103; 3A-C, p. 105) shows the high complexity and thickness of the foot wall and the pedal plexus in combination with a relative small pedal hemocoel, as already described by Plate (1892). In contrast, gadiliids show a much bigger foot lumen and weaker longitudinal foot muscles (Steiner 1992a). According to Kier and Smith (1985) and Kier (1988), this demonstrates that muscle antagonism is the main driving force for dentaliid foot expansion, while hydraulic activities, similar to those in bivalves, are regarded as the main driving force for foot protraction in gadilids.

The question whether the dentaliidan muscular-hydrostat system or the gadilidan combined muscular retraction and hydraulic expansion system is basal for the Scaphopoda

remains unsolved because scaphopod phylogeny as a whole is still unclear (Steiner 1992b, 1996, Reynolds 1997, Reynolds and Okusu 1999).

The musculature and functionality of the cephalic tentacles

The cephalic tentacles of gastropods and scaphopods, named "captacula" in the latter taxon, are both cerebrally innervated (Salvini-Plawen 1981, Ivanov 1991) and develop from cells of the cephalic region (III: 2, p. 82). This identifies them both as cephalic derivatives and they are thus considered homologous. Homology with the arms of cephalopods, however, is still uncertain. The captacula of *Antalis* have very narrow hemolymphic spaces and prominent longitudinal retractors in combination with a dense muscular grid (IV: 2K-L, p. 103; 3G-H, p. 105), indicating that their extension is based on a similar muscle antagonist system as found in the foot. The same mechanism is generally found in the arms of cephalopods and the cephalic tentacles of prosobranch gastropods and is therefore considered basal for the Gastropoda (I: p. 40-55). In the cephalic tentacles of euthyneuran gastropods, however, muscular and hydraulic activities are combined (see Kier 1988).

4.3. Shell development in the Mollusca and comparative *engrailed* expression patterns

Early development of conchiferan embryonic shells starts with an initial stage of shell field invagination (Kniprath 1981). This is followed by shell field evagination and the migration of the shell secreting cells towards the mantle edge (Kniprath 1981, Waller 1981, Moore 1983). In *Antalis*, *engrailed* is expressed in these cells of the mantle margin that secrete the embryonic shell. During its entire morphogenesis, the embryonic shell of *Antalis* remains univalved. These data are in accordance with the first and to date sole studies by Lacaze-Duthiers (1857) and Kowalevsky (1883) and disprove an earlier hypothesis proposed by Runnegar and Pojeta (1974), which comprised the Scaphopoda and Bivalvia to a supertaxon Diasoma. This idea was based on the unproved assumption that the scaphopod shell undergoes an early ontogenetic bilobed stage, which herein is shown to be not the case (see above and III: p. 75-95).

Recent studies showed that the homeobox gene *engrailed* is involved in shell (plate) and spicule formation in chitons, bivalves, and gastropods (Moshel et al. 1998, Jacobs et al. 2000). The present work shows that *engrailed* is expressed in mantle margin cells of *Antalis* that secrete the protoconch but not in cells that contribute to teleoconch formation. This indicates that the genetic backgrounds that underlie protoconch and

teleoconch formation are different and therefore both organs may not be considered homologous (see III: p. 75-95). A comparison of *engrailed* expression patterns in bivalves and scaphopods clearly demonstrates the differences of early protoconch formation in both taxa. In bivalves, protoconch formation starts with two centers of calcification (Kniprath 1981, Waller 1981) which correspond to two distinct clusters of *engrailed* positive cells (Jacobs et al. 2000). In contrast, the scaphopod embryonic shell field is a single structure which is surrounded by marginal *engrailed* expressing cells (III: p. 75-95). This expression pattern supports the SEM observation of a unipartite scaphopod shell. Consequently, the Diasoma concept should be abandoned and, based on data regarding scaphopod muscle development (see above and IV: p. 96-118), the Scaphopoda are more likely the sister taxon of the Gastropoda + Cephalopoda rather than of the Bivalvia (cf. Haszprunar 2000 and IV: 5, p. 109).

In addition to protoconch morphogenesis, *engrailed* plays an important role in nervous system patterning in at least scaphopod (III: p. 75-95) and probably polyplacophoran molluscs (Jacobs et al. 2000), as well as in annelids (e.g., Shain et al. 2000), arthropods (e.g., Abzhanov and Kaufman 2000), echinoderms (Lowe and Wray 1997), and chordates (e.g., Patel et al. 1989, Holland et al. 1997, Hanks et al. 1998). This is regarded as its basal function within the Bilateria (Lowe and Wray 1997). Further independent gain-of-function events of *engrailed* include compartment formation in annelids and arthropods ("segmentation gene"; see Kornberg 1981, Lans et al. 1993, De Robertis 1997, Dahmann and Basler 1999, Abzhanov and Kaufman 2000, Marie and Bacon 2000), skeletogenesis in echinoderms (Lowe and Wray 1997) and molluscs (Moshel et al. 1998, Jacobs et al. 2000, this work), maybe gametogenesis in sea urchins (Dolecki and Humphreys 1988), and limb development in vertebrates (Loomis et al. 1996, Logan et al. 1997, Hanks et al. 1998). Thus, comparative gene expression pattern analyses of *engrailed* demonstrate the high evolvability and plasticity of gene functions during animal evolution.

5. CONCLUSIONS

The data presented herein enable significant conclusions regarding molluscan evolution:

(1) The ancestral condition of the molluscan myo-groundplan include a 3-layered body wall musculature and multiple sets of serially arranged and ventrally intercrossing dorso-ventral muscle fibers as expressed in the recent Solenogastres and partly in the Caudofoveata and the polyplacophoran larva.

(2) Morphogenesis of the dorso-ventral shell plate musculature in Polyplacophora undergoes an initial stage of multiple seriality. The 8-metamerism as found in the adult is a secondary condition which thus contradicts earlier hypotheses which tried to derive polyplacophorans and the entire Mollusca from an annelid-like segmented ancestor.

(3) Due to the introduction of a stable exoskeleton (shell [plates]), the body wall musculature is lost and the dorso-ventral musculature is subsequently concentrated and numerically reduced within the Conchifera.

(4) Specific larval retractor systems, which are lost before, during, or shortly after metamorphosis, belong to the groundplan of the Gastropoda.

(5) Torsion in gastropods is originally an entirely larval process which is mainly caused by muscular activity of the asymmetric larval retractor muscles.

(6) In addition to its basal function in the Bilateria as a nervous system patterning gene, *engrailed* plays a significant role in (embryonic) shell (plate) and spicule formation in Polyplacophora, Bivalvia, Scaphopoda, and Gastropoda and most probably generally in the Mollusca.

(7) In contrast to the Bivalvia, the scaphopod shell is univalved throughout ontogeny.

(8) The existence of distinct cephalic retractors suggests a novel clade which comprises the Scaphopoda and the Gastropoda + Cephalopoda. The Diasoma concept is thus abandoned.

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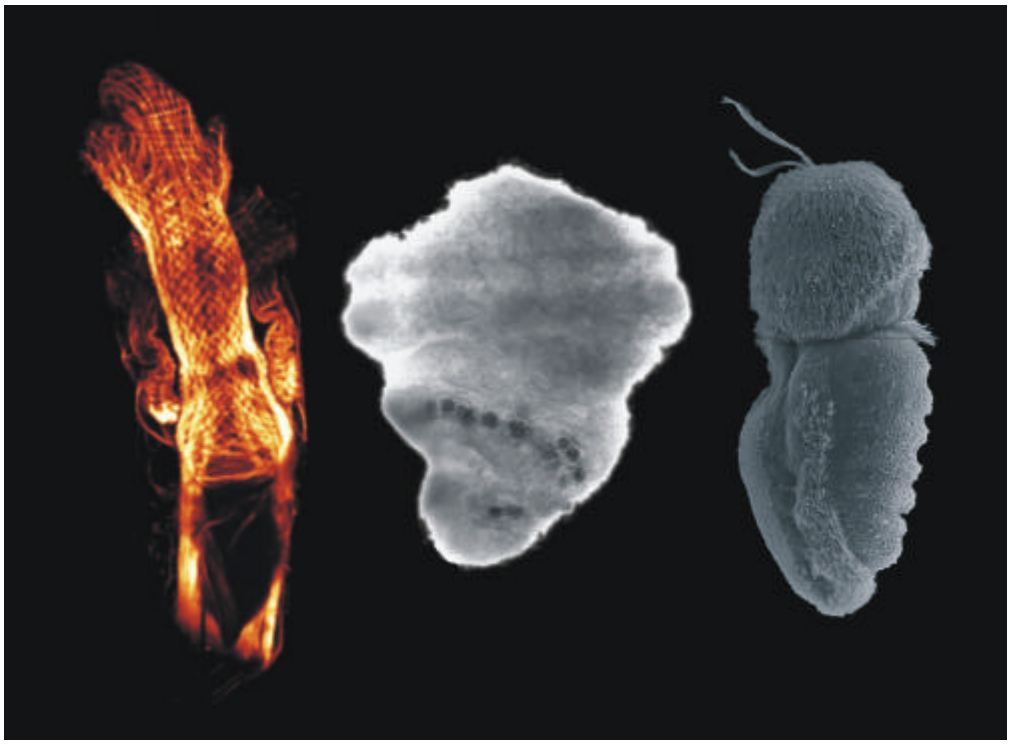
APPENDIX



APPENDIX I

Molluscan muscle systems in development and evolution

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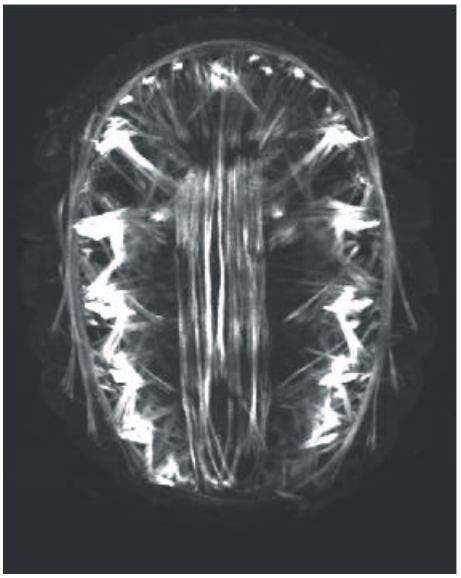

Abstract. The evolutionary history of the various molluscan muscle systems reflects drastic modifications and reductions as well as true innovations. No less than eight main and independent muscle systems of the Mollusca are described and, based on the current understanding of molluscan phylogeny, their evolutionary histories are outlined.

New data on the myogenesis of the Polyplacophora by means of fluorescence-staining and image analysis by Confocal Laser Scanning Microscopy show that the pre-oral region recapitulates a "worm-grid", and that the dorso-ventral musculature passes a stage of multiple seriality as found in adult Solenogastres. Old and new data on bivalves and recent studies on primitive gastropods provide clear evidence that the larval musculature of both groups (and thus possibly of all conchiferans) is entirely independent from the adult condition.

The growth of shell-inserted muscles always necessitates substantial renewal of myocytes which is still poorly understood. Though very promising for phylogenetic purposes, the understanding of the developmental genetics of the various molluscan muscle systems is still in their infancy.

INTRODUCTION

All biological systems can and should be studied and understood from two major points of view: both eco-functional and historical-phylogenetic aspects have in principle the same value and necessity. This is also very true for the various muscle systems in the Mollusca (e.g., Kier 1988; Salvini-Plawen 1988).

Because of the high importance for the extensive fossil record of molluscs, most studies on molluscan muscle systems focused on the various shell muscles, the scars of which are regularly retained and often represent the only traces of the animal's soft body. Shell muscles include the so-called dorso-ventral musculature or the pedal retractors or the spindle muscle respectively, the various adductor systems particularly in bivalves, and (rarely) the mantle retractor system in various conchiferan taxa. In addition, there are several thorough studies on the functional morphology as well as on comparative aspects of the buccal system in various molluscan taxa (e.g., Starmühlner 1969; Graham 1973; Nisbet 1973; Deimel 1982; Wingstrand 1985; Nixon 1988). However, such comparative studies have been rarely used as data bases for phylogenetic considerations (but see, e.g., Sasaki 1998) and are largely missing even in certain major groups such as Solenogastres or Scaphopoda.

The progress in phylogenetic methodology and theoretical foundation during the last decades finally has led to testable trees for several molluscan classes[1] (eg., Haszprunar 1988; Salvini-Plawen and Steiner 1996; Steiner 1996; Reynolds 1997; Ponder and Lindberg 1997; Waller 1998; Reynolds and Okusu 1999) as well as for Mollusca as a whole (Salvini-Plawen and Steiner 1996; Haszprunar 1996, 2000). These basic phylogenetic analyses allow now to "tell the tree" also with respect to the various molluscan muscle systems.

Up to recently ontogenetic aspects of molluscan musculature have been solely based on light microscopical data, which are usually superficial and (sometimes) even inaccurate. Modern methodological progress, particularly fine-structural studies and fluorescence staining procedures, enable a much more detailed framework of embryonic and larval musculature. It will be shown that these data sets have strong implications for theories on the evolution of the various muscle system within the Mollusca.

Thus, the present contribution provides a review on the various molluscan muscle systems concerning their evolutionary history as well as ontogenetic aspects in order to provide a better understanding for these crucial organ systems. Certain general aspects of muscle development and growth are added.

COMPARATIVE ANATOMY AND PHYLOGENY

Body wall musculature

There is little doubt that the molluscan ancestor had worm-like appearance and accordingly was provided with a layer of body wall musculature consisting of outer ring, intermediate oblique and inner longitudinal fibers (e.g., Salvini-Plawen 1991; Haszprunar 1992b). Among the extant Mollusca the aplacophoran taxa Solenogastres and Caudofoveata alone have retained these original body wall muscles (Fig. 1A). The Solenogastres alone still exhibit the molluscan-diagnostic gap above the gliding sole, whereas Caudofoveata have secondarily lost this sole and show strong elaboration of the longitudinal fibers for burrowing activity in soft sediments.

Secondary "worms" have repeatedly evolved within the conchiferan classes namely in bivalves (e.g., ship-worms) and in various gastropod lines, particularly in opisthobranchs, where genus names like *Pseudovermis* or *Helminthope* perfectly reflect this type of external

[1]We use here the category "class" in the traditional sense, but want to make clear that we regard categories as pure expression of relative hierarchy without any meaning for importance, diversity or age.

appearance. Detailed studies on these clearly secondary body wall muscle systems have not been outlined up to now.

Whereas most molluscs extend their body or its appendages mainly by means of haemolymphic pressure, prosobranch gastropods and cephalopods are provided with a so-called muscular hydrostatic system similar to the vertebrate tongue. Here, extension of the body as a whole or of its appendages is provided by muscular contraction and thus much faster than by haemolymphic pressure (e.g., Kier 1988). However, euthyneuran Gastropoda have reestablished the original type of body extension by haemolymphic pressure.

Buccal musculature

Although there are several thorough and comparative studies concerning the anatomy of the buccal musculature in Mollusca, detailed assumptions on phylogenetic steps and direct homologies are rare and are restricted to smaller groupings (e.g., Sasaki 1998). An exception concerns once more Polyplacophora and Tryblidia, where Wingstrand (1985) thoroughly pointed out that even details of the buccal musculature agree in the neopilinids *Neopilina* and *Vema* and the polyplacophoran *Acanthopleura*. A specific buccal retractor occurring in both groups has a highly significant shell scar and is thus also detectable in the fossil record: This

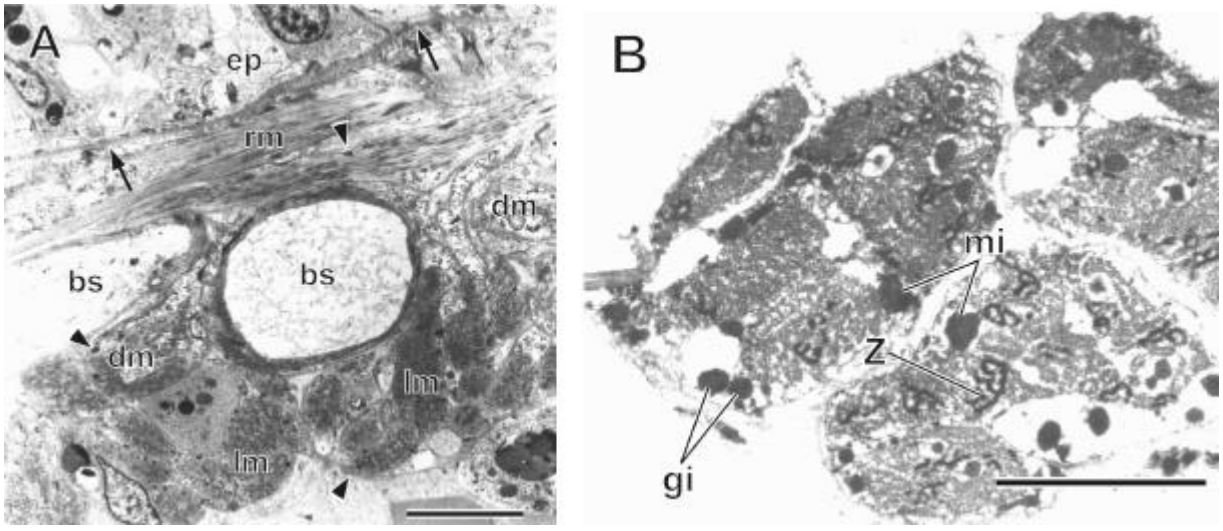

Fig. 1. TEM: **(A)** Cross section through the body wall musculature of the solenogaster *Dondersia* sp. with outer ring (rm), intermediate diagonal (dm) and inner longitudinal fibers (lm) below the epidermis (ep) and its basal matrix (arrows). Arrowheads mark the presence of hemidesmosomes of the myocytes; bs - blood sinus. **(B)** Cross section of the buccal muscles of the neopilinid *Laevipilina antarctica* showing myocytes with few mitochondria (mi) and distinct patterns of myofibrillar arrangement (Z-stripes) being surrounded by cells of the glio-interstitial system with large vesicles (gi). Scale bars = 5 mm.

muscle scar has spotted appearance and is situated immediately inwards of the most anterior bundle of the dorso-ventral shell muscles.

The buccal musculature of molluscs with stereoglossate radula (i.e., a simple rasp; Polyplacophora, Tryblidia, Patellogastropoda) also shows cytological-physiological deviations from regular muscle cells. There is myoglobin (e.g., Terwilliger and Read 1970; Graham 1973; Nisbet 1973) resulting in a bloody red appearance in the living animal. In addition, many buccal muscles are cross-striated (e.g., Nisbet 1973; Haszprunar and Schaefer 1997; Fig. 1B).

Dorso-ventral musculature (shell muscles) (Fig. 2)

Because of its nearly overall presence in shelled forms the dorso-ventral musculature has received most interest by malacologists. Most authors agree that the original state of this organ system was a non-individualized, multiple seriality of dorso-ventral fibers, a condition, which is solely retained in Solenogastres and (anteriorly) in certain Caudofoveata. The medio-ventral inter-crossing of the innermost fibers is diagnostic for Mollusca and contradicts all attempts of direct homologization with dorso-ventral muscle fibers of any supposed outgroup.

The conditions in Polyplacophora and Tryblidia (Neopilinida) require specific discussion. In these taxa the dorso-ventral shell musculature is organized in two times eight (Polyplacophora) or eight (Tryblidia) pairs of muscles each of which appears even as an individual homologue (Wingstrand 1985). Thus, at this point of molluscan evolution there is serial homology among the various muscle pairs in a single animal and suprataxic homology when for instance the seventh muscle bundle between a chiton and *Neopilina* or is compared (see Haszprunar (1992a) for a discussion of homology types). Whereas the eight-pattern in the Polyplacophora is directly understood by the presence of eight shell plates, the same condition in the "mono-placophoran" Tryblidia is generally accepted as an example of recapitulation (e.g., Salvini-Plawen 1981; but see below).

The basic phylogenetic assumption of concentration of the dorso-ventral musculature from a multiple seriality to a set of eight bundles is supported by two lines of evidence: (1) Among the Bivalvia there is further reduction in the number of retained dorso-ventral muscles (i.e., pedal retractors) from originally five to six to finally three pairs. Further reduction (or concentration) occurs in the remaining conchiferan classes: Scaphopoda show one or two pairs of shell muscles (pedal retractors). Cephalopoda and Gastropoda are provided with a single pair or the retained (post-torsional) left "spindle" muscle plus an additional pair of head retractors, the latter are considered to be a synapomorphy of both taxa correlated with the

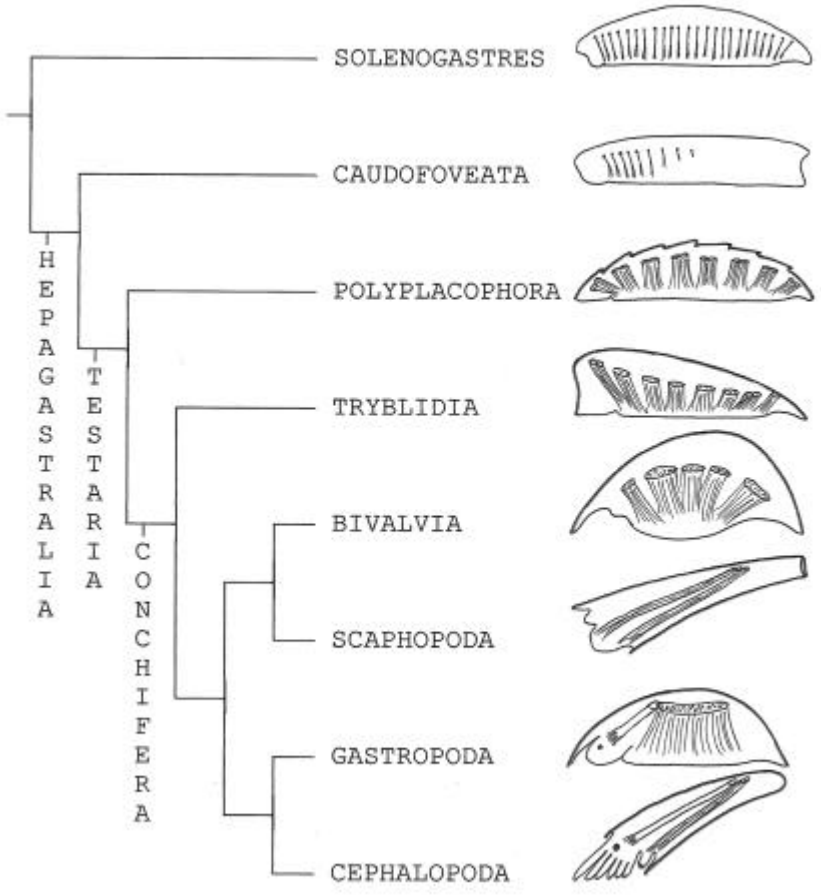

Fig. 2. Most parsimonious tree of the Mollusca (after Haszprunar 2000) with schematic expression of the dorso-ventral and head musculature of each terminal taxon.

formation of a freely moveable head. Unfortunately, there is yet no evidence to individualize the retained muscle bundles compared with the polyplacophoran-tryblidian stage respectively to trace which of the original eight pairs are retained. (2) Myogenesis of the shell muscles in the Polyplacophora recapitulates a multiple serial stage (see below).

Secondary subdivision of shell muscles occur in various gastropod taxa particularly in limpets with U-shaped shell muscles. In all cases investigated a single pair or a single left muscle is present earlier in ontogeny (e.g., Wanninger et al. 1999a, b; see below).

Mantle retractor system (pallial line)

Contrary to the aculiferan taxa, all shelled conchiferans show a specific muscle system to contract the outer mantle. This system is most prominent in the Bivalvia, where its usually continuous scar-line is often named the "pallial line". Tryblidia and certain early bivalves (e.g., the early Cambrian *Pojetaia runnegari*; cf. Runnegar and Bentley 1983) show separated bundles of this muscular system. In limpet-like gastropods (e.g., *Patella*) a "pallial line" is

most obvious in the anterior part of the shell, but the retractor system in fact surrounds the whole animal, and its scar line is situated adjacent to the outer border of the U-shaped shell muscle.

Muscular layer of the gut

Rarely mentioned or even figured (e.g., Haszprunar and Schaefer, 1997), the molluscan gut is generally provided with a distinct muscular layer forming a grid with longitudindally and transversally orientated muscle fibers. Aside from the ciliation of the gut this musculature is responsible for the transport of the food. Contrary to eucoelomate animals (Echiura, Sipuncula, Annelida, Phoronida, Brachiopoda, Deuterostomia), where the muscular layer of the gut is always formed by the inner wall of the coelom, the gut musculature of the Mollusca has nothing to do with any coelomatic cavity (Salvini-Plawen and Bartolomaeus 1995).

Extra-ocular eye muscles of Cephalopoda

As recently reviewed by Budelmann et al. (1997) Cephalopoda alone show a highly complex system of extra-ocular muscles which produce eye-ball movements. There are distinct differences in the arrangement of these muscles between *Nautilus*, Decabrachia and Octopoda, and many muscles can be individually homologized.

Adductor systems

Contrary to all muscle systems described above adductor systems are a matter of convergence within the Mollusca and are correlated with the functional necessity to close a bivalved shell. Certain opisthobranchs (*Akera*, *Ascobulla*, *Cylindrobulla*, and bivalved sacoglossans) show a single, more or less centrally placed adductor muscle (e.g., Kawaguti and Yamasu 1960a, b), and this muscle might be a synapomorphic character of the mentioned taxa or a symplesiomorphic feature of all Sacoglossa (Mikkelsen 1996, 1998; Jensen 1996).

There is little doubt that a dimyarian (anterior-posterior) adductor muscle system is a synapomorphic character of the Bivalvia. Likewise doubtless, however, is the multiple shift towards anisomyar and finally monomyar conditions within the various bivalvian clades, where usually the posterior adductor is retained (Yonge 1953).

Enrolling muscles

Enrolling muscles have been described for Solenogastres, certain Caudofoveata, and all Polyplacophora. Salvini-Plawen (1981, 1985) considered these muscles and the ring muscle

in the foot of Neopilindae as true homologues. However, this view is problematic and can only partly be supported.

There is no doubt that the enrolling muscles of Solenogastres and Caudofoveata are a specialized, latero-ventral part of the longitudinal layer of the body wall musculature. Thus, direct homology appears possible, although direct evidence (versus parallelism) by ontogenetic data is still missing. Homologization of the enrolling muscle of the Polyplacophora with those of the aplacophoran taxa seems improbable, although the innervation of both systems emerges from the lateral (visceral) cords. However, recent studies on the myogenesis of the chiton *Mopalia muscosa* revealed that the enrolling muscle is in principle a ring-system independent of the original body wall muscle grid, and that the enrolling function is provided by the transverse stiffness of the shell-plates. Therefore the enrolling muscle is more likely considered as an autapomorphy of the Polyplacophora.

Whereas the enrolling muscle of Solenogastres, Caudofoveata, and Polyplacophora is laterally (viscerally) innervated, the ring muscle in the foot of neopilinids is a pedal organ. In particular the positional comparison with the enrolling muscle of the Polyplacophora, where the muscle lies outwards of the circumpedal mantle cavity clearly contradicts hypotheses of a direct homology, although both muscle represent a ring system (see above).

MYOGENESIS IN THE MOLLUSCA

Methodological progress

Recent applications of staining procedures of actin filaments for muscular microanatomy plus new methods concerning the visualization (e.g., Confocal Laser Scanning Microscopy) of such preparations have caused remarkable progress in the understanding of molluscan myogenesis.

Myogenesis in Polyplacophora

Preliminary data on the myogenesis of the polyplacophoran species *Mopalia muscosa* revealed several features with high significance for phylogenetic considerations: (1) The anterior, pretrochal part of the chiton trochophore-like larva shows a muscular grid similar to the body-wall musculature of worm-like organisms such as turbellarian flatworms (Fig. 3A) . Based on the molluscan tree, where the two aplacophoran taxa with such body wall muscles are basally placed (Haszprunar 2000; Fig. 2), we regard this feature as a recapitulative event of the original body wall musculature. (2) The fibers of the dorso-ventral muscles occur

simultaneously, contradicting once more earlier suggestions of a segmented nature of Mollusca. (3) The dorso-ventral muscles first show multiple seriality (Fig. 3A) with later concentration into distinct bundles (Fig. 3B). Again this can be reasonably interpreted as direct recapitulation of the phylogenetic transition from the aplacophoran to the polyplacophoran (testarian) level of molluscan evolution. (4) The enrolling muscle occurs early in ontogeny as does its counterpart, the dorso-longitudinal *musculus rectus* (Fig. 3A, B). Being a circular system rather than a longitudinal one as in the aplacophoran taxa and because of its entire independence from the "worm-grid", homology with the enrolling muscle of the latter groups appears improbable. These data are part of a forthcoming study on chiton myogenesis (Wanninger and Haszprunar in prep.).

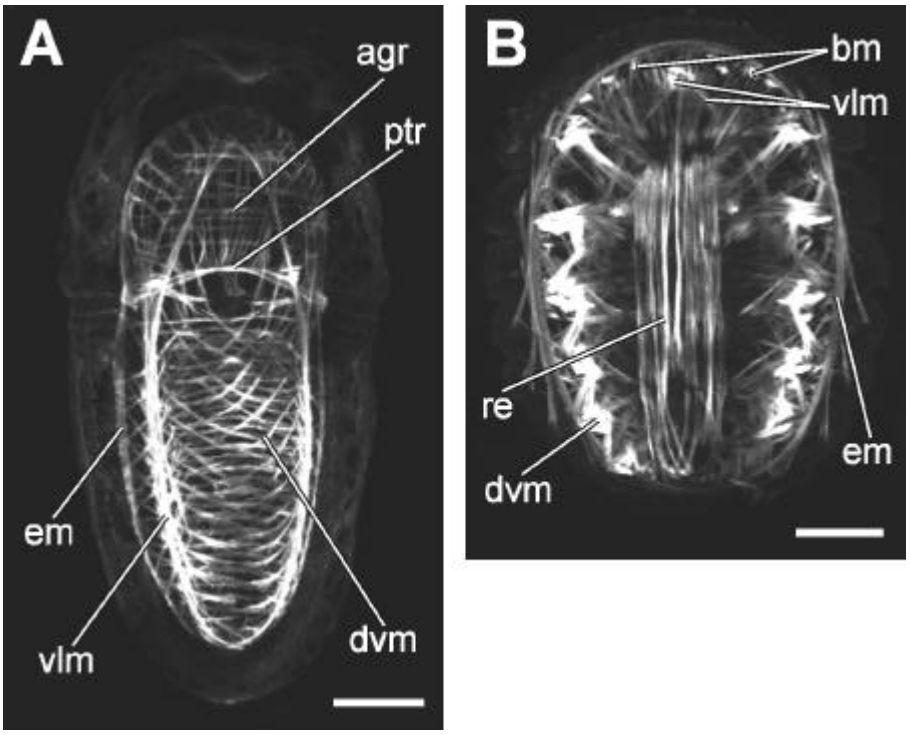

Fig. 3. Myogenesis in the polyplacophoran *Mopalia muscosa* by means of FITC-conjugated phalloidin fluorescence preparations and Confocal Laser-Scanning Microscopy (CLSM). **(A)** Ventral view of a late trochophore-stage with an anteriorly placed muscular grid (agr), a prototrochal ring (ptr), the multiple serial dorso-ventral fibers (dvm), the ventro-lateral longitudinal muscle (vlm), and the ring-system of the enrolling muscle (em). **(B)** Dorsal view of an early juvenile with buccal muscles (bm), the dorso-median rectus muscle (re), a distinct ventro-lateral muscle (vlm), concentrated bundles of dorso-ventral muscles (dvm), and the enrolling muscle (em). Scale bars = 50 mm.

Myogenesis in Gastropoda and Bivalvia

Several recent studies have contributed to our understanding of gastropod myogenesis. Page's (1997) fine-structural studies on larval muscles of *Haliotis kamtschatkana*, the whole-mount preparations of metamorphic *Haliotis rufescens* by Degnan et al. (1997), and combined studies on the myogenesis in *Patella* species (Wanninger et al. 1999a, b) agree in that there exists a distinct larval shell musculature which is entirely independent from that of the adult animal (Fig. 4). Accordingly, there is no ontogenetic homology (cf. Haszprunar 1992a) between any larval or adult shell muscle(s), and also the ontogenetic torsion process in gastropods has nothing to do with any pattern of the adult musculature (Wanninger et al. 2000).

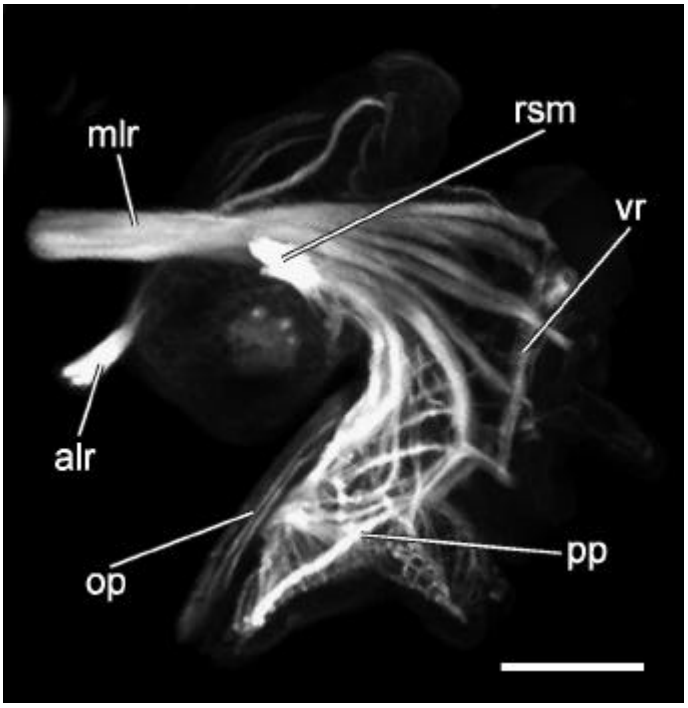

Fig. 4. Myogenesis in the limpet *Patella caerulea* by means of FITC-conjugated phalloidin fluorescence preparations and Confocal Laser-Scanning Microscopy (CLSM). Lateral view from the right of a late larva showing an operculum (op), the main (mlr) and the accessory (alr) larval retractor, the right shell muscle (rsm), the pedal plexus (pp), and the velum ring (vr). Note the separated insertion areas of the larval retractors (mlr, alr) and of the adult shell muscle (rsm). Scale bars = 50 mm.

Although current data are still very scarce, there is good evidence that different clades of higher gastropods differ with respect to myogenesis and the nature of their larval main retractor (spindle muscle): Whereas the larval retractor is the original (obliquely striated) larval shell muscle in planktotrophic larva of nudibranchs (Page 1995), the later planktotrophic veliger of the caenogastropod *Polinices lewisii* already has the (smooth) adult

spindle muscle. This clear difference suggests polyphyly of the planktotrophic mode of development in higher gastropods, but more data are needed to confirm this assumption.

There are no very recent data on the myogenesis of bivalves. However, thorough studies by means of light (e.g., Meisenheimer 1901) or electron microscopy (e.g., Cragg 1985; Cragg and Crisp 1991) again show a distinct, at least partly striated, larval shell musculature (mainly a velum retractor system consisting of several bundles) which is entirely independent of the (smooth) adult pedal retractors. Since the ontogeny of cephalopods is highly derived and because data on the myogenesis of Scaphopoda and Tryblidia are highly incomplete or still entirely missing, it is currently unclear whether or not the character "specific larval shell muscles" occurred independently in Gastropoda and Bivalvia or is a synapomorphy of (higher?) Conchifera.

Although the data presented by Degnan et al. (1997) provided a first insight into the developmental genetics of molluscan (gastropod) musculature, our understanding of the epigenetic system concerning the larval and adult muscles is still in its infancy.

Muscular growth

It is largely overlooked or ignored by authors that regular growth of shell-inserted muscles always necessitates substantial renewal of myocytes, since the region of the muscle scar cannot be directly moved or shifted. Consequently, simple growth of the animal causes the resorption or modification of myocytes at the inner edge of the respective insertion area (line) and the constant production of new muscle fibers at its outer margin. It is still unknown whether this renewal process takes place by invading and differentiating stem-cells or is the result of direct cell division of the already differentiated myocytes *in situ*.

Because of this highly dynamic process throughout the animal's life - most molluscs grow until their death - it is very improbable that a muscle can "survive" phylogenetically, if there is no functional need for it. Accordingly, the combination of a highly complicated shell musculature with a very simple shell in the Tryblidia remains an enigma worth future studies.

CONCLUSIONS

Recent progress in molluscan phylogenetics enables a clearer and better framework for the understanding of the evolution of the various muscle systems by "telling the tree". In addition, modern studies on myogenesis provided very useful insights in myo-phylogeny. However, concerning developmental genetics or the cytological dynamics of muscular growth, we have just begun to understand molluscan musculature.

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APPENDIX II

Chiton myogenesis: Perspectives for the development and evolution of larval and adult muscle systems in molluscs

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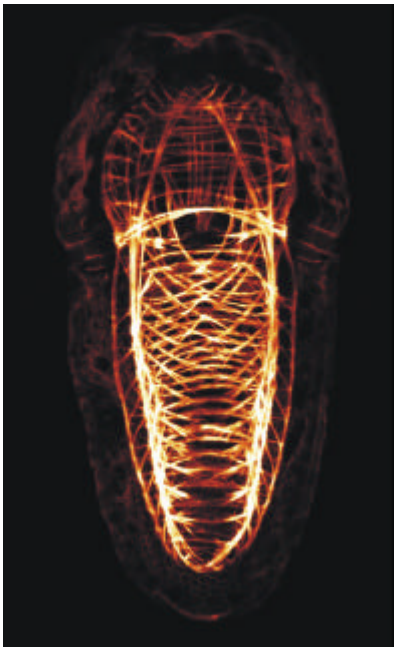

Abstract. We investigated muscle development in two chiton species, *Mopalia muscosa* and *Chiton olivaceus*, from embryo hatching until ten days after metamorphosis. The anlagen of the dorsal longitudinal rectus muscle and a larval prototroch muscle ring are the first detectable muscle structures in the early trochophore-like larva. Slightly later, a ventro-laterally situated pair of longitudinal muscles appears, which persists through metamorphosis. In addition, the anlagen of the putative dorso-ventral shell musculature and the first fibers of a muscular grid, which is restricted to the pre-trochal region and consists of outer ring and inner diagonal muscle fibers, are generated. Subsequently, transversal muscle fibers form underneath each future shell plate and the ventro-lateral enrolling muscle is established. At metamorphic competence, the dorso-ventral shell musculature consists of numerous serially repeated, intercrossing muscle fibers. Their concentration into seven (and later eight) functional shell plate muscle bundles starts after the completion of metamorphosis. The larval prototroch ring and the pre-trochal muscle grid are lost at metamorphosis.

The structure of the apical grid and its atrophy during metamorphosis suggest ontogenetic repetition of (parts of) the original body-wall musculature of a proposed worm-shaped molluscan ancestor. Moreover, our data show that the "segmented" character of the polyplacophoran shell musculature is a secondary condition, thus contradicting earlier theories that regarded the Polyplacophora (and thus the entire phylum Mollusca) as primarily eumetameric (annelid-like). Instead, we propose an unsegmented trochozoan ancestor at the base of molluscan evolution.

INTRODUCTION

Adult polyplacophorans show a complicated system of eight sets of paired dorso-ventral shell muscles that correspond to the eight distinct shell plates in the adult animal. In addition, a ventro-laterally positioned circular enrolling muscle, an unpaired dorsal longitudinal "rectus" muscle, the buccal apparatus, and transversal and oblique muscles underneath each shell plate are present (see, e.g., Sampson, 1895; Plate, 1897; Henrici, 1913; Wingstrand, 1985). Despite numerous detailed studies on the anatomy of the adult polyplacophoran musculature, no data on its ontogenetic development exist until today. Several recent papers (Page, 1995, 1997a,b, 1998; Degnan et al., 1997; Wanninger et al., 1999a,b) as well as earlier studies (e.g., Meisenheimer, 1901; Smith, 1935; Crofts, 1937, 1955; Cole, 1938; Anderson, 1965; Smith, 1967; Cragg, 1985; Cragg and Crisp, 1991) showed that specific larval retractor systems do exist in several gastropod and bivalve clades. These data raise the question whether the

existence of independent larval retractor(s) may either be (syn)apomorphic for the entire phylum Mollusca, solely for the Conchifera, or evolved independently within the several molluscan taxa. In order to answer this question, knowledge of the polyplacophoran condition is crucial.

The Polyplacophora have retained numerous characters that are considered plesiomorphic for the Mollusca, e.g., a chitinous cuticle with calcareous spicules, lack of jaws, bipectinate ctenidia, and a cord-like tetraneuran nervous system with a suprarectal commissure and serial pedal commissures. Therefore, they are phylogenetically regarded as either generally primitive (Scheltema, 1996) or as linking the aplacophoran clades Solenogastres and Caudofoveata to the Conchifera (Monoplacophora, Gastropoda, Cephalopoda, Bivalvia, Scaphopoda) (Boettger, 1955; Salvini-Plawen, 1980; Salvini-Plawen and Steiner, 1996). However, the prominent feature of seriality of shell plates, muscles, and ctenidia has often been and still is used to argue in favor of a primary segmented molluscan ancestor (Götting, 1980; Ghiselin, 1988; Lake, 1990; Nielsen, 1995; but see Russell-Hunter, 1988).

In order to solve the question of an independent larval musculature and to provide new data for the discussion of the "segmentation problem" in the Mollusca, we analyzed the ontogeny of the shell plate musculature in two chiton species, *Chiton olivaceus* and *Mopalia muscosa*, by means of fluorescence staining of F-actin as well as by scanning and transmission electron microscopy.

MATERIALS AND METHODS

Animal cultures

Adult specimens of *Chiton olivaceus* Spengler 1797 were collected on the rocky shore near the STARESO marine station in Calvi/Corsica. Individuals of both sexes spawned during the evening after collection. The eggs were rinsed in sea water and fertilized immediately. Embryos and larvae were kept in glass dishes at 24-27°C.

Breeding of the mossy chiton, *Mopalia muscosa* Gould 1846, was carried out at the Friday Harbor Laboratories/WA, USA. Adult individuals were found near Argyle Creek, San Juan Island, and transported to the laboratory, where some of them immediately released gametes. After insemination, the embryos and larvae were maintained in Millipore-filtered seawater (MFSW) in small custard dishes within a temperature range of 10-12°C. To avoid bacterial or fungal infection, 60 mg penicillin and 50 mg streptomycin were added per liter MFSW.

Metamorphosis was induced by adding either small rocks covered with encrusting corralline red algae or stones from which adult specimens had been removed to the culture dishes with metamorphic competent larvae. Thus, most animals induced at the age of 215 hours post fertilization (hpf) or older settled at the bottom of the culture dish within a few hours after the rocks had been added and the first metamorphosed animals were found at 24-48 hours after induction (cf. Leise, 1986; Strathmann and Eernisse, 1987). Juveniles were cultured until ten days after metamorphosis, bearing seven well developed shell plates but still lacking the eighth plate.

F-actin staining

Animals were relaxed by adding drops of 7% $MgCl_2$ to the MFSW and fixed overnight at 4°C in 4% paraformaldehyde in 0.1M PBS with 10% sucrose. Late larval and juvenile stages were decalcified in 2% EDTA for two hours prior to staining. Staining of filamentous F-actin was performed with Oregon Green 488 phalloidin (Molecular Probes) and followed the detailed description of Wanninger et al. (1999a). Analyses were done using confocal laser scanning microscopy (CLSM) on a Leica DM IRBE microscope with Leica TCS NT software.

Scanning and transmission electron microscopy

Relaxation (see above), fixations and all further preparations and analyses exactly followed the procedures described by Wanninger et al. (1999a).

RESULTS

General remarks

Myogenesis followed the same chronological patterns in *Chiton olivaceus* and *Mopalia muscosa*. However, due to lower rearing temperatures, the timing of development was more synchronous and could be followed more easily in *Mopalia muscosa*. Thus, the data presented herein were obtained from *Mopalia* cultures under the conditions mentioned above, if not stated otherwise.

Please note that herein the term "trochophore" is used in the broad sense as proposed by Rouse (1999), which characterizes all spiralian larval types that bear a prototroch and thus defines the taxon Trochozoa.

Myogenesis

In *Mopalia muscosa*, hatching of the embryos starts at around 21 hpf at 10-12°C. The first myocytes are formed at 74 hpf (Figs. 1A, 2A). Dorsally, myogenesis starts with the anlagen of the prototroch muscle ring and the first two myocytes of the putative rectus muscle, which arise along the median body axis underneath the prototroch and ventrally cross the prototroch muscle ring (Fig. 2A left). A yet delicate, paired longitudinal muscle appears ventro-laterally on both sides of the larva and starts to extend post-trochally (Fig. 2A right). Relative to the rectus muscle, the myocytes of the prototroch ring are situated more dorsally. During subsequent development, the fibers of the prototroch muscle ring and the ventro-lateral longitudinal muscles gain strength and the two myofibrils of the rectus muscle grow both towards the anterior and the posterior pole of the larva. Ventrally, the anlage of the dorso-ventral musculature becomes visible and the fibers of the ventro-lateral longitudinal muscle pair start to expand into the pre-trochal region. At this stage, the first ring muscles of the pre-trochal muscle grid become visible on the dorsal and ventral side (Fig. 2B).

These muscle systems grow subsequently. New myocytes of the rectus muscle are formed laterally on both sides, resulting in a bilaterally symmetrical, prominent muscular system. However, these newly formed fibrils strongly diverge towards the anterior pole of the larva with only the two earliest formed fibers marking a strict anterior-posterior axis through the animal by running parallel to each other along the middle of the larval body. In addition, ring muscles extend throughout the whole pre-trochal region and form a muscular meshwork around the fibers of the rectus muscle (Figs. 2C-D, 3A-B, 4A). This "apical grid" is engulfed laterally by a still weak, circular muscle that later becomes the ventral enrolling muscle. In the post-trochal body region, transversal muscle fibers are formed that are situated immediately underneath the epithelium of each putative shell plate, i.e., dorsal of the fibers of the rectus muscle (Figs. 2C-D, 3A-B, 4B).

As larval development proceeds, proportions of the larval body plan change, resulting in an elongated post-trochal area relative to the pre-trochal region at metamorphic competence (cf. Figs. 1A-C, 2, 3A-B). The anlagen of the putative first seven shell plates are already present in the late trochophore larva. In both species, *Mopalia muscosa* (Fig. 1B-C) and *Chiton olivaceus* (not shown), the anlage of the first plate (head valve) extends into the pre-trochal region.

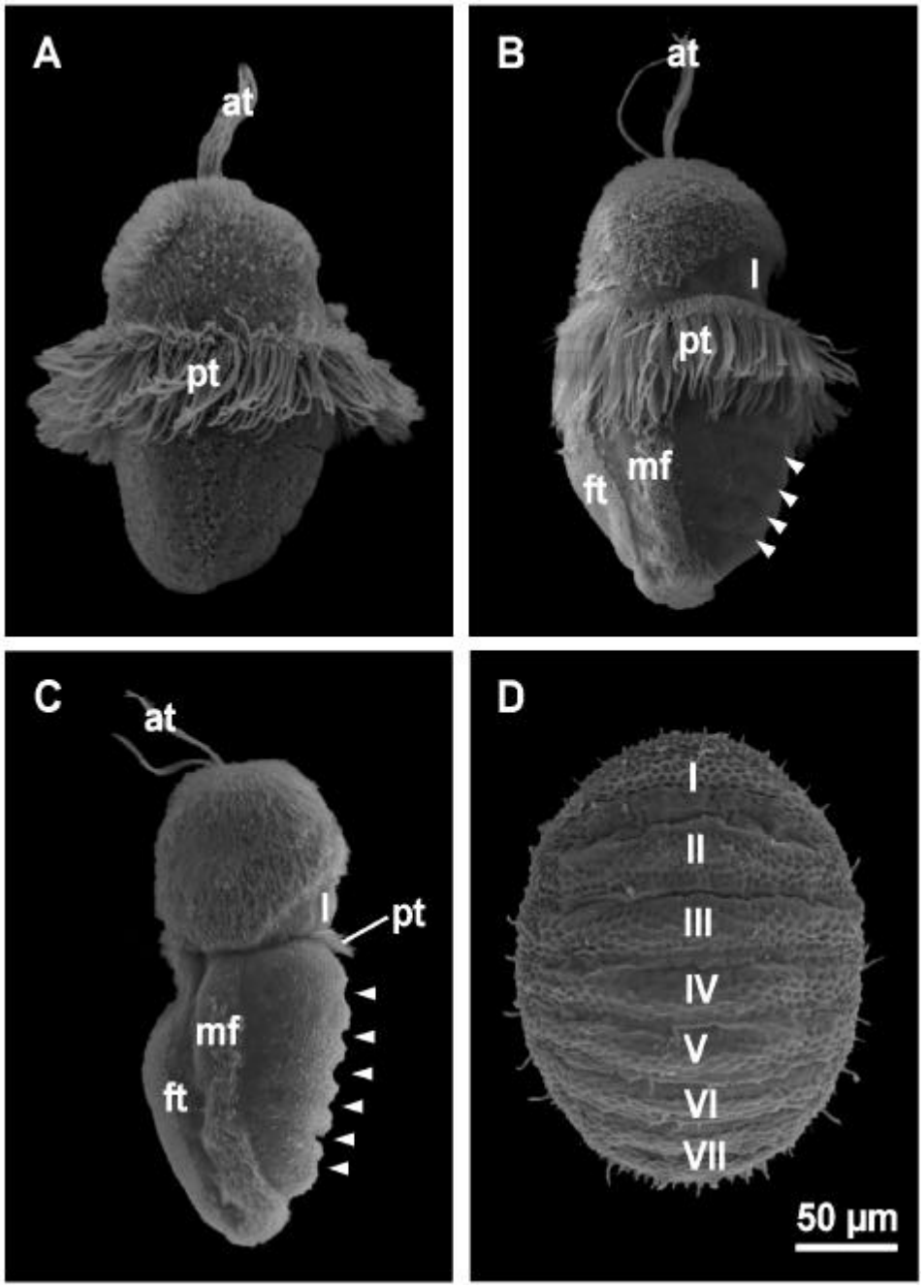

Fig. 1. SEM of the larval development of *Mopalia muscosa*. A. Early trochophore-like larva at the beginning of myogenesis with well defined prototroch (pt) and apical tuft (at), lateral view. Age: 74.25 hpf. B. Late trochophore, dorso-lateral view. Note the pre-trochally extending anlage of the first shell plate (I) and the post-trochal transversal dorsal depressions of the subsequent shell fields (arrowheads). The foot (ft) and mantle fold (mf) start to form. Age: 142 hpf. C. Late trochophore during metamorphosis, lateral view. Note the partially shed prototroch (pt). Age: 240 hpf. D. Early juvenile, approximately 2 days after metamorphosis with 7 well developed shell plates (I-VII), dorsal view.

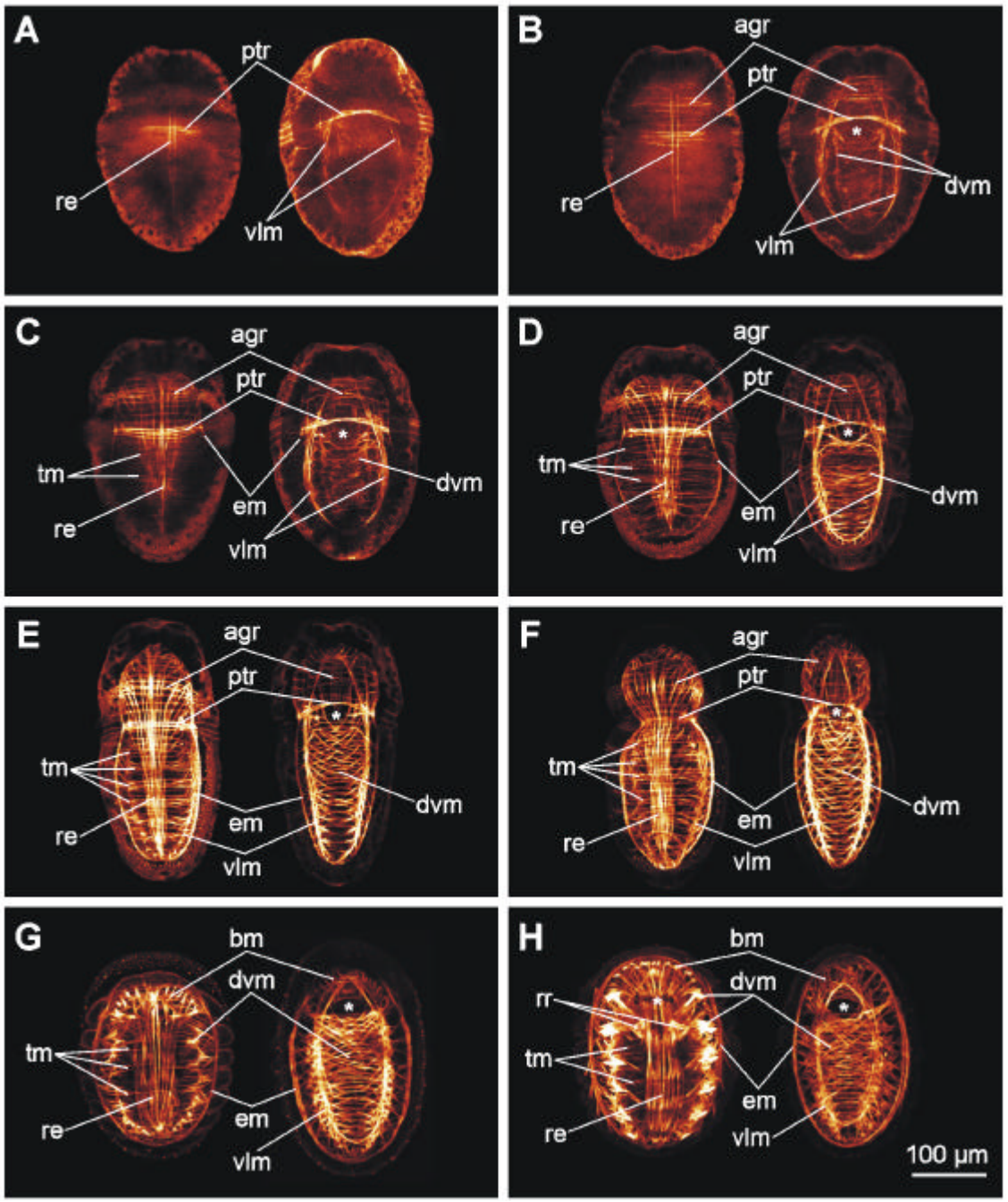

Fig. 2. Myogenesis in *Mopalia muscosa*, CLSM. Each pair of fluorescence images shows a dorsal (left) and a ventral (right) view of the respective developmental stage. Ages are given in hours post fertilization (hpf) or days post metamorphosis (dpm) at 10-12°C. Asterisks mark the mouth opening. A. Early trochophore stage, showing the first 2 fibers of the dorsal rectus muscle (re), fine myofibrils of the prototroch ring (ptr) and the paired ventro-lateral longitudinal muscle (vlm). Age: 74.25 hpf (left), 82.25 hpf (right). B. The fibers of the rectus muscle (re) and ventro-lateral longitudinal muscle (vlm) elongate and the first anlagen of the apical grid (agr) and the dorso-ventral (shell) musculature (dvm) are formed. Age: 86.25 hpf (left), 93 hpf (right). C. Further differentiation of all muscle systems; the enrolling muscle (em) and transversal myofibrils (tm) in the region of the putative shell plates start to form. Age: 108 hpf (left), 96 hpf (right). D-F. Subsequent development of all pre-metamorphically occurring muscle systems until metamorphic competence. Note the 3-dimensional apical grid (agr) in the pre-trochal area and the prominent ventro-lateral longitudinal muscles (vlm). Age: 129 hpf (D, left), 142 hpf (D, right), 161.15 hpf (E, left and right), 239.75 hpf (F, left and right). G-H. Post-metamorphic juvenile stages at 1 dpm (G) and 10 dpm (H). The buccal musculature (bm) forms soon after metamorphosis and attaches at the first shell plate. The rearrangement of the dorso-ventral shell muscles (dvm) into paired functional units has started in G (cf. their relative position to the weakly stained rims of the first 7 shell plates), but is fully achieved only in later stages (H). The radula retractors (rr) are the last muscles to be formed. Note the still prominent staining of the ventral longitudinal muscles (vlm).

At around 129 hpf, the various muscle systems have reached an intermediate stage of differentiation: the rectus muscle forms a predominant, dorsal, longitudinal unit and extends antero-laterally, while the apical grid surrounds the pre-trochal body part as a three-dimensional muscular net, consisting of distinct outer ring and inner diagonal muscle fibers. This network encircles the fibers of the rectus muscle, and some of them bifurcate at their anterior end. The prototroch ring is a solid band of muscle fibers located directly underneath the prototroch. In addition, a layer of post-trochal transversal myofibrils is found under each of the seven shell plates, which have already started to calcify. Laterally, the enrolling muscle encircles all other muscle systems, forming a border against the outer mantle. The ventro-lateral longitudinal muscle pair lies more ventral and medial to the latter muscle and consists of two distinct muscle strands that do not form anterior contact. This ventro-lateral longitudinal muscle interconnects on both sides with the dorso-ventral musculature via numerous short muscle fibers (Figs. 2D, 3A). The dorso-ventral musculature appears as a multiple repetition of minute myofibrils that intercross in the pedal region (Figs. 2D, 3A-B).

During subsequent larval (i.e., pre-metamorphic) development from approximately 145 hpf until metamorphic competence at around 210-215 hpf the only major changes regarding myogenesis are the growing number of myofibrils and the increasing thickness of the muscle bundles of the respective muscle systems (Fig. 3A-B). At metamorphic competence (Figs. 1C, 3B), all muscles show a bright fluorescent signal, indicating that no muscular atrophy has taken place so far (cf. Fig. 3A-B).

During metamorphosis, the larval prototroch muscle ring and the apical muscle grid degenerate (Fig. 3C-D). The buccal musculature arises immediately after metamorphosis and consists of numerous fibers that insert symmetrically on the first shell plate. The former distinct, delicate dorso-ventral muscle fibers start to concentrate (Fig. 3C), and ten days after metamorphosis, the paired shell muscle bundles are already differentiated under each shell plate. Additionally, the radular retractor muscles are formed. They insert on the second shell plate and are situated on both sides of the rectus muscle (Fig. 3D). The paired ventral longitudinal muscle persists in the juvenile animal, although it has not yet been described for any adult polyplacophoran species (see Discussion). The circular enrolling muscle is already functional in early juvenile animals (i.e., at one day after metamorphosis, see Fig. 3C), enabling the animal to protect its soft body parts on the ventral side if separated from the substratum.

The myofibrils of the dorsal rectus muscle undergo considerable rearrangement during larval life and especially at metamorphosis: their strong anterior divergence ceases (cf. Figs. 2C-D,

3A-B) and after metamorphosis all fibers follow the longitudinal anterior-posterior orientation of the first two myocytes, which are still situated on the medio-dorsal line of the animal (cf. Figs. 2A-B, 3C).

Ultrastructure of muscle systems

Nearly all larval and adult muscle systems in *Mopalia muscosa* and *Chiton olivaceus* are smooth (Fig. 4) except for the obliquely striated buccal musculature. Tendon cells, which form the shell attachment junctions of various gastropod shell muscles (see Page, 1995, 1998; Wanninger et al., 1999a) and contain a high density of F-actin fibers, were not found in the larvae of the two polyplacophoran species investigated. The outer ring muscles of the apical grid and the post-trochal transversal muscles under the shell plates lie dorsad of the rectus muscle (Fig. 4A-B).

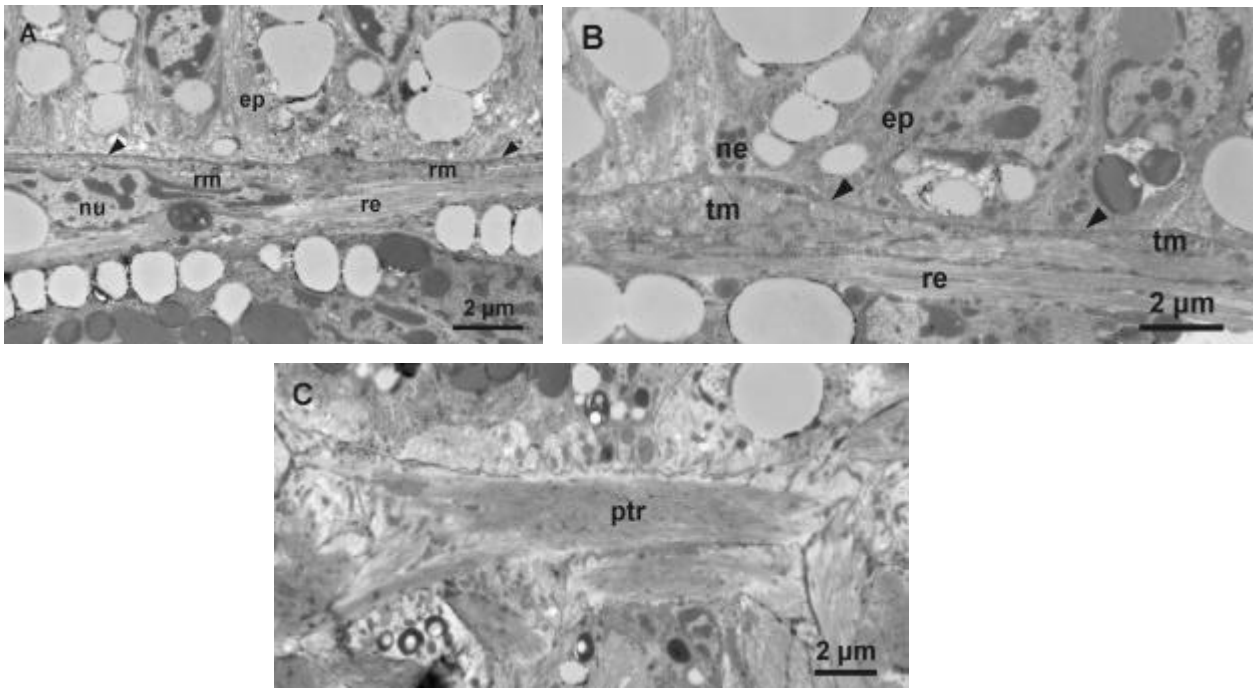

Fig. 3. Ultrastructure of several smooth muscle systems in larvae of *Mopalia muscosa*. Dorsal side faces upwards in A and B and to the right in C. A. Longitudinal section of the apical area of a late trochophore stage. The myocytes of the ring musculature (rm, with its adjacent nucleus (nu)) of the apical muscle grid lie directly underneath the basal membrane (arrowheads) of the dorsal epidermis (ep), thus engulfing the fibers of the rectus muscle (re). B. Longitudinal section of the post-trochal region of the same specimen as in A, with intraepithelial neural projection (ne). The transversal muscle fibers (tm), which underlie each shell plate, are ventrally bordered by the rectus muscle (re) while the basal membrane (arrowheads) of the dorsal epidermis (ep) lies on their dorsal side. C. Longitudinal section of the smooth larval prototroch muscle ring (ptr).

DISCUSSION

General notes on polyplacophoran larval development

As in many animal taxa with a biphasic life cycle, the transition from a free-swimming larval to a benthic juvenile stage involves dramatic changes of their gross morphology. In the Polyplacophora, the dorso-ventral axis flattens considerably and the post-metamorphic juvenile chiton becomes typically oval shaped. At the same time, the animal sheds its prototroch cells and parts of the pre-trochal area are lost (Fig. 1).

As shown on SEM micrographs of earlier studies on *Lepidochitona thomasi* (Eernisse, 1988: fig. 7C; Eernisse and Reynolds, 1994: fig. 5A) and confirmed by our observations on *Mopalia muscosa* and *Chiton olivaceus* (see above), the first shell plate extends pre-trochally, thus contradicting former statements on the sole post-trochal origin of all shell plates in the Polyplacophora (Kniprath, 1980; Eernisse and Reynolds, 1994). This raises doubts on the homology of the polyplacophoran shell plates and the conchiferan shell, since the latter is entirely of post-trochal origin and position (Kniprath, 1981) and because shell (plate) secretion is different in conchiferan and polyplacophoran larvae (Haas, 1981). Moreover, shell plate ontogeny in the Polyplacophora does not show a stage of shell field invagination as found in the Conchifera (Kniprath, 1981). The very gradual and, compared to gastropods and bivalves, slow establishment of the eventual juvenile body plan seems to be a general feature in polyplacophoran ontogeny. This is indicated by the fact that organs like gills, aesthetes, and the final shell plate are usually formed weeks after metamorphosis. On the other hand, several larval structures such as protonephridia and larval eyes are carried over into the post-metamorphic stage (Heath, 1904; Grave, 1932; Creese, 1986; Strathmann and Eernisse, 1987: p. 213).

Recent studies on the myogenesis in the Gastropoda (Page, 1995, 1997a,b, 1998; Degnan et al., 1997; Wanninger et al., 1999a,b) as well as earlier works on several bivalves (Meisenheimer, 1901; Smith, 1935; Crofts, 1937, 1955; Cole, 1938; Anderson, 1965; Smith, 1967; Cragg, 1985; Cragg and Crisp, 1991) and the data presented herein, allow a comparison of the various muscle systems and the mechanisms involved in molluscan myogenesis.

The prototroch muscle ring

As in the basal gastropod *Patella* (Wanninger et al., 1999a), both polyplacophoran species investigated show a smooth muscular ring (see Fig. 4C) that is situated directly underneath the ciliated prototroch cells and that is lost during metamorphosis. These positional, structural, and ontogenetic similarities in both groups suggest supraspecific homology of this

larval muscle system for the Gastropoda and the Polyplacophora. No similar structure has yet been described for either higher planctotrophic gastropod larvae with a much more complicated velum or any bivalve. Thus, it may be a molluscan plesiomorphy that is conserved only in some of the basal lecithotrophic molluscan larvae that possess a "simple" prototroch rather then a highly specialized and complicated velum.

Polyplacophoran vs. aplacophoran enrolling muscles

The data presented herein raise doubts on the homology of the enrolling muscles of chitons and aplacophoran molluscs as proposed by Salvini-Plawen (1972). The enrolling muscle in the Polyplacophora clearly represents a single circular muscle system, while it is longitudinally paired in adult Caudofoveata and Solenogastres (Salvini-Plawen, 1972). In addition, the enrolling muscle is a strengthened part of the longitudinal body-wall musculature in the aplacophoran taxa, but an independent system in chitons. However, data on the myogenesis in aplacophorans are necessary to finally solve this problem.

The fate and function of the paired ventral longitudinal muscle in *Mopalia muscosa* and *Chiton olivaceus*, which is retained in the juvenile animal (see Fig. 3D), remains enigmatic. Since it has not been found in any of the numerous detailed anatomical studies of adult chitons, it is very likely that this muscle disappears during subsequent development. Functionally, it may support the still relatively weak enrolling muscle, although its early ontogenetic appearance seems to contradict this hypothesis.

Larval and adult shell (plate) muscles

Larval velar and mantle retractor muscles that disappear through or shortly after metamorphosis are common throughout the Gastropoda (e.g., Smith, 1935; Smith, 1967; Fretter, 1972; Bonar and Hadfield, 1974; Page, 1995, 1997a, 1998; Degnan et al., 1997; Wanninger et al., 1999a,b) and are also found in several bivalves (Meisenheimer, 1901; Cragg, 1985; Cragg and Crisp, 1991). The absence of such larval shell muscles in the Polyplacophora indicates that they are probably not a part of the ancestral molluscan bauplan, although a secondary loss at the base of the polyplacophoran line cannot be completely ruled out. The restriction of larval retractor systems to those molluscan taxa that possess a protective shell in the early larval stages suggests co-evolution of larval retractors and a functional larval or heterochronically shifted adult shell. Thus, the presence of specific larval retractor system(s) neither seems to be characteristic for the entire Mollusca, nor for the Testaria (Polyplacophora + Conchifera), but may be so for the Conchifera. However,

preliminary data on the myogenesis in Scaphopoda (pers. obs.) makes independent evolution of larval retractors in gastropods and bivalves equally possible.

Compared to the conditions found in gastropods and bivalves (see Meisenheimer, 1901; Cragg, 1985; Cragg and Crisp, 1991; Page, 1995, 1997a,b, 1998; Wanninger et al., 1999a,b), the formation of the adult shell musculature in the Polyplacophora shows striking differences. In free-swimming larvae of several gastropod and bivalve taxa, the adult shell muscles arise after the functional establishment of the larval retractor systems. In these groups, the adult shell musculature is formed very fast (in basal gastropods after the completion of torsion) and, with the exception of steady growth, does not undergo major morphological rearrangement during its ontogeny. In *Mopalia* and *Chiton*, however, their generation and ultimate functional arrangement appears as a much more gradual process starting in the early trochophore-like larva with continuous elaboration until considerable time after metamorphosis (see Fig. 3).

The dorso-ventral musculature and the "segmentation problem"

The polyplacophoran dorso-ventral musculature, inserting on the shell plates in post-metamorphic animals, starts to form as numerous distinct, serially repeated muscle fibers along the whole post-trochal larval body. The adult morphological and functional arrangement in seven (and later eight) sets of paired shell muscles is clearly a secondary condition that starts after the completion of metamorphosis. The latter condition is thus not indicative for a proposed segmented bauplan in chitons as previously proposed (e.g., Götting, 1980; Lake, 1990). Instead, these findings argue in favor of recapitulation as proposed by Salvini-Plawen (1969, 1981), who regarded the shell (plate) musculature as having evolved from serially arranged dorso-ventral muscle fibers as found in adult Solenogastres. Accordingly, the testarian shell muscles evolved by subsequent concentration of such fibers, a condition which can still be traced ontogenetically in the recent Polyplacophora (see above).

Recently, gene expression pattern analyses of the homeobox gene *engrailed*, which is involved in arthropod segment formation, showed that this gene plays an important role in embryonic shell morphogenesis in gastropods (Moshel et al., 1998), bivalves (Jacobs et al., 2000), and scaphopods (Wanninger, pers. obs.) as well as in shell plate and spicule formation in polyplacophorans (Jacobs et al., 2000). Thus, the serial expression of *engrailed* in seven transversal stripes in the dorsal ectoderm of late chiton larvae reflects the function of "exoskeleton" formation of this gene in molluscs rather than proving their annelid-like "segmented" character.

Microanatomical and ontogenetic studies on the partly paedomorphic monoplacophoran *Micropilina arntzi* (Haszprunar and Schaefer, 1997) also suggest a non-segmented body plan for the Monoplacophora, mainly because the ontogenetic formation of several organ systems such as ctenidia and gonads occurs from posterior to anterior, not vice versa as in the Annelida. The fundamental differences regarding the coelomic conditions in the Annelida and the Mollusca support this hypothesis: the coelomic cavities in the Mollusca are restricted to two sacs, one around the heart (pericardial cavity) and one enclosing the gonad, while they appear as multiple paired sacs along the anterior-posterior axis of the Annelida, which defines true segmentation or eumetamerism. Comparative analyses of the ontogeny of these epithelially lined cavities even suggest their diphyletic origin between molluscs and the eucoelomate taxa, thus making the possibility of secondary loss of segmentation within the Mollusca very improbable (see Salvini-Plawen and Bartolomaeus, 1995). In addition, most authors nowadays (e.g., Salvini-Plawen and Steiner, 1996) consider the aplacophoran taxa (i.e., Solenogastres and Caudofoveata) as most basal clades of the Mollusca, and neither their adult body plan nor ontogenetic data on the Solenogastre *Neomenia carinata* (Thompson, 1960) show any trace of eumetamerism in these groups.

The ancestral condition: from worm to mollusc

The adult dorso-ventral musculature of the Mollusca, which intercrosses just dorsal of the foot sole, is phylogenetically distinct from that of all other phyla. Thus, the molluscan dorso-ventral musculature can be regarded as apomorphic for the phylum (e.g., Salvini-Plawen, 1980; Haszprunar, 1988; Haszprunar and Wanninger, 2000). Platyhelminthes, however, also express dorso-ventral muscles different from that of molluscs and distinct from the typical worm-like body-wall musculature (Tyler and Rieger, 1999). The body-wall musculature of worm-shaped groups like annelids, platyhelminths, or nemerteans mainly consists of three layers of ring, diagonal, and longitudinal muscles (e.g., Rieger et al., 1994; Reiter et al., 1996; Hooge and Tyler, 1999). The Solenogastres and Caudofoveata are the only major molluscan taxa which express in their adult body plan a three-layered body-wall musculature similar to the phyla mentioned above (Salvini-Plawen, 1972, 1981; Scheltema et al., 1994; Haszprunar and Wanninger, 2000). Our results suggest that the fibers of the apical muscle grid in the chiton larva may be vestiges of such body-wall muscles of a proposed worm-shaped molluscan ancestor. Due to the evolution of protective larval and adult shells in the Conchifera, the original body-wall muscles were completely reduced in this clade. Instead, the conchiferans elaborated the dorso-ventral musculature as the main adult shell muscle system

(note that gastropods and bivalves possess distinct larval retractors which are independent of the adult shell muscles; see above). Indeed, all gastropods with a planctonic veliger stage investigated so far show a larval shell and larval retractor systems early in development, but no "worm-like" body-wall muscles are present. In cases of shell reduction, however, a secondary "worm body" is found (e.g., nudibranchs, slugs, ship-worms). The Polyplacophora, which are phylogenetically situated at the interface of the primary worm-shaped aplacophorans and the Conchifera, lack a distinct larval shell and the adult shell plates are not protective before metamorphosis, but relics of such ancestral body-wall muscles (i.e., the apical grid) are present. However, in both chiton species investigated, *Mopalia muscosa* and *Chiton olivaceus,* longitudinal muscles were not observed in the apical grid. Thus, it seems that the longitudinal fibers are replaced by the diverging rectus muscle fibers in the chiton larva, which is indicated by the fact that after metamorphosis (i.e., after the loss of the apical grid) the rectus muscle appears as a solid median band of parallel longitudinal myocytes. However, the question whether this is a result of myofibrillar rearrangement and/or cell death of these fibers remains open. Accordingly, two evolutionary pathways appear equally possible: (1) assuming recapitulation of an ancestral body-wall musculature, the longitudinal fibers of the apical grid are completely lost in the Polyplacophora, or (2) the original longitudinal muscles are modified and contribute to (parts of) the rectus muscle. The latter assumption infers that at least parts of the rectus muscle are homologous to the longitudinal body-wall muscles of aplacophoran molluscs (see Salvini-Plawen, 1972). For further insights, ontogenetic data on the myogenesis of aplacophoran molluscs and the cytological mechanisms on the myofibrillar rearrangement of the rectus muscle fibers during chiton metamorphosis are crucial.

The transversal muscle fibers under the shell plates are most likely a polyplacophoran apomorphy which co-evolved with the shell plates. The strictly transversal character throughout their ontogenetic development makes a derivation from the body-wall ring muscles of a molluscan ancestor unlikely.

CONCLUSIONS

Myogenesis in the Polyplacophora involves several mechanisms in the transition from the larval planctonic to the juvenile benthic life style: (1) Degeneration of larval muscle systems (prototroch muscle ring, apical grid, and probably the paired ventral longitudinal muscle), (2) *de novo* generation of the buccal musculature including the paired radular retractors after

metamorphosis, and (3) gradual morphological rearrangement of the dorso-ventral shell musculature and the dorsal rectus muscle. The cytological mechanisms and epigenetic background of these muscular dynamics, however, remain unknown but are highly promising for future studies.

Our study supports the concept that that the "segmented" character of the adult polyplacophoran shell musculature is a secondary condition, contradicting previous attempts to derive the Polyplacophora (and the entire phylum Mollusca) from a primarily segmented stem species. The data currently available suggest their descent from an unsegmented, non-eucoelomate trochozoan ancestor (cf. Haszprunar, 1996).

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APPENDIX III

The role of *engrailed* in larval development and shell formation of the tusk-shell, *Antalis entalis* (Mollusca, Scaphopoda)

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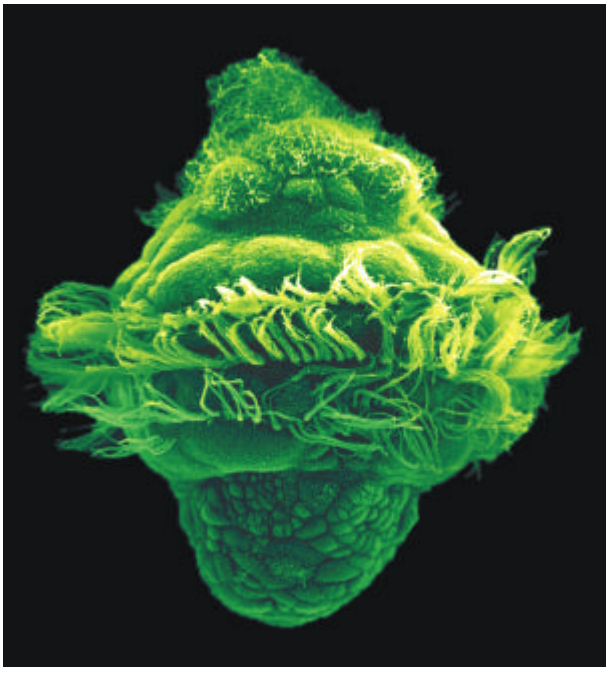

Abstract. This study presents the first detailed account of the larval and early post-metamorphic development of a scaphopod species, *Antalis entalis*, since 1883. Special reference is given to the role of *engrailed* in the formation of the embryonic (protoconch) and adult shell (teleoconch). We found that in the trochophore-like larva *engrailed* is expressed in shell secreting cells at the margin of the protoconch close to the mantle edge. During metamorphosis the growth of the protoconch and *engrailed* expression along its margin stop and the teleoconch starts to form. After metamorphosis *engrailed* is mainly expressed in cells of the putative central nervous system.

These data suggest a different genetic background regarding protoconch and teleoconch formation in the Scaphopoda and possibly all Conchifera, thus inferring a different evolutionary origin of both organs. The single anlage of the scaphopod protoconch contradicts earlier hypotheses of a monophyletic taxon Diasoma (Scaphopoda + Bivalvia) which has been mainly based on the assumption of a primarily bilobed shell in both taxa.

Comparative data on *engrailed* expression patterns suggest nervous system patterning as the basic function of *engrailed* in the Bilateria. However, there are several independent gain-of-function events, namely segment compartmentation in the Annelida and Arthropoda, protoconch, shell plate, and spicule formation in the Mollusca, skeletogenesis in the Echinodermata, and limb formation in vertebrates. These findings provide further evidence that homologous genes may act in very different pathways of bilaterian body plan formation in various animal phyla.

INTRODUCTION

Conchiferan Mollusca (i.e., Monoplacophora, Gastropoda, Cephalopoda, Bivalvia, Scaphopoda) undergo several ontogenetic stages of shell formation. In the trochophore- or veliger-like free-swimming or intracapsular larva, a so-called embryonic shell (protoconch I, also named prodissoconch I in bivalves) is secreted by an embryonic shell field (see Kniprath 1981). This protoconch ontogenetically precedes the adult shell (teleoconch), which starts to form after metamorphosis and usually keeps growing until the individual dies, thus showing typical growth lines which allow its distinction from the embryonic shell (e.g., Hadfield and Strathmann 1990, Wanninger et al. 1999a). Certain caenogastropods (formerly meso- and neogastropods) and bivalves show an additional shell stage, the larval shell (protoconch II or prodissoconch II in bivalves), which is often ornamented and occurs in the differentiated

veliger larva after the establishment of the protoconch I and prior to teleoconch morphogenesis (e.g., Bandel 1975, Haszprunar et al. 1995, Morse and Zardus 1997).

While there is little doubt about supraspecific homology of the conchiferan embryonic as well as adult shells, their direct ontogenetic homology (see Haszprunar 1992 for definition) remains questionable. Despite their structural differences, all 3 shell stages are formed by similar cells (or their progenitors) that at first invaginate (thus forming the "shell gland") and later evaginate (forming the "shell field"), which argues in favor of direct ontogenetic homology of all 3 shell stages (see Kniprath 1981 for extensive review). However, other authors (Thiriot-Quievreux 1972, Haszprunar et al. 1995) regarded the "shell field" (*sensu* Kniprath 1981; referred to as "shell gland" by Haszprunar et al. 1995), which secretes the embryonic shell, as morphologically distinct from the mantle margin that produces the larval and adult shell, thus rendering embryonic and adult shells as of independent evolutionary origin.

Recently, it has been shown that the homeobox gene *engrailed*, which is known to play an important role in neurogenesis and segment formation in annelids and arthropods (e.g., Morata and Lawrence 1975, Weisblat et al. 1980, Kornberg 1981, Fjöse et al. 1985, Weisblat et al. 1988, Patel et al. 1989, Wedeen and Weisblat 1991, Lans et al. 1993, Abzhanov and Kaufman 2000, Shain et al. 2000, Marie and Bacon 2000), is involved in the development of the embryonic shell in gastropods and bivalves as well as in shell plate and spicule formation in polyplacophorans (Moshel et al. 1998, Jacobs et al. 2000). To date, these remain the only molecular approaches to resolve the genetic background that underlies molluscan shell formation. Since the analyses on gastropods and bivalves exclusively focused on protoconch I morphogenesis, no comparative data on embryonic and adult molluscan shell development are available. In order to shed new light on the homology hypothesis of the embryonic and adult molluscan shell, we analyzed the gene expression pattern of the homeobox gene *engrailed* in larval and early juvenile stages of the scaphopod *Antalis entalis*.

The Diasoma concept proposed by Runnegar and Pojeta (1974, Pojeta and Runnegar 1985) comprises the Scaphopoda and the Bivalvia as direct sister taxa, mainly based on the assumption that both clades possess a primarily bivalved shell. While this unquestionably holds true for the Bivalvia, the only thorough studies on scaphopod ontogeny, dating from the 19th century, stated an unpaired origin of the scaphopod shell (Lacaze-Duthiers 1857, Kowalevsky 1883). In the light of recent cladistic analyses, which argue in favor of a close relationship of Scaphopoda and Gastropoda + Cephalopoda, rather than Scaphopoda +

Bivalvia (Waller 1998, Haszprunar 2000), the present study also contributes to the question of the sister-group-relationships of the Scaphopoda.

MATERIALS AND METHODS

Animals

Adult *Antalis entalis* (Jeffreys, 1869) were collected from about 30 m depth by SCUBA diving off the Atlantic coast near Roscoff, France during July 1999. The specimens were transported to the laboratory of the Station Biologique de Roscoff and were kept in running seawater on a layer of coarse shell gravel from the native habitat. To induce spawning, single individuals were placed in small petri dishes containing Millipore filtered seawater (MFSW) without substratum and treated with repeated temperature shocks by alternating incubations for 1-2 hours at 4°C and 25°C, respectively. Freshly released eggs were rinsed in MFSW, transferred to small glass dishes, and immediately inseminated. To avoid bacterial or fungal infections, 50 mg streptomycin sulfate and 60 mg penicillin G were added per liter MFSW to the cultures with free-swimming larvae. The water was changed daily (see Wanninger et al. 1999b). In culture dishes containing post-metamorphic animals, antibiotics were omitted and the water changes reduced to once every 2-3 days, to allow microbial growth as food resource for the juveniles.

Metamorphosis was very effectively induced by adding single pieces of shell gravel from the substratum of the adult habitat to the cultures. All cultures were kept within a temperature range of 17.5°C-19.5°C.

Scanning electron microscopy (SEM)

Specimens were fixed in intervals of 3-4 hours from hatching until metamorphic competence, as well as 3, 7, 41, 61 hours after metamorphosis (hpm) and 13 days after metamorphosis (dpm). From 24.5 hours post fertilization (hpf) onwards, larvae were anaesthetized by adding drops of 7% $MgCl_2$ prior to fixation. Late larval and post-metamorphic stages were immersed in 3.5% $MgCl_2$ for up to 15 minutes to assure full relaxation.

Fixation was carried out in 2 different ways. For better tissue preservation, a solution of 4 % glutaraldehyde in 0.2M sodium cacodylate buffer with 0.1M NaCl and 0.35M sucrose was used as primary fixative. After 3 washes of 15 minutes in 0.2M sodium cacodylate buffer with 0.1M NaCl and 0.35M sucrose, the specimens were postfixed with 1% OsO_4 in 0.2M sodium

cacodylate buffer with 0.3M NaCl for 2 hours, followed by 3 washes (15 minutes) in 0.2M sodium cacodylate buffer with 0.3M NaCl.

Alternatively, larval and post-metamorphic stages were fixed in 1% OsO_4 in distilled water to avoid decalcification of the embryonic and juvenile shells.

Subsequently, all samples fixed for SEM were dehydrated in an acetone series, critical point dried, sputter coated with gold, and observed with a Philips XL 20 SEM.

Staining of the *engrailed* protein

After relaxation, the specimens were fixed in 4% paraformaldehyde in 0.1M phosphate buffer solution (PBS) for 2-6 hours at room temperature or over night at 4°C. After 3 washes of 10-15 minutes in PBS, the animals were stored in PBS with 0.1% sodium azide (NaN_3) at 4°C. All further treatments were carried out at room temperature, except where stated otherwise. Specimens aged 42.5 hpf or older were decalcified in 2% EDTA for up to 1 hour. This was followed by 3 washes (15 minutes each) in PBS containing 0.4% Triton X-100 (PBT) and by incubation in the blocking agent (PBT with 5% normal goat serum [Jackson ImmunoResearch], and 0.2% bovine serum albumin [Sigma]; blocking-PBT) for 1 hour.

The *engrailed* protein was detected by using a monoclonal antibody raised against the 4D9 transcript (Mab4D9) as primary antibody, which was obtained from the Developmental Studies Hybridoma Bank, University of Iowa, USA. This antibody has been shown to selectively bind to the *engrailed* protein of chitons, bivalves, and gastropods (Moshel et al. 1998, Jacobs et al. 2000), and the existence of an *engrailed* homologue in scaphopods has been described earlier (Wray et al. 1995). The probes were incubated in a 1:5 dilution of the Mab4D9 in blocking-PBT over night (18-24 hours) at 4°C. After 3 washes (10-15 minutes) in PBT, the specimens were transferred into the undiluted secondary antibody solution (EnVision+ HRP goat anti-rabbit, DAKO Diagnostika) for 5 hours. 3 additional washes in PBT were followed by the staining reaction (5-20 minutes), which was done using a 3,3'-Diaminobenzide (Sigma Fast^{TM}, Sigma) solution which contained 0.07% nickel-ammoniumsulfate. After 3 washes in PBS and one change in distilled water, the specimens were dehydrated in a graded ethanol series, cleared in a 2:1 solution of benzyl benzoate and benzyl alcohol (BBA), and mounted on glass slides. Negative controls were carried out by omitting either incubation in the primary or the secondary antibody and yielded no signal.

For fluorescence counter-staining of cell nuclei, the staining reaction was followed by 3 washes in PBT, with 2 drops of 4',6-Diamidino-2-phenylindole (DAPI, Sigma) added (2-3 minutes). After dehydration and clearing, DAPI stained specimens were mounted in BBA

containing 0.1% n-propylgallate to reduce bleaching. The preparations were viewed under a Leica DM RBE microscope equipped with an epifluorescence unit and the pictures recorded using a digital imaging system (Kappa DX 30).

RESULTS

Terminology

Regarding shell and shell field/shell gland terminology, we use the definitions as mentioned in the introductory section (see also Kniprath 1981, Haszprunar et al. 1995, Morse and Zardus 1997, Wanninger et al. 1999a). Since the existence of a distinct larval shell (protoconch II) in *Antalis* can neither be confirmed nor completely be ruled out (see below), we use the neutral term "protoconch" for the pre-metamorphic shell in *Antalis*. In the figures of early larvae of *Antalis entalis*, with still ventrally open mantle and embryonic shell field, the region of the anlage of the embryonic shell is indicated by "α". The apical organ defines all pretrochal cells bearing cilia that contribute to the apical ciliary tuft.

Larval development and formation of the embryonic shell (protoconch)

The embryos of *Antalis entalis* start hatching at around 17-19 hours post fertilization (hpf) and about half an hour later the first free-swimming larvae are observed in the water column. However, the prototroch cilia are not yet fully developed and the prototroch cells are not arranged in the typical 3 parallel rows as found in slightly older individuals (cf. Fig. 1A, B). The cells of the apical organ are already distinct and bear numerous long, fine cilia, which form the apical ciliary tuft. The post-trochal area is hardly differentiated and consists of a small cluster of cells. At 32 hpf the prototroch is fully established and the pre-trochal area remains predominant relative to the still poorly differentiated post-trochal region (Fig. 1B). From approximately 35 hpf onwards, dramatic morphological transformations occur until metamorphic competence, which is achieved at 94 hpf (Fig. 1C-I). The size of the prototroch cells ceases and, from around 54 hpf onwards, the prototroch is recognized as a narrow band of ciliated cells which encircles the anterior region of the animal (Fig. 1F-I). The reduction of the larval apical organ starts at around 35 hpf and is indicated by its atrophying ciliated cells (Fig. 1C, E, F, G). Considerable time before metamorphic competence, the apical organ, including its neural components (Wanninger, pers. obs.), is completely lost. This feature will be dealt with in greater detail elsewhere.

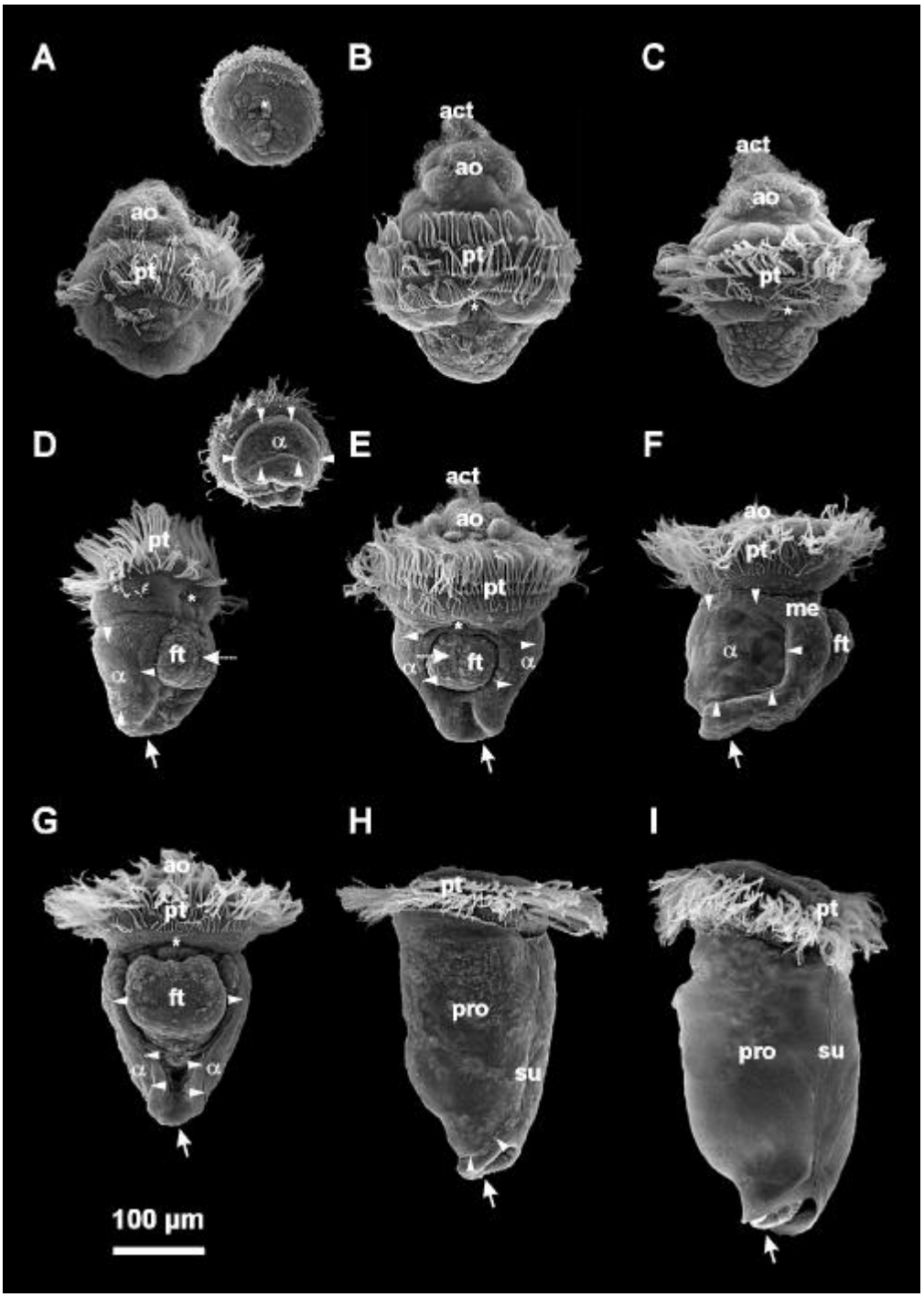

Fig. 1. Larval development of *Antalis entalis*, SEM, anterior faces upwards. (A) Early trochophore immediately after hatching, with prominent apical organ (ao) and not yet fully established prototroch (pt); dorsal view. Inset: posterior view showing blastopore (asterisk). Age: 20.5 hpf. (B) Trochophore with symmetrical prototroch (pt) and large apical organ (ao) with apical ciliary tuft (act). Asterisk marks the mouth opening; ventral view. Age: 32 hpf. (C) Larva with differentiating post-trochal region. The prototroch (pt) and apical organ (ao) start to decline; ventral view. Age: 36.75 hpf. (D) Larva with bilobed foot (ft) anlage with distinct midline (stippled arrow) and embryonic shell field (α). Arrowheads point to the margin of the embryonic shell field, arrow marks the future posterior mantle opening ["pavillon", see Lacaze-Duthiers (1857), Steiner (1991)]; ventro-lateral right view. Inset: Postero-dorsal view. Age: 48.5 hpf. (E and F) Larvae with growing embryonic shell field (α), mantle epithelium (me) and decreasing apical organ (ao); ventral (E) and lateral right view (F). Age: 54 hpf. (G) Slightly older specimen; ventral view. Age: 62 hpf. (H and I) Larva prior to (H) and at metamorphic competence (I). Note the ventrally closed mantle and protoconch (pro) with distinct ventral fusion line (suture; su), as well as the completely reduced apical organ. Both ventro-lateral right view. Age: 74.5 hpf (H) and 95 hpf (I).

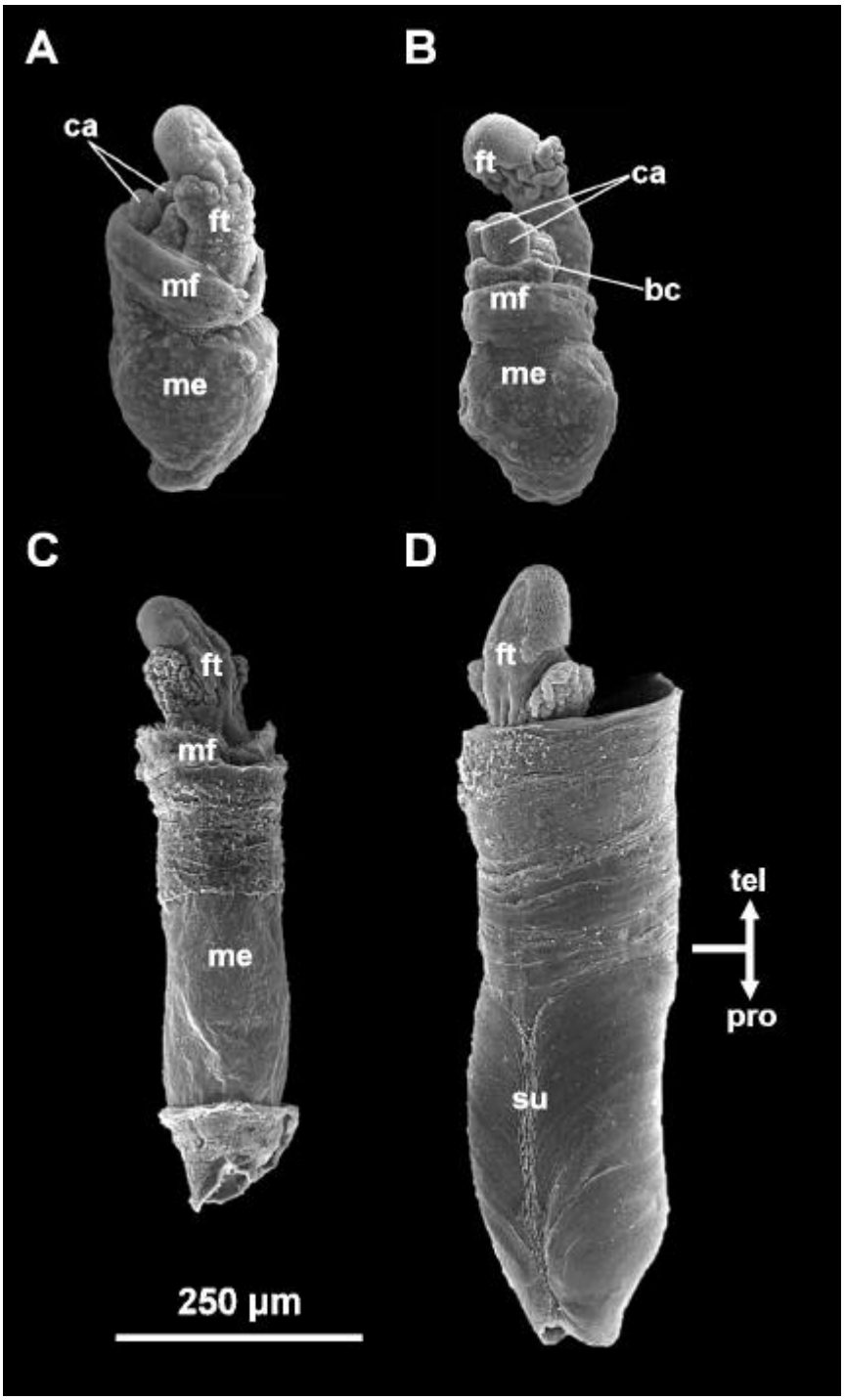

Fig. 2. Post-metamorphic juveniles of *Antalis entalis*, SEM, anterior faces upwards. (A) Early juvenile at 7 hours post metamorphosis (hpm), showing the paired anlage of the captacula (ca), and the foot (ft) with apical ciliation. Decalcified specimen, thus the anterior mantle fold (mf) and the mantle epithelium (me) are visible. Lateral right view. (B) 61 hpm old individual, decalcified, dorso-lateral right view with buccal cone (bc) bearing the paired anlage of the captacula (ca). (C and D) Juveniles at 13 dpm. Note the 3-lobed foot (ft). (C) Decalcified, lateral right view. (D) Specimen showing the distinct protoconch (pro) with suture (su), and the teleoconch (tel) bearing numerous growth lines. Ventro-lateral left view.

In contrast, the post-trochal area starts to expand posteriorly, and at 39 hpf the calcified, single primordium of the embryonic shell is visible under polarized light (not shown). Likewise, the anlagen of the foot, the mantle, and the embryonic shell field become visible by SEM (Fig. 1D). The formation of both, the mantle epithelium and the embryonic shell field, starts dorsally and both structures grow in anterior and ventral direction. At 64 hpf the first specimens with ventrally closed mantle and embryonic shell are found. The successive calcification of the protoconch in dorso-ventral direction and its anterior growth until metamorphic competence is indicated by the suture, the ventral fusion line of the embryonic shell (Fig. 1H, I). The surface of the protoconch is completely smooth, without any distinct growth lines.

The foot anlage appears as a symmetrical hump on the ventral side, consisting of 2 equally sized halves which are separated by a distinct midline (Fig. 1D, E). The foot increases its length but remains non-functional until metamorphic competence. In late larvae, it is completely buried in the mantle cavity which is formed by the ventrally closed mantle epithelium (Fig. 1H, I).

Metamorphosis and early post-metamorphic development

Larvae induced at 94 hpf or later performed metamorphosis within 2 hours at a rate of almost 100% in all cultures. The major morphological changes from the larval to the early juvenile body plan were completed within these 2 hours from induction.

During metamorphosis, the prototroch is lost and the paired anlage of the first captacula (anterior tentacles of the adult for the capture of prey) is formed dorso-laterally in the cephalic region of the juvenile (Fig. 2A, B). The foot differentiates and develops its characteristic three-lobed morphology, which is retained through adulthood. Accordingly, the animal switches from a planctic free-swimming to a benthic creeping-burrowing locomotion. The anterior tip of the predominant central lobe of the foot is densely ciliated and forms the foot sole, while its lateral sides lack ciliation (Fig. 2A-D). The formation of the protoconch stops at the onset of metamorphosis and the adult shell (teleoconch) is generated. In contrast to the embryonic shell, the teleoconch is gradually secreted by marginal cells of the anterior mantle epithelium, leaving striking growth lines in posterior-anterior direction. Due to this different mode of shell formation, the adult shell lacks a suture. These differences enable easy distinction of the both shell types in the post-metamorphic juvenile scaphopod (Fig. 2D).

Engrailed expression during larval and early juvenile life

The *engrailed* transcript is first localized in early trochophores aged 28.5 hpf. Morphologically, no distinct shell field is recognized by that time. *Engrailed* is expressed in 2 unequally sized clusters of cells of the dorsal ectoderm. The much larger anterior cluster consists of approximately 15 cells which are arranged in a semi-circle just behind the prototroch around the putative anlage of the embryonic shell field. The second *engrailed* positive cluster is formed by a mere 3 cells, situated close to the posterior pole of the larva (Fig. 3A). Slightly later, *engrailed* is found in cells surrounding the anlage of the embryonic shell (protoconch), which has now started to differenciate between the 2 former *engrailed* clusters (Fig. 3B: α). During subsequent development, the embryonic shell field expands ventro-laterally with *engrailed* expressing cells forming the margin towards the outer mantle epithelium (Figs. 3C-E, 4). The ventral view of young trochophores shows *engrailed* positive cells at the left and right lateral margins of the not yet ventrally closed embryonic shell field, flanking the centrally positioned anlage of the foot (Figs. 3E, 4B). Figure 4 combines the findings of the *engrailed* expression pattern and larval morphogenesis in trochophorae prior to the ventral closure of the mantle and the embryonic shell field. Both, the gross morphology of the shell field and the pattern of *engrailed* expression, clearly reflect the primary univalved character of the protoconch (Fig. 4C). After ventral closure of the mantle and the embryonic shell field, the protoconch is established and the *engrailed* expressing cells are found around both the anterior and the posterior edges of the shell field (Fig. 5A, B, E).

In 80.5 hpf old specimens the *engrailed* protein is also found in a few single cells of the body mass (Fig. 5C, D, arrows). Although we were not able to clearly identify these cells *in situ*, preliminary data on the neurogenesis in *Antalis entalis* suggest that they are part of the developing adult nervous system. At metamorphic competence the morphogenesis of the protoconch is complete and the shell stops growing. Likewise, *engrailed* expression disappears at the margins of the shell field. In contrast, expression in the cells of the larval body increases and is now found in several body regions, namely in the anterior region of the putative cephalic ganglia which form the adult central nervous system (Fig. 6C, stippled arrow), in 2 centers of the mid-body (Fig. 6A, C, arrows), the foot (Fig. 6B, C), and in a cell cluster of the visceral mass, which probably forms the anlage of the adult visceral ganglion (Fig. 6D, white arrow).

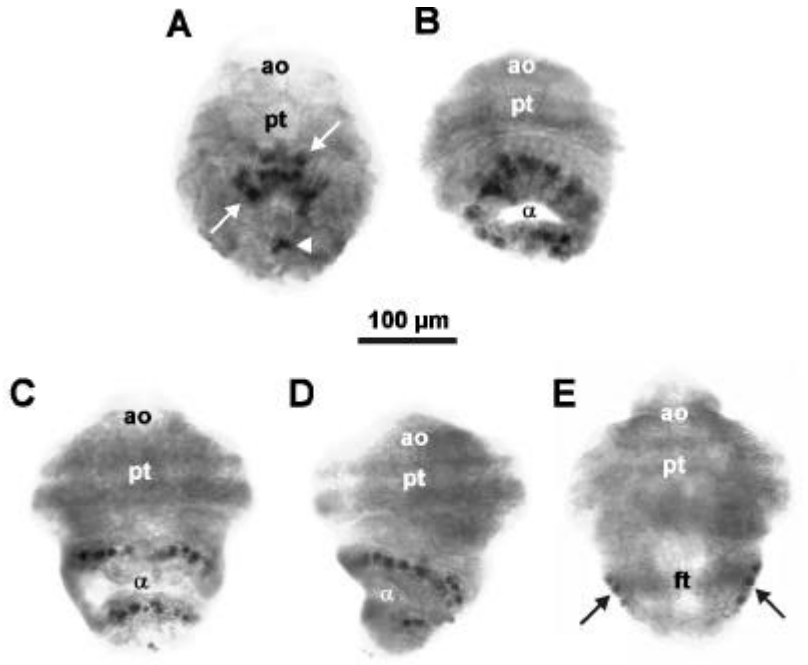

Fig. 3. *Engrailed* expression in early trochophorae, anterior faces upwards. (A) Dorsal view of a larva with first detectable signal at 28.5 hpf. Note both, the large cell cluster (arrows), situated just behind the apical region with apical organ (ao) and prototroch (pt), and the smaller posterior cluster (arrowhead). (B) Dorsal view at 32 hpf with expression around the early anlage of the embryonic shell field (α). (C-E) Expression pattern in 39 hpf old larvae. (C) Dorsal view. *En* positive cells are localized at the anterior and posterior border of the embryonic shell field (α). (D) Lateral right view. (E) Ventral view showing 3 *en* expressing cells on each side of the ventral margin of the embryonic shell field (arrows) which flanks the anlage of the foot (ft).

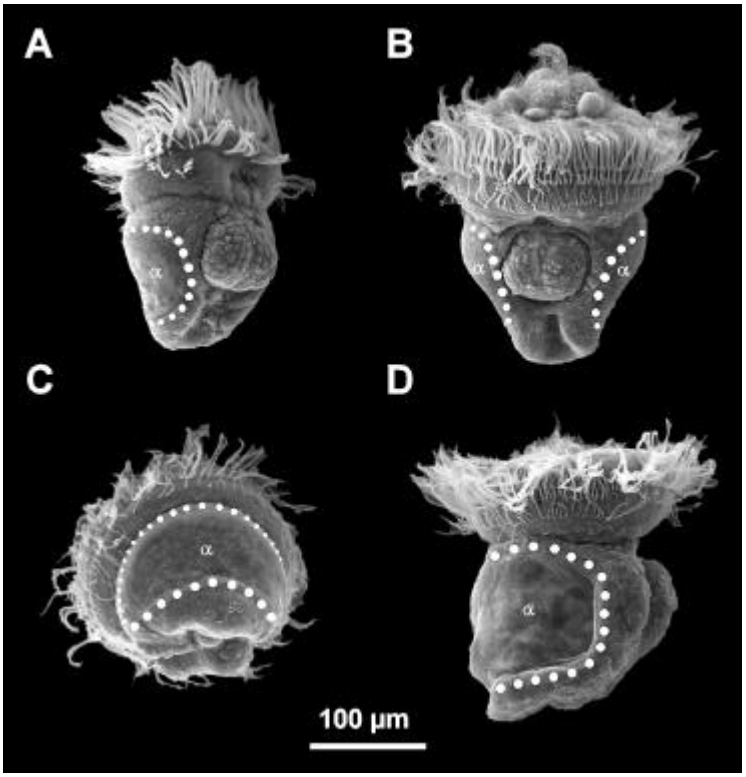

Fig. 4. Patterning of *engrailed* expression (white dots) plotted on SEM's of 48.5 hpf (A and C) and 54 hpf (B and D) old larvae with ventrally open anlage of the embryonic shell (α). The localization of *en* positive cells is exclusively found at the interface of the embryonic shell field (α) and the adjacent mantle epithelium. See also Figs. 1D-F, 3C-E. (A) Ventro-lateral right view, (B) ventral view, (C) postero-dorsal view, (D) lateral right view.

At 61 hours after metamorphosis, *engrailed* expression is restricted to few cells that contribute to the adult cerebral ganglion (Fig. 7, arrow). All other regions, where the transcript was formerly found, remain without signal. *Engrailed* is not expressed in cells that are involved in teleoconch (adult shell) formation (cf. Figs. 2D, 7). We did not find any *engrailed* expressing cells in individuals aged 13 days post metamorphosis.

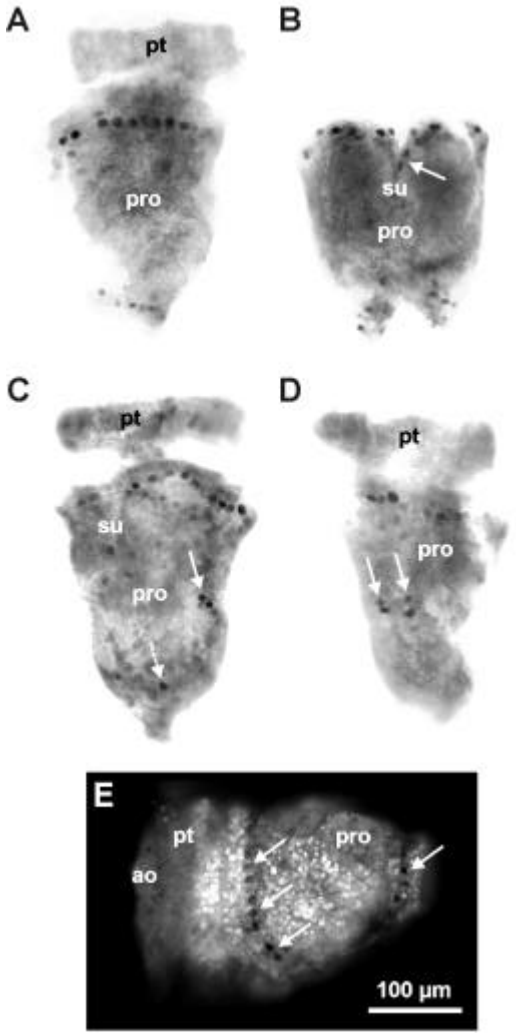

Fig. 5. *Engrailed* expression in larvae with fully developed protoconch (pro) at 72.5 hpf (A, B, E) and 80.5 hpf (C and D). Anterior faces upwards in A-D and to the left in E. (A) Lateral left view showing the signal in cells at the anterior and posterior margin of the protoconch (pro; pt – prototroch). (B) Ventral view of a retracted specimen, indicating that *en* is also found at the edges of the suture (su) (arrow). (C and D) Ventro-lateral left view (C) and lateral right view (D) with the first signal in cells located in the central (C and D, arrows) and visceral (C, stippled arrow) body region. (E) Specimen counter-stained with DAPI to illustrate the relative low number of *en* positive cells (dark spots, arrows) compared to fluorescent nuclei (bright spots) of non-expressing cells; lateral left view.

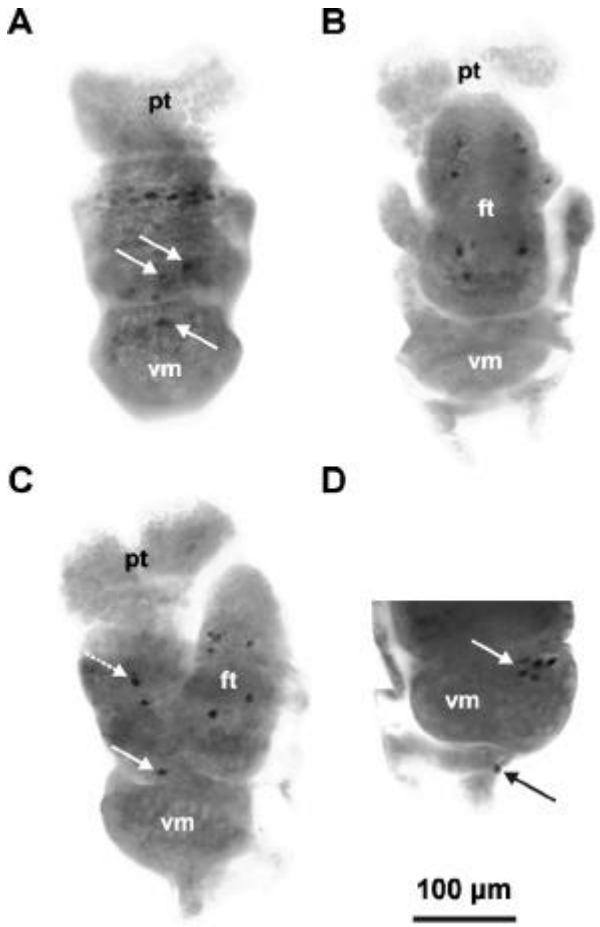

Fig. 6. Localization of *engrailed* in metamorphic competent larvae (age: 100 hpf), anterior faces upwards. Scale bar refers to A-C and equals 200 µm in D. (A) Dorsal view showing few *en* positive cells at the anterior edge of the protoconch, as well as in the visceral mass (vm) and the central body region (arrows; pt - prototroch). (B) Ventral view of a specimen with *en* expressing cells in the foot (ft). (C) Lateral right view revealing expression in the foot (ft), in the anlage of the adult cephalic ganglion (stippled arrow), and in the visceral mass (vm) (arrow). (D) Lateral left view showing a close-up of the visceral body region (vm) with *en* positive cell cluster (white arrow) and a single remaining *en* expressing cell at the posterior margin of the protoconch (black arrow).

Fig. 7. Post-metamorphic individual at 61 hpm, lateral left view, anterior faces upwards. *En* expression is restricted to a few number of cells situated in the region of the adult cephalic ganglion (arrow). No expression in either the foot (ft) or the visceral mass (vm). ca – anlage of the adult captacula.

DISCUSSION

General scaphopod development

The prototroch of *Antalis entalis* consists of 3 rows of large ciliated cells, which arrange some time after hatching and continuously move towards each other, but are retained until the onset of metamorphosis (Fig. 1). This is in accordance with Kowalevsky (1883) and Cather and Verdonk (1979), but differs from earlier results by Lacaze-Duthiers (1857: pl. 7), who observed 6 initial rows of ciliated cells which are subsequently reduced to a single row in late larvae. Compared to larvae of other molluscan clades (see, e.g., Nielsen 1995), *Antalis* shows a unique type of molluscan prototroch, thus reflecting the high phenotypic plasticity of molluscan "trochophores". The foot develops from a primarily bi-lobed hump, similar to the early pedal anlage of gastropods (see, e.g., Patten 1886, Arnolds et al. 1983, Damen and Dictus 1996: fig. 1E, Wanninger et al. 2000). Its 3-lobed functional appearance is obtained before or slightly after metamorphosis (cf. Fig. 2 herein and Lacaze-Duthiers 1857: pl. 7, fig. 8), with the ciliated foot sole being restricted to the anterior-most tip of the central foot lobe (Fig. 2). Contrary to the gastropod cephalic tentacles, the scaphopod cephalic captacula are of entire post-metamorphic origin (cf. Fig. 2 herein and Lacaze-Duthiers 1857: p. 236 and pl. 8, fig. 2).

Shell formation

It has been shown earlier for nearly all major conchiferan groups, that embryonic shell formation starts with an initial stage of shell field invagination (see Kniprath 1981). Functionally, this has been explained by the fact that only the cells that are situated at the edge of the embryonic shell field are able to secrete the organic matrix and the periostracum, which need to be formed prior to calcification. By invagination of the shell field, these cells are brought closely together to avoid the formation of a hole during the earliest phase of calcification (Ziegler 1885, Kniprath 1977, 1979, 1981). After evagination, the shell forming cells migrate laterally and eventually become situated at the mantle edge (Kniprath 1981, Waller 1981, Moore 1983). As in gastropods and bivalves (Hadfield and Strathmann 1990, Morse and Zardus 1997, Wanninger et al. 1999b), the surface of the scaphopod protoconch is relatively smooth compared to the teleoconch which shows distinct growth lines (Fig. 2D; see also Engeser et al. 1993 for similar findings in fossilized Scaphopoda). In contrast to lecithotrophic Gastropoda and Bivalvia, we found that the scaphopod protoconch undergoes

significant growth until metamorphic competence, which is thus not formed "at once" as in the former clades (e.g., Haszprunar et al. 1995).

We found *engrailed* being expressed in the cells of the shell field and, later, in the cells of the mantle margin which contribute to protoconch formation. During its entire morphogenesis, the shell remains univalved in *Antalis entalis*. These data not only coincide with the original data on scaphopod shell formation (Lacaze-Duthiers 1857, Kowalevsky 1883), but also with the findings of Moshel et al. (1998) on *engrailed* expression in the gastropod *Ilyanassa obsoleta*. In contrast, after invagination of the single shell field, bivalve embryonic shell (prodissoconch I) formation starts with 2 centers of calcification, which are separated by an uncalcified ridge which later becomes the hinge (Kniprath 1981, Waller 1981). Gene expression pattern analyses revealed *engrailed* positive cells in both calcification centers and in the putative shell hinge area (Jacobs et al. 2000). These results strongly argue in favor of a primary univalved (embryonic) shell as plesiomorphic for the Conchifera, while a secondary bivalved shell is regarded as apomorphy for the Bivalvia. Thus, and because of the results of recent cladistic analyses (Waller 1998, Haszprunar 2000), we propose to abandon the Diasoma concept. The lack of *engrailed* expression in cells that form the teleoconch indicates different genetic mechanisms being involved in embryonic and adult shell morphogenesis, which should thus not be regarded (ontogenetic) homologous.

Comparative expression patterns of *engrailed*

As in annelids (e.g., Shain et al. 2000), arthropods (e.g., Abzhanov and Kaufman 2000), echinoderms (Lowe and Wray 1997), and chordates (e.g., Patel et al. 1989, Holland et al. 1997, Hanks et al. 1998), the homeobox gene *engrailed* appears to play a role in the neurogenesis of the adult central nervous system in the Scaphopoda. In annelids and arthropods, *engrailed* and its homologues also serve as "compartment" genes by being involved in segment formation (Kornberg 1981, Lans et al. 1993, De Robertis 1997, Dahmann and Basler 1999, Abzhanov and Kaufman 2000, Marie and Bacon 2000).

Aside from the present study, the only expression data on *engrailed* in the Mollusca are provided by Moshel et al. (1998) and Jacobs et al. (2000). Wray et al. (1995) showed that *engrailed* homologues are also present in the Cephalopoda. In this taxon, however, *engrailed* was only found in the basal, external shell bearing *Nautilus*, while *Loligo*, with reduced, non-mineralized internal shell (gladius), lacks an *engrailed* homologue altogether. These findings, in combination with our data, allow the assumption that *engrailed* was part of a set of genes present in the ancestral testarian (Conchifera + Polyplacophora; cf. Haszprunar 2000)

bauplan, required to form shell (plates?). Furthermore, the results presented herein indicate that *engrailed* only contributes to the formation of the conchiferan protoconch but not to the adult shell. However, future detailed investigations of basal bivalve, polyplacophoran, and especially aplacophoran taxa should significantly enhance phylogenetic insights regarding shell morphogenesis and shell homologies in the Mollusca.

Gene expression pattern analyses in deuterostomes revealed additional functional innovations of *engrailed*. In echinoderms, *engrailed* is also involved in the formation of the calcitic, ectodermal endoskeleton (Lowe and Wray 1997). Moreover, in the sea urchin *Tripneustes gratilla*, the *engrailed* transcript has also been found in the coelomocyte as well as in the ovary and testis of adult individuals, but not in early embryonic or larval stages, suggesting regulative functions of *engrailed* during gametogenesis (Dolecki and Humphreys 1988). Finally, one of the 2 vertebrate *engrailed* homologues (*en-1*) appears to play an important role in limb development (Loomis et al. 1996, Logan et al. 1997, Hanks et al. 1998).

In summary, the available data suggest that the basic function of *engrailed* in the bilaterian ancestor was to regulate (adult) neurogenesis, a feature that has been conserved throughout the Bilateria (cf. Lowe and Wray 1997). However, *engrailed* shows several independent functional innovations, which at least include segment compartmentation in annelids and arthropods, biomineralization in molluscs and echinoderms, limb development in vertebrates, and maybe gametogenesis in sea urchins. Thus, *engrailed* may serve as an example which demonstrates the evolvability and plasticity of gene functions during evolution. Accordingly, the co-expression of orthologous genes in organs of species of different phyla alone can not solve the question whether or not these organs are indeed homologous (see Abouheif et al. 1997).

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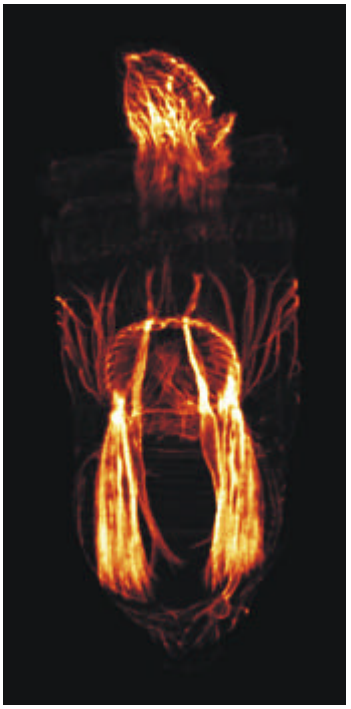
APPENDIX IV

Muscle development in *Antalis entalis* (Mollusca, Scaphopoda) and its significance for scaphopod relationships

***Journal of Morphology*: submitted.**



Abstract. We applied fluorescence staining of F-actin, confocal laser scanning microscopy, as well as light microscopy, SEM, and TEM to examine myogenesis in larval and early juvenile stages of the tusk-shell, *Antalis entalis*. Myogenesis follows a strict bilateral symmetrical pattern without distinct larval muscle systems. The paired cephalic and foot retractors appear synchronously in the early trochophore-like larva. In late larvae, both retractors form additional fibers which project into the anterior region, thus enabling retraction of the larval prototroch. These fibers, together with the prototroch, disappear during metamorphosis. The anlagen of the putative foot musculature, mantle retractors, and buccal musculature are formed in late larval stages. The cephalic captacula and their musculature are of post-metamorphic origin. Development of the foot musculature is dramatically pronounced after metamorphosis, which results in a dense muscular grid consisting of outer ring, intermediate diagonal, and inner longitudinal fibers. This is in accordance with the proposed function of the foot as a burrowing organ based on muscle-antagonistic activity. The existence of a distinct pair of cephalic retractors, which is also found in basal gastropods and cephalopods, as well as new data on scaphopod shell morphogenesis and recent cladistic analyses, indicate that the Scaphopoda may be closer related to the Gastropoda and Cephalopoda rather than to the Bivalvia.

INTRODUCTION

At the beginning of the 21st century, the traditional proposal of a direct bivalve-scaphopod relationship still remains controversal (Waller, 1998; Haszprunar, 2000). Despite the lack of clear syn-apomorphies, the Scaphopoda have traditionally been comprised with the Bivalvia, especially since Runnegar and Pojeta (1974) proposed the Diasoma concept. This hypothesis was based on the assumption of a primarily bi-lobed shell in both groups. Recently, Wanninger and Haszprunar (2001a) rejected such a taxon by revealing an entire univalved character of the scaphopod shell during development of the tusk shell, *Antalis*. So, if the "Diasoma" fail, where in the molluscan tree and according to which syn-apomorphies should the Scaphopoda be placed?

With molecular methodologies rapidly improving during the last 15 years, questions regarding animal phylogeny were tackled on a comparative genetic level. While the first molecular trees of the animal kingdom yielded results that were mostly in complete discordance with the morphological ones, improvement of both, molecular techniques and data processing (e.g., computer analysis software), led to fascinating alternatives to the

current believes of animal relationships (e. g., Field et al., 1988; Yang, 1996; Aguinaldo et al., 1997; Ruiz-Trillo et al., 1999). In the course of this phylogenetic renaissance, ontogenetic data were increasingly considered useful for phylogenetic analyses. This created the currently booming field of evolutionary developmental biology (evo-devo), which revived the Haeckelian tradition of the 19th century. While, in the dawn of evo-devo, data generation mainly concentrated on revealing gene homologies, comparative gene expression pattern analysis is the current state of the art. Focusing on these sub-cellular criteria, however, data regarding morphological aspects of developmental biology are still innumerous, although these have produced highly relevant insights in the mechanisms of animal evolution and phylogeny (Eernisse et al., 1992; Nielsen, 1995; Salvini-Plawen and Bartolomaeus, 1995; Ponder and Lindberg, 1997; Haszprunar and Wanninger, 2000; Wanninger et al., 2000; Wanninger and Haszprunar, 2001a, b). In the framework of an extensive comparative study on muscle development in the Mollusca, we applied fluorescence labeling of F-actin combined with confocal laser scanning microscopy and computer image analysis, as well as SEM and TEM, in order to elucidate the developmental mechanisms which underlie molluscan myogenesis. New data sets on muscle morphogenesis may answer developmental, phylogenetic, and eco-functional questions in the Mollusca and their possible outgroups.

In this paper, we present the first data on muscle development in a scaphopod, *Antalis entalis* (Jeffreys, 1869), and their implications for phylogenetic considerations within the Mollusca. Special reference is given to the scaphopod-bivalve question, the sister-group relationship of which has recently been questioned (Waller, 1998; Haszprunar, 2000; Wanninger and Haszprunar, 2001a). In addition, a comprehensive review of the current state of knowledge regarding molluscan myogenesis is provided.

MATERIALS AND METHODS

Animals

Adult *Antalis entalis* (Jeffreys, 1869) were collected from 30m depth along the Atlantic coast near Roscoff, France during July 1999. Single individuals were placed in petri dishes and spawning was induced by alternating incubations at 4°C and 25°C. Embryos, larvae, and juveniles were cultured at 17.5°C-19.5°C in Millipore filtered seawater (MFSW) with 50 mg streptomycin sulfate and 60 mg penicillin G added per litre to avoid bacterial or fungal infections. Metamorphosis was induced by addition of pieces of shell gravel from

the substratum of the adult habitat to the cultures. For further details see Wanninger and Haszprunar (2001a).

F-actin labeling and confocal laser scanning microscopy (CLSM)

A detailed description of the protocol for F-actin staining is given in Wanninger et al. (1999a). In brief, animals were relaxed by addition of 7% $MgCl_2$ to the MFSW and fixed for 4 hours at room temperature in 4% paraformaldehyde in 0.1M phosphate buffer (PBS), followed by 3 washes in the same buffer. For F-actin labeling, the specimens were incubated in PBS with 0.2% Triton X-100 (PBS-T) for 1 hour and stained in a 1:40 dilution of Oregon Green 488 phalloidin (Molecular Probes) in PBS-T (1 hour). After 3 washes in PBS whole-mount preparations were obtained by mounting the stained specimens in Vecta Shield (Vector) antifade mounting medium on glass slides. For digital image generation CLSM was performed by using a Leica DM IRBE microscope with Leica TCS NT software.

Scanning electron microscopy (SEM)

After relaxation, standard protocols were applied as described by Wanninger and Haszprunar (2001a). To preserve the delicate protoconch, larvae were fixed in 1% OsO_4 in distilled water. For better tissue conservation, 4% glutaraldehyde in 0.2M sodium cacodylate buffer with 0.1M NaCl and 0.35M sucrose was applied for several hours, followed by 3 washes in the same buffer, post-fixation in 1% OsO_4 in 0.2M sodium cacodylate buffer with 0.3M NaCl (2 hours), and 3 final washes in 0.2M sodium cacodylate buffer with 0.3M NaCl.

All SEM samples were dehydrated in an acetone series, critical point dried, sputter coated with gold, and observed with a Philips XL 20 SEM.

Sectioning, light microscopy (LM) and transmission electron microscopy (TEM)

Animals were fixed as described for SEM, dehydrated in a graded ethanol series, and embedded in low viscosity resin (Spurr, 1969). For LM, ribboned semi-thin serial sections were obtained with glass knives and stained with methylene-blue-azure II (Richardson et al., 1960). These sections were analyzed using a Leica DM RBE microscope and relevant pictures recorded with a Kappa DX 30 digital imaging system. For TEM, ultra-thin sections were cut with diamond knives, stained with uranyl-acetate and lead-citrate (see Reynolds, 1963), and investigated using a Philips CM 10 TEM.

RESULTS

General outline of scaphopod larval development

A detailed description of scaphopod larval and post-metamorphic development is given by Wanninger and Haszprunar (2001a). Thus, we only refer to the main developmental stages herein, which are summarized in figure 1. Around 2 days after hatching, the anlage of the foot is clearly visible and the mantle epithelium has started to differentiate. However, the lateral mantle edges have not yet ventrally fused. Although this represents a quite early stage of the lecithotrophic trochophore-like larva, the cells of the apical organ have already started to decay (Fig. 1A). During subsequent development, the mantle and the protoconch (embryonic shell secreted by mantle margin cells) fuse ventrally, leaving a characteristic median ventral fusion line (suture) on the embryonic shell. The whole animal grows in anterior direction until metamorphic competence (Fig. 1B). During metamorphosis, the larval prototroch is shed, the protoconch stops growing, and the typical features of the adult scaphopod body plan such as teleoconch (adult shell), 3-lobed foot with ciliated tip of the central lobe, cephalic captacula, and buccal apparatus start to form (Fig. 1C). These and all other organ systems are continuously elaborated and the juvenile starts feeding several days after metamorphosis (Fig. 1D).

Myogenesis

Muscle development in *Antalis* starts at around 50 hours post fertilization (hpf). By this time, the mantle epithelium and the protoconch have already started to develop, but are still ventrally open (cf. Figs. 1A, 2A). Two dorso-laterally positioned, F-actin positive regions are stained in the posterior third of the larval body. These areas are bilaterally symmetrically arranged and the anlagen of the putative head and foot retractors are not yet distinctive (Fig. 2A). Slightly later, both retractors have started to form distinct fibers. The myofibrils of the putative pedal retractors run dorsally from their posterior shell attachment site into the mid-body region. The anlagen of the cephalic retractors, likewise situated in the dorsal area, continue into the anterior third of the larval body and consist of fewer fibers than the foot retractor (Fig. 2B). After ventral closure of the protoconch, the pedal retractor fibers project ventrally into the foot rudiment and interconnect with the newly formed myocytes of the pedal plexus. In contrast, the cephalic retractors run in anterior direction towards the mantle fold. Both, the pedal and the cephalic retractors show additional fibers which penetrate the prototroch area thus serving as prototroch retractor muscles (Fig. 2C).

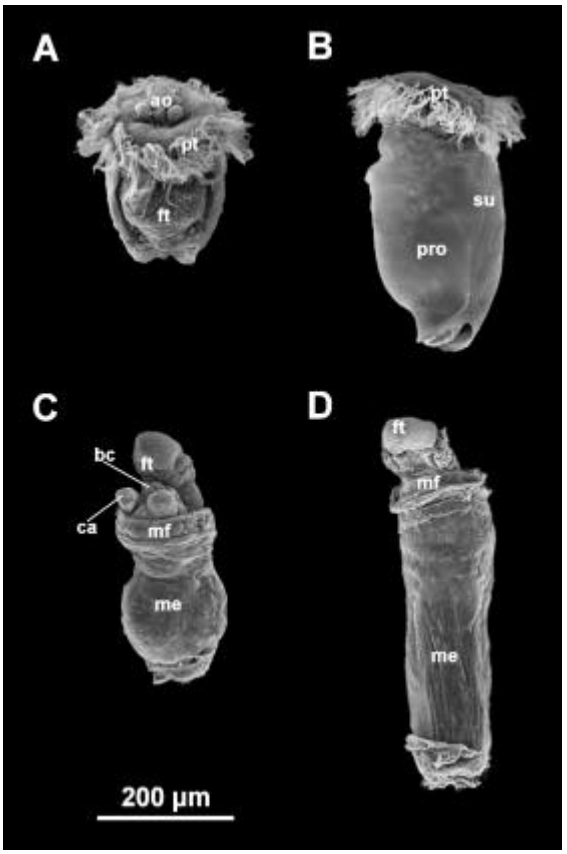


Fig. 1. Larval and early juvenile development of *Antalis entalis*, SEM, anterior faces upwards. A, C, D, decalcified, B with intact protoconch (pro). (A) Larva with the ventrally open mantle, thus the foot (ft) visible. Note the already degenerating apical organ (ao) and prototroch (pt). Age: 62 hours post fertilization (hpf), antero-ventral view. (B) Metamorphic competent specimen showing ventral fusion line (suture, su) of the embryonic shell (protoconch, pro). Age: 95 hpf, ventro-lateral right view. (C) Early juvenile with well developed foot (ft) and visceral body including mantle epithelium (me) and mantle fold (mf). The anlagen of the buccal cone (bc) and captacula (ca) are visible. Age 7 hours post metamorphosis, dorso-lateral right view. (D) Later juvenile with prominent visceral body region. Age: 13 days post metamorphosis, dorsal view.

However, these muscle portions are not independent but are connected to either the cephalic or the pedal retractors. No prototroch muscle ring is present during the entire larval development of *Antalis*. At metamorphic competence, 2 additional muscle systems have emerged, namely the buccal musculature, which is represented by a muscular ring which encircles the region of the foregut in the anterior-most third of the larval main body, and the mantle retracting fibers, which start to develop laterally on both sides (Fig. 2D). The latter muscles consist of a left and right yet little differentiated cluster of muscle cells. The fibers of the buccal ring run just ventral to the cephalic retractor and at the inner side of the mantle retractor muscles.

Due to the morphological changes at metamorphosis, which include shedding of the larval prototroch (see Fig. 1), the prototroch retracting muscle projections of the pedal and cephalic retractors are resorbed. In contrast, the mantle retractors have started to arrange antero-

laterally (Fig. 2E). Both the pedal and cephalic retractors increase in thickness, with the pedal retractors forming the most prominent muscle system in the early juvenile scaphopod (Fig. 2F, G). The foot musculature (pedal plexus) starts to arrange post-metamorphically and at around 60 hours post metamorphosis (hpm) its middle piece consists of circular, longitudinal, and diagonal fibers which form a 3-dimensional muscular grid. The musculature of the 3-partite anterior part of the foot is not yet fully differentiated (Fig. 2G, H). Due to the anterior growth of the animal the mantle retractor muscles increase in length and the buccal musculature is continuously elaborated. The shell attachment sites of both the pedal and the cephalic retractors lie dorso-laterally just anterior to the posterior shell opening (porus). From here, the pedal retractors run slightly more dorsal and more lateral to the cephalic retractors into the mid-body of the juvenile, until they reach the buccal muscle ring. (Fig. 2H, I). From the inner sides of this ring, both foot retractor muscles run in ventral direction into both sides of the foot, thus forming the lateral longitudinal myofibrils which penetrate the whole length of the foot (Fig. 2K, L). The cephalic retractors run from their postero-dorsal origin in anterior direction until they reach the buccal ring, which they cross dorsally. Then, they continue slightly ventrally into the buccal region of the animal (cf. Fig. 1C, 2H).

The structural differentiation of the foot musculature is best recognizable in whole mount fluorescence preparations and cross-sections of juveniles aged several days after metamorphosis (Figs. 2K, L, 3). The central (main) lobe of the anterior part mainly consists of outer circular and inner longitudinal muscle fibers with only occasionally present diagonal myocytes (Fig. 3B, D, E). The 2 lateral lobes bear few diagonal, several longitudinal, and additional circular muscle fibers, with which they are connected to the central foot lobe (Fig. 3B, E). In contrast, the middle piece (which we define as the region extending from the basis of the 2 lateral lobes back to the slightly broadened foot basis) and the foot basis are formed by a dense muscular meshwork, which mainly consists of longitudinal and intercrossing diagonal muscles (Fig. 3B, F). Although the captacula start to form during or immediately after metamorphosis (cf. Fig. 1C), their musculature starts to develop several days after metamorphosis (dpm). However, in the oldest specimens investigated (13 dpm), the captacula muscles were already able to retract the captacula into the mantle cavity (Figs. 2L, 3). Cross-sections of the 2 pairs of captacula in 13 dpm old specimens illustrate their strongly developed musculature which hardly leaves space for a distinct captacular cavity. (Fig. 3G, H).

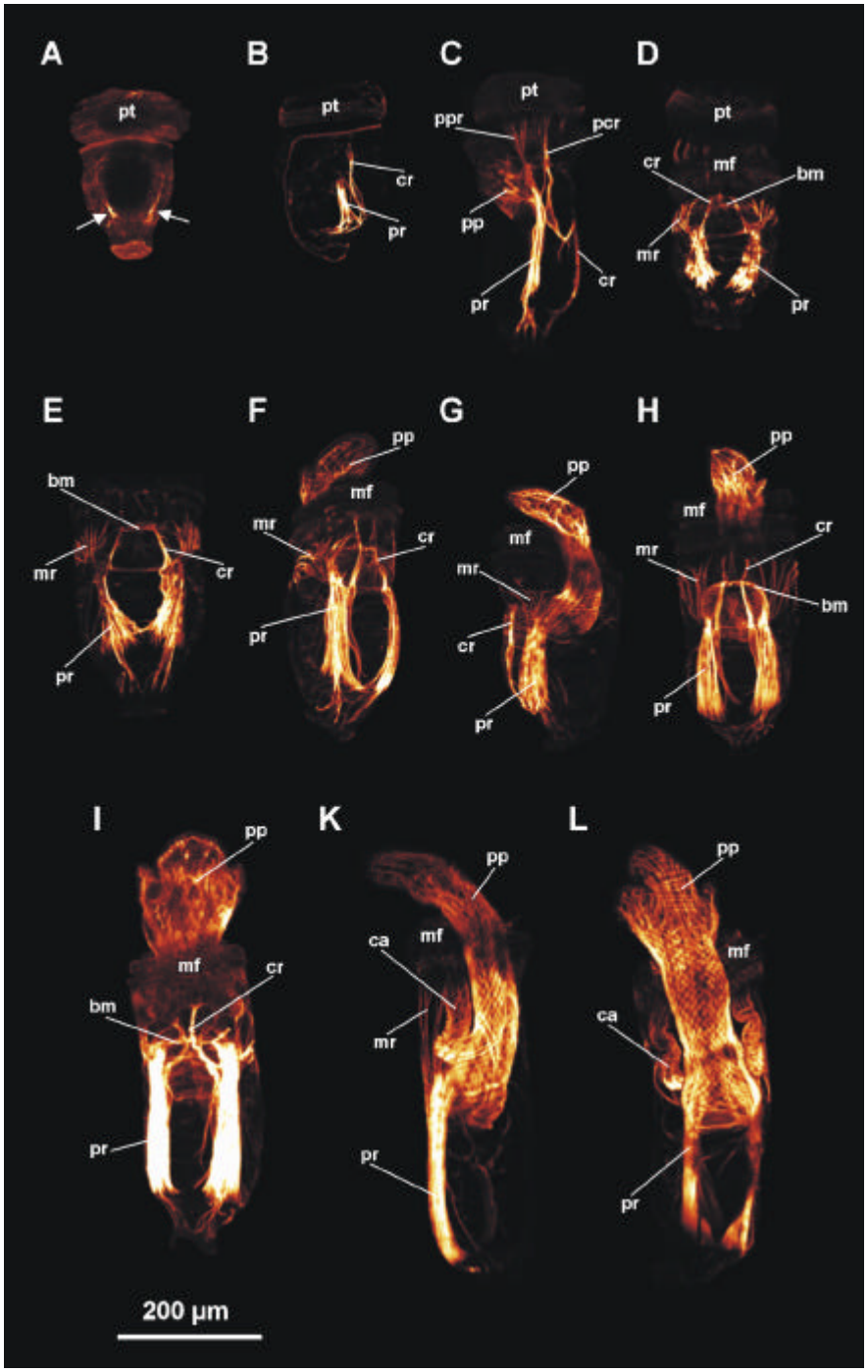


Fig. 2. CLSM micrographs of myogenesis in *Antalis entalis*, anterior faces upwards. (A-D) Developmental sequence from the first detectable signal until metamorphic competence. (A) Earliest, paired anlage of the putative retractors in the dorso-posterior region of the larva (arrows) (pt, prototroch). Age: 53 hpf, dorsal view. (B) The pedal retractors (pr) and cephalic retractors (cr) have started to form distinct muscle fibers. Age: 62 hpm, lateral left view. (C) The pedal retractors (pr) run into the foot and interconnect with the pedal plexus (pp) but also form fibers that project into the anterior region of the prototroch (pt) ("prototrochal projections of the pedal retractor", ppr). The more delicate cephalic retractors (cr) run along the dorsal mantle edge with their distinct prototrochal projections (pcr) also inserting in the prototrochal region. Age: 90 hpf, lateral left view. (D) Specimen showing both retractor systems (pr, cr) as well as the early anlagen of the mantle retractors (mr) and the buccal muscle ring (bm), both situated in the region behind the mantle fold (mf). Age: 95 hpf (metamorphic competent), dorsal view. (E-H) Juveniles aged several hours post metamorphosis (hpm) until 25 days post metamorphosis (dpm). (E) Early juvenile with shed prototroch, lost prototrochal muscle projections, and retracted foot, showing the close proximity of both retractor systems (pr, cr). Age: 5 hpm, dorsal view. (F) Relaxed specimen demonstrating the independence of the pedal retractors (pr) and cephalic retractors (cr). Note the pedal plexus (pp) which starts to elaborate. Age: 9 hpm, dorso-lateral left view. (G) Specimen that illustrates

the absolute and relative thickness of the pedal (pr) and cephalic retractors (cr) as well as the muscular meshwork of the foot (pp). Age: 42 hpm, lateral right view. (H) Overview of all muscles in an early juvenile. Relative to the pedal retractors (pr), the cephalic retractors (cr) lie closer to the dorsal mid-line of the animal. Posteriorly, the cephalic retractors (cr) start slightly more ventrally of the pedal retractors (pr), run in anterior direction until the reach the buccal muscle ring (bm), bend again ventrally and insert in the cephalic region. The pedal retractors (pr) run dorsally until they reach the buccal muscle ring (bm), from where they project laterally into both sides of the foot. Age: 61 hpm, dorsal view. (I-L) 13 dpm old specimens. (I) Dorsal view, showing the increasing dominance of the pedal retractors (pr) relative to the cephalic retractors (cr). (K) Lateral right view. Note the right pedal retractor (pr) running laterally into the foot, as well as the fine, slender mantle retracting fibers (mr), the well developed musculature of the cephalic captacula (ca), and the massive foot musculature (pp). (L) Ventral view which illustrates the meshwork-like myo-pattern of the pedal plexus (pp), the relative position of the captacula (ca) in the juvenile body, and the penetration sites of the pedal retractors (pr) into the foot basis.

Ultrastructure

All identified muscle systems in *Antalis entalis* are smooth (i.e., non-striated) (Fig. 4). This is also true for the prototrochal projections of the cephalic and the foot retractors and for the buccal musculature.

DISCUSSION

Molluscan muscle systems and their phylogenetic significance

Until recently, molluscan myogenesis has received little to no attention for phylogenetic considerations. New data on the muscle development in basal gastropods (Degnan et al., 1997; Page, 1997, 1998; Wanninger et al. 1999a, b), Nudibranchia (Page, 1995), pulmonates (Ruthensteiner, pers. comm.), Polyplacophora (Wanninger and Haszprunar, 2001b), and Scaphopoda (this paper), as well as earlier works on bivalves (Hatschek, 1880; Meisenheimer, 1901; Cragg, 1985; Cragg and Crisp, 1991) enable a broad comparison across the Mollusca, especially since their adult myo-anatomy is fairly well known (cf. Table 1).

Body wall and dorso-ventral (shell) musculature

It is now widely accepted that the aplacophoran taxa Caudofoveata and Solenogastres form the most basal molluscan classes, with the Solenogastres being probably the earliest offshoot of the phylum (Haszprunar, 2000; Fig. 5 herein). Their numerous plesiomorphic characters include multiple, serially repeated dorso-ventral muscle fibers and a 3-layered body wall musculature which consists of outer ring, intermediate diagonal, and inner longitudinal muscles (Salvini-Plawen, 1969, 1972; Scheltema, 1993; Scheltema et al., 1994; Haszprunar and Wanninger, 2000; cf. also Table 1).

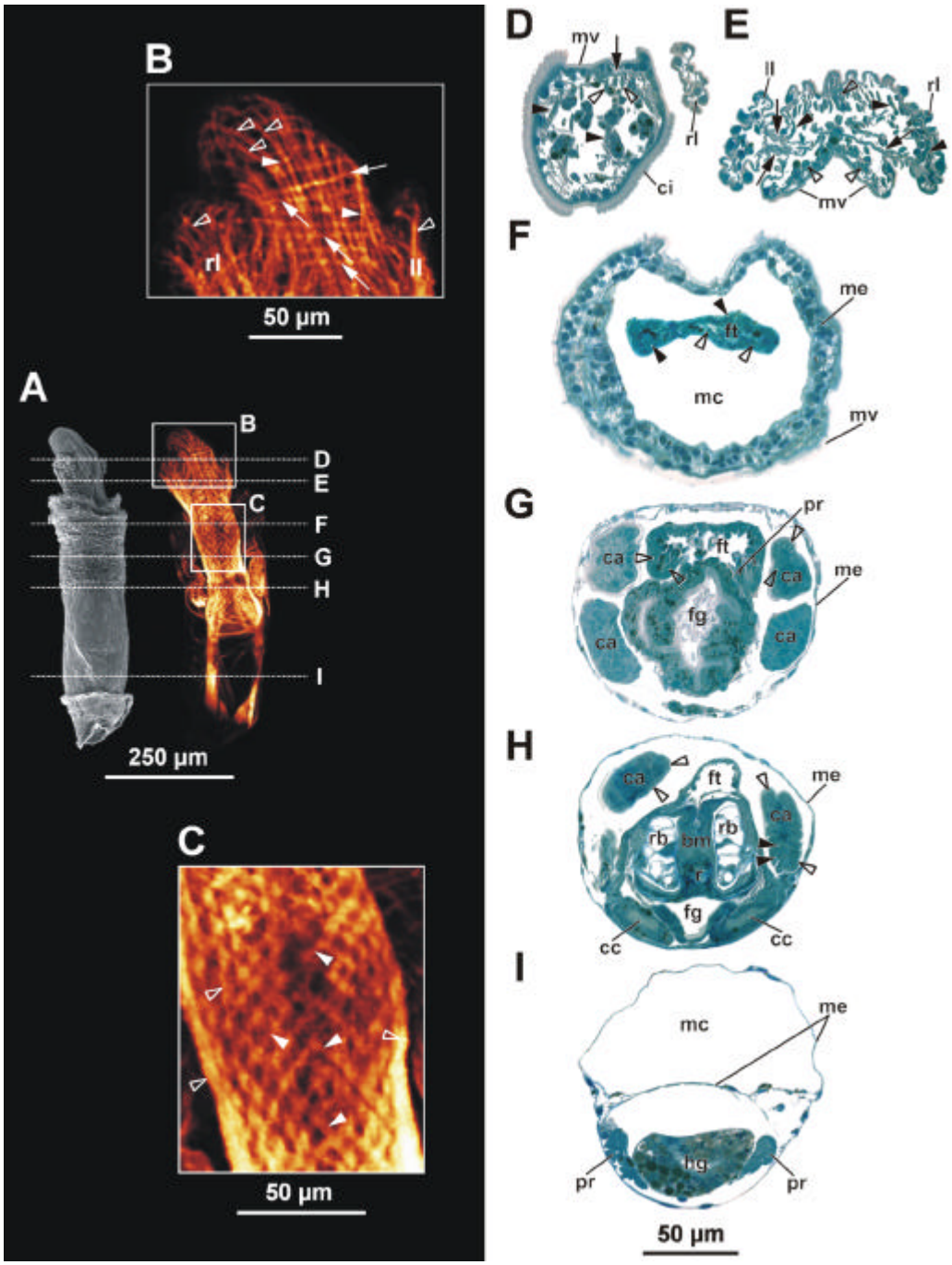


Fig. 3. The myo-anatomy of juvenile *Antalis entalis* specimens as revealed by SEM, fluorescence staining of F-actin, and serial semi-thin cross sections. Boxed areas are enlarged in B and C, stippled lines mark the sectioning planes of D-I. All images obtained from 13 dpm old specimens. Orientation is with anterior up in A-C and with ventral up in D-I. (A) SEM (left, ventro-lateral right view) and CLSM (right, ventral view) image to illustrate the position of the body regions shown in B-I. (B) Anterior tip of the foot with numerous longitudinal muscle fibers (open arrowheads), circular muscles (arrows), and few diagonal myocytes (full arrowheads). Note the similar patterning in the central (main) foot lobe and the left (ll) and right lateral lobes (rl). (C) Detail of the central foot region revealing the typical pattern due to the predominant signal of the diagonal muscle fibers (full arrowheads). The longitudinal muscles (open arrowheads) are mainly visible in the lateral foot regions. (D-I) Serial cross sections of different body regions along the antero-posterior axis. All sections are slightly shifted to the right. (D) Central lobe in the anterior, ciliated (ci) region with anterior-most part of the right lateral lobe (rl). The diagonal (full arrowheads), longitudinal (open arrowheads), and circular muscle fibers (arrow) are loosely arranged leaving a small lumen in the foot tip (mv, epidermis with microvilli). (E) Area at the basis of the lateral lobes (rl, ll), showing that circular muscle fibers (arrows) connect the lateral lobes to the central main foot part. (F) Section in the region where the foot (ft) is already buried in the mantle cavity (mc), slightly anterior of the captacula. The foot musculature is very densely packed, leaves no space for a distinct cavity, and contains

massive diagonal (full arrowheads) and longitudinal muscle bundles (open arrowheads). Note the relatively thick mantle epithelium (me). (G) Section in the anterior foregut (fg) region, where the pedal retractors (pr; here only right one visible) project into the foot. Note the 2 pairs of captacula (ca) with numerous cell nuclei and muscle bundles but without lumen. (H) Section through the region of the cerebral commissure (cc) and the radula (r), revealing the large, multi-chambered radula bolsters (rb), which are embedded in the surrounding buccal musculature (bm). (I) The posterior body area is characterized by a spacious mantle cavity (mc) and by a body cavity which bears the paired pedal retractors (pr) (and cephalic retractors, which are not distinguishable by semi-thin section analysis; cf. Fig. 2) and the hindgut (hg).

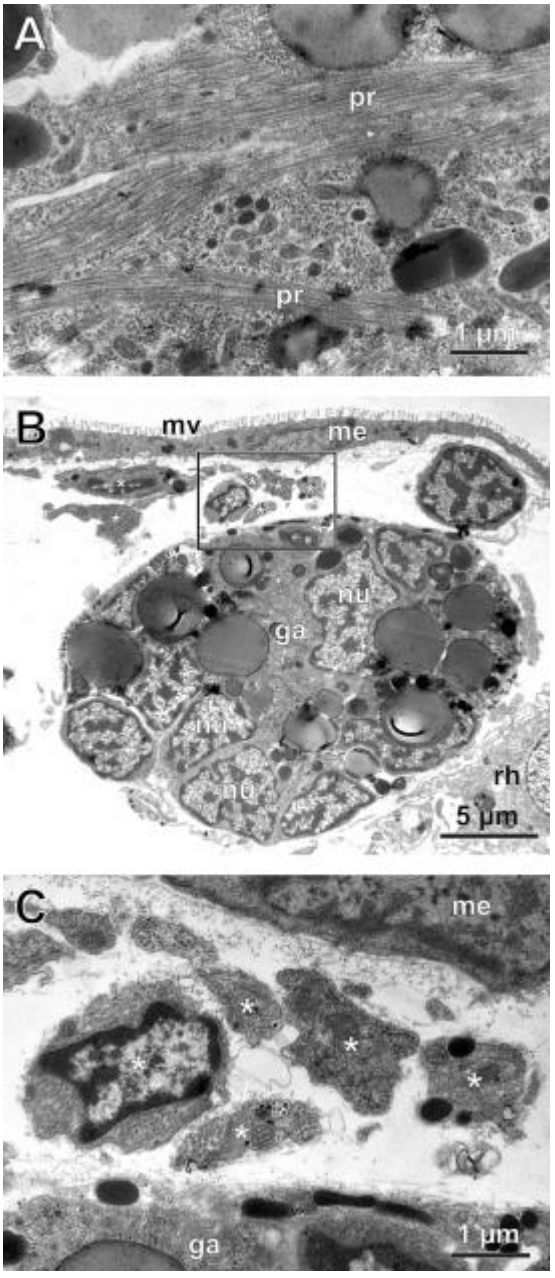


Fig. 4. Ultrastructure of muscle systems of a juvenile specimen (13 dpm). (A) Longitudinal section demonstrating the smooth character of the pedal retractor fibers (pr). (B) Cross section in the region of the buccal cone showing myofibers of the cephalic retractor (asterisks) which are situated between the dorsal mantle epithelium (me; with outer microvillous border, mv) and a more ventrally positioned ganglion (ga) of the central nervous system (nu, nucleus; rh, rhogocyte). Boxed area is enlarged in C. (C) Detail of cross section of cephalic retractor fibers (asterisks).

Table 1. Major currently available data regarding the occurrence of the main muscle systems in the various classes of the Mollusca.

CLASS	"worm-like" body wall musculature	larval retractors	prototroch/ velum ring	adult cephalic retractors	number of sets of adult dorso-ventral (shell) muscles	Reference
SOLENOGASTRES	+ (adult)	?	?	-	multiple	Salvini-Plawen, 1985; Scheltema, 1993; Scheltema et al., 1994; Haszprunar and Wanninger, 2000
CAUDOFOVEATA	+ (adult)	? (-)	? (-)	-	reduced, several in anterior body region	Salvini-Plawen, 1972, 1985; Scheltema et al., 1994; Wanninger, pers. obs.
POLYPLACOPHORA	+ (larva, pre-trochal)	-	+	-	8 (formed by multiple, serially arranged fibers)	Wanninger and Haszprunar, 2001b
TRYBLIDIA	-	?	?	-	8	Lemche and Wingstrand, 1959; Wingstrand, 1985; Haszprunar and Schaefer, 1997a, b
BIVALVIA	- (+ secondary in ship worms)	+	+ (basal ?)	-	3-8	Hatschek, 1880; Meisenheimer, 1901; Cragg, 1985; Cragg and Crisp, 1991; Wanninger, pers. obs.
SCAPHOPODA	-	-	-	+ (1 pair)	1-2	Steiner, 1992a; this paper
GASTROPODA	- (+ secondary in slugs)	+	+	+ (1 pair)	1	Wanninger et al., 1999a, b; Degnan et al., 1997; Page, 1995, 1997, 1998; Ruthensteiner, pers. comm.
CEPHALOPODA	-	(-)	(-)	+ (1 pair)	1 ("depressor infundibuli")	Lang, 1900; Wells, 1988

These are the main features which are correlated their "worm-like" gross morphology, and although the molluscan sister-phylum still needs to be determined (Haszprunar, 1996, 2000; Waller, 1998), these data imply a "worm-shaped" ancestor at the base of molluscan phylogeny (see also Haszprunar and Wanninger, 2000). Regarding the evolution of the molluscan musculature, Wanninger and Haszprunar (2001b) showed that the Polyplacophora represent a link between the aplacophoran worm shape and the conchiferan condition of concentrated and numerically reduced shell muscles (Table 1, Fig. 5). Evidence for this is twofold. First, there is a "worm grid" in the pre-trochal region of the chiton larva, which is regarded as ontogenetic recapitulation of the ancestral body wall musculature as found in adult aplacophorans. Second, the development of the dorso-ventral musculature undergoes an initial stage of multiple seriality which corresponds to the situation in adult Solenogastres, thus rendering the typical chiton-like 8-metamerism of the adult dorso-ventral shell musculature a secondary condition. Since larval stages of the Tryblidia (monoplacophorans) are still unknown, the question whether these relics are ontogenetically present in basal conchiferans remains speculative. Despite this, the data currently available clearly suggest a link of 2 evolutionary trends within the Mollusca, which can be traced from the basal aplacophoran to the derived gastropod-cephalopod condition. These are the evolution of protective epidermal structures – the homology of which still being uncertain - from calcareous spicules (Solenogastres and Caudofoveata) via shell plates (Polyplacophora) to a univalved shell (Conchifera; the bivalve shell represents an apomorphy for the Bivalvia, see Wanninger and Haszprunar, 2001a), which coincides with a subsequent concentration and numeric reduction of the dorso-ventral shell musculature. Additionally, with the functional innovation of a stable "exoskeleton" (shell), the 3-layered, ancestral body wall musculature, important in the aplacophorans for maintaining the body shape as antagonist against the body pressure, lost its original function and disappeared in the Conchifera (Fig. 5).

Other muscle systems and the scaphopod-bivalve relationship

A recent overview on all muscle systems present in the Mollusca is given by Haszprunar and Wanninger (2000). Here, we focus on the ontogenetically and phylogenetically most relevant ones, i.e., the larval retractor systems, the prototroch/ velum muscle ring, and the adult cephalic retractors (cf. Table 1). Larval retractor muscles, which are characterized by distinct shell insertion areas and an (oblique) striation pattern, have been found in certain planctotrophic bivalve taxa more than 100 years ago (Hatschek, 1880; Meisenheimer, 1901; Cragg, 1985; Cragg and Crisp, 1991).

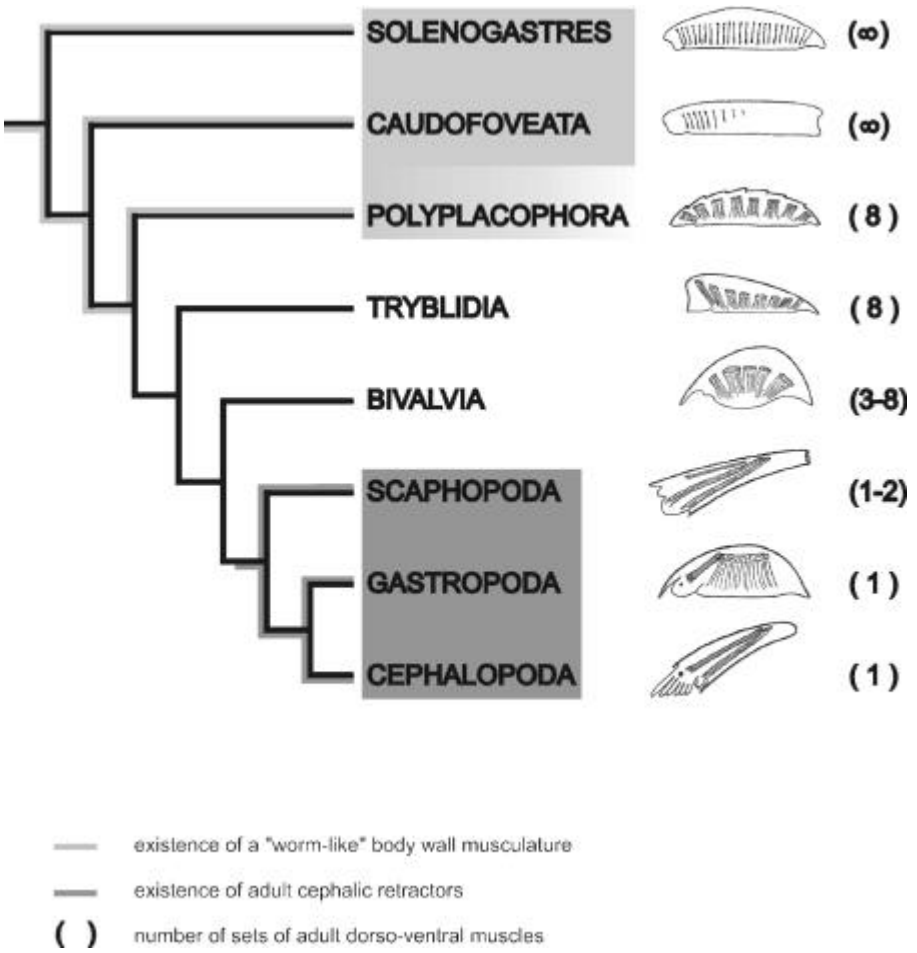


Fig. 5. Phylogenetic tree of the Mollusca (after Haszprunar, 2000) as inferred from the currently available data sets including the new data on myogenesis. Thus, a typical worm-shaped ancestor with 3-layered body wall musculature and serially repeated dorso-ventral muscles, as found in adult Solenogastres and Caudofoveata as well as in larvae of the Polyplacophora, is part of the ancestral molluscan bauplan. The introduction of a distinct cephalic retractor system at the base of the scaphopod-gastropod-cephalopod line is diagnostic for such a supertaxon and contradicts the earlier proposed Diasoma concept which comprised the Scaphopoda and the Bivalvia ("Diasoma") as sister taxon of the Gastropoda and Cephalopoda ("Cyrtosoma"). The evolutionary origin of larval retractors and the velum/ prototroch muscle ring remain phylogenetically ambiguous due to the lack of data for the Solenogastres, Caudofoveata, and Tryblidia and because of the derived development of the Cephalopoda (see text).

However, the presence of such retractors in the bivalve groundplan remains debatable since the basal Bivalvia (protobranchs) show a lecithotrophic test-cell larva, which is nowadays considered a derived larval type diagnostic for protobranchs, with their larval myo-anatomy still being unknown. Since the larval condition in the Tryblidia, Caudofoveata, and Solenogastres remains obscure and the cephalopod development is highly derived, only the polyplacophorans, scaphopods, and gastropods provide data relevant for discussion. The lack of distinct larval retractors in the Polyplacophora and Scaphopoda - the prototrochal muscle projections of which are smooth and lack distinct shell insertion sites, see above - and their

presence in the basal gastropod bauplan (Degnan et al., 1997; Page, 1997, 1998; Wanninger et al., 1999a; see also Table 1) makes their evolutionary origin at the interface of the Bivalvia versus Scaphopoda, Gastropoda, and Cephalopoda (inferring secondary loss in the Scaphopoda) equally parsimonious with their twice independent evolution in the Bivalvia and the Gastropoda (cf. Fig. 5). A similar problem occurs regarding the phylogenetic origin of the velar/ prototrochal muscle ring, which is found in the Polyplacophora, (basal ?) Bivalvia, and all Gastropoda with a larval stage, including Nudibranchia (Page, 1995) and pulmonates (Ruthensteiner, pers. comm.), but not in the Scaphopoda (Table 1). Applying the parsimony principle at the current state of knowledge, the available data suggest its evolution at the polyplacophoran-conchiferan interface (secondary loss in the Scaphopoda), but due to the missing data for numerous taxa (see above), especially the aplacophorans and tryblidians, this issue requires further investigation.

Until this study, the existence of adult (i.e., post-metamorphic) cephalic retractors were only reported for the Gastropoda and Cephalopoda (Salvini-Plawen and Steiner, 1996; Haszprunar, 2000). However, Lacaze-Duthiers (1857: pp. 233-234 and pl. 9, fig. 2) already stated that one of the 2 retractor pairs in juvenile *Dentalium* projects into the antero-dorsal region of the animal close to the mouth ("... où il s'unit au corps en arrière de la bouche." [p. 233]). Despite this and the illustration in his plate 9, fig. 2, which indicates the insertion of this muscle within the buccal cone, he interprets this muscle as a mantle retractor ("... rétracteurs ... du manteau." [p. 233]). However, as outlined above, the dentaliid mantle retracting system origins much more anteriorly (close to the buccal apparatus) and consists of numerous loosely arranged muscle fibers rather then a solid muscle bundle (cf. Fig. 2E, H herein and Lacaze-Duthiers, 1857: pl. 9, fig. 2).

It is important to note that all other Mollusca, although lacking a free movable head which is apomorphic for gastropods and cephalopods (Salvini-Plawen and Steiner, 1996; but see Waller, 1998, who interpreted the scaphopod buccal cone as a similar free movable head structure), do, however, have a distinct "cephalic region". This is characterized by a buccal apparatus (which is secondarily lost in the Bivalvia) and distinct cerebral ganglia with commissure. Herein, we demonstrate that the Scaphopoda show an independent, paired retractor system, which projects into the dorsal region of the buccal cone but is not associated with the buccal cartilages (Figs. 1, 2C). In contrast to gastropods, where the cephalic retractors are of entire post-metamorphic origin (cephalopods lack a truly larval stage; see above), these muscles start forming very early in scaphopod development and appear synchronously with the foot retractors. This reflects the more direct character of larval

development in scaphopods compared to polyplacophorans, bivalves, or gastropods, and it seems likely that the origin of the cephalic retractors has been heterochronically shifted into the early larval stages in scaphopods. However, the presence of larval protonephridia in post-metamorphic juveniles already bearing the adult excretory system (Ruthensteiner et al., 2001) demonstrate that the ontogeny of *Antalis* comprises mechanisms of accelerated as well as delayed development (note also the pre-metamorphic anlage of the cephalic tentacles in gastropods versus the post-metamorphic origin of the captacula in scaphopods; Fig. 2C and Wanninger and Haszprunar, 2001a).

Because of positional, structural, and functional similarities, we regard the cephalic retractor system as suprataxic homologous and thus diagnostic for a supertaxon comprising Scaphopoda and Gastropoda + Cephalopoda. The lack of definite apomorphies for a diasome clade (Waller, 1998; Haszprunar, 2000; Wanninger and Haszprunar, 2001a) strengthens this hypothesis.

Functionality of the scaphopod foot and captacula

The main features of the current believes regarding functional anatomy of the molluscan foot is reviewed and updated in Kier (1988). Generally, there are 2 ways of how animal appendages may function. One is by a so-called muscular-hydrostat system, which exclusively relies on antagonistic muscle activity and thus requires a complex and often massive 3-dimensional myo-pattern which usually leaves little or no space for hemolymphic cavities. Such systems are often found in body regions which require fast movements such as the cephalopod arms (for the capture of prey) or the squid mantle for producing the jet propulsion (Trueman, 1980; Kier, 1988). In contrast, a pure hydrostatic system, which is based on a combination of hemolymphatic pressure (for relaxation) and muscular activity (for contraction), is found in body regions which produce a steady force, such as the burrowing foot of many bivalves (Trueman, 1966, 1967), or in organs that do not require fast expansion movements, such as the cephalic tentacles of euthyneuran gastropods. Thus, a distinct and often wide lumen is present in these organs. In scaphopods, both foot types are expressed, following scaphopod phylogenetic classification in the 2 sub-classes Gadilida and Dentaliida (Steiner, 1992a). Accordingly, the elongation of the gadilidan foot is caused by hydraulic pressure alone, while its dentaliidan counterpart, with stronger longitudinal foot muscles and a general smaller pedal sinus relative to the foot wall, is said to stretch by combined hydraulic and muscular-hydrostat activities (see Steiner, 1992a for details). These results are in striking

contrast to those of Morton (1959) and Trueman (1968), who concluded that purely hydraulic mechanisms are responsible for foot and captacula protraction in *Dentalium entalis* (=*Antalis entalis*). However, whole mount preparations of juvenile *Antalis entalis* (Figs. 2G-L, 3A-C) as well as semi-thin cross sections of several regions along the foot (Fig. 3D-G herein; Steiner, 1992a: fig. 3) show the high complexity and thickness of the 3-dimensional muscular meshwork of the foot wall in combination with a relative small pedal hemolymphic cavity especially in the mid-part of the foot of *Antalis*. These results are in accordance with an earlier study by Plate (1892), who found that the dentaliidan foot bears a 3-layered musculature consisting of outer ring, intermediate diagonal, and exceptional massive inner longitudinal muscles, thus significantly limiting the volume of the foot lumen, while several gadilidan species investigated show a much smaller foot wall-foot lumen ratio, mainly due to relatively weak longitudinal foot muscles (Steiner, 1992a). According to the muscular-hydrostat system (Kier and Smith, 1985; Kier, 1988), this clearly demonstrates that muscle antagonism is likely to play a significant role in the expansion of the dentaliidan foot while hydraulic activities, similar to those in bivalves, are regarded as the main driving force for gadilidan foot protraction. The cephalic captacula of *Antalis*, too, lack a distinct hemolymphic cavity but possess massive longitudinal retractors (Fig. 3G, H), indicating that their extension is also based on a muscle antagonist system as found in the foot. Thus, the captacula of *Antalis*, both in anatomy and function, resemble the arms of cephalopods and the cephalic tentacles of prosobranch gastropods, but differ from the euthyneuran cephalic tentacles or the bivalve siphons, which combine muscular and hydraulic activities (see Kier, 1988). Ontogenetically, the gastropod tentacles pre-date the captacula of *Antalis*, since the former are already formed in the late veliger larva (e.g., Wanninger et al., 1999a), while the scaphopod captacula are of entire post-metamorphic origin (Wanninger and Haszprunar, 2001a).

The question whether the dentaliidan muscular-hydrostat system or the gadilidan combined muscular retraction vs. hydraulic expansion system represents the basal scaphopod condition remains unsolved, also because scaphopod phylogeny as a whole is still unclear (Steiner, 1992b, 1996; Reynolds, 1997; Reynolds and Okusu, 1999).

CONCLUSIONS

The current data on molluscan myogenesis enable significant conclusions regarding the evolution of the Mollusca:

(1) The ancestral condition of the molluscan myo-groundplan include a 3-layered body wall musculature and multiple sets of serially arranged and ventrally intercrossing dorso-ventral muscle fibers as expressed in the recent Solenogastres and partly in the Caudofoveata and the polyplacophoran larva.
(2) Due to the introduction of a stable exoskeleton (shell [plates]), the body wall musculature is lost and the dorso-ventral musculature is subsequently concentrated and numerically reduced within the Conchifera.
(3) Dentaliid scaphopods show a muscular-hydrostat system in their foot and captacula as found in Gastropoda and Cephalopoda but not in Bivalvia.
(4) The existence of distinct cephalic retractors proposes a novel supertaxon comprising the Scaphopoda and the Gastropoda + Cephalopoda.

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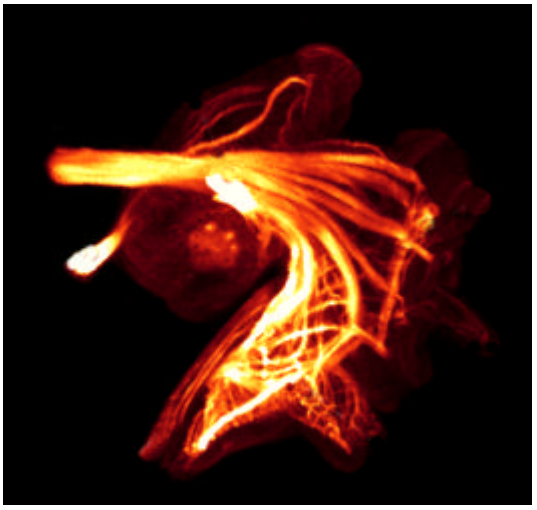
APPENDIX V

On the muscle development in the limpet *Patella* (Mollusca, Patellogastropoda)

Published as parts of the article "Development of the musculature in the limpet *Patella* (Mollusca, Patellogastropoda)" in: *Development, Genes, and Evolution* 209 (1999): 226-238.



Abstract. Whole mount technique using fluorescent-labelled phalloidin for actin staining and confocal laser scanning microscopy as well as semithin serial sectioning, SEM and TEM were applied to investigate the ontogeny of the various muscular systems during larval development in the limpets *Patella vulgata* L. and *Patella caerulea* L. In contrast to earlier studies, which described a single or two larval shell muscles, the pretorsional trochophore-like larva shows no less than four different muscle systems, namely the asymmetrical main head/foot larval retractor muscle, an accessory larval retractor with distinct insertion area, a circular prototroch/velar system, and a plexus-like pedal muscle system. In both *Patella* species only posttorsional larvae are able to retract into the shell and to close the aperture by means of the operculum. Shortly after torsion the two adult shell muscles originate independently in lateral positions, starting with two fine muscle fibres which insert at the operculum and laterally at the shell. During late larval development the main larval retractor and the accessory larval retractor become reduced and the velar muscle system is shed. In contrast, the paired adult shell muscles and the pedal muscle plexus increase in volume, and a new mantle musculature, the tentacular muscle system, and the buccal musculature arise. Because the adult shell muscles are entirely independent from the various larval muscular systems, several current hypotheses on the ontogeny and phylogeny of the early gastropod muscle system have to be reconsidered.

INTRODUCTION

The origin and homologies of larval and adult shell muscles in gastropods have been a matter of debate for over a century. Because earlier studies (Smith 1935; Crofts 1937, 1955) have considered the larval shell muscles as being primarily responsible for ontogenetic torsion (but see Bandel (1982) for contrary view), these questions are intimately associated with the problem of origin and definition of the class Gastropoda as a whole. Up to recently most authorities have held that there is continuity and thus direct ontogenetic homology (see Haszprunar (1992) for definition) between the main larval retractor muscle(s) and (one of) the adult shell muscle(s) in gastropods (Table 1).

In *Patella vulgata*, Smith (1935) identified two asymmetrically positioned larval shell muscles which were described as being independent of the two symmetrical adult shell muscles, the latter finally forming a horse-shoe shaped organ consisting of several distinct muscle bundles being interrupted by blood sinuses to the mantle. Anderson (1965) described similar conditions in lottiid patellogastropods.

The fundamental paper by Crofts (1937) on the ontogeny of the zeugobranch vetigastropod *Haliotis tuberculata* and her second big contribution (Crofts 1955) on haliotids, patellids and trochids have become the main data-basis for most hypotheses on this subject. Her data on *Haliotis tuberculata* suggest that the main larval retractor is continued by the adult left shell muscle. The data from the second contribution by Crofts (1955), where muscle ontogenesis in haliotids, patellids and trochids were described, were taken for granted by Fretter & Graham (1962; Fretter 1969) and remained nearly undisputed until recently. Unfortunately, notable counter-evidence such as Smith's (1967) study on the shelled opisthobranch *Retusa obtusa*, where the adult shell (columellar) muscle arises independently from the larval musculature, has been ignored by most subsequent authors (but see Ponder & Lindberg 1997).

Contrary to Crofts (1955), Bandel (1982) claimed a continuity between the main larval retractor and the (left) adult shell muscle in trochids. Haszprunar (1985) showed on the basis of shell muscle innervation, that the typical spindle muscle of Trochidae, Caenogastropoda and Heterobranchia is homologous to the left shell muscle of primitive gastropod taxa.

However, more recent investigations (Voltzow 1987, 1996; Collins 1996; Page 1997b) have expressed serious doubts on the correctness and accuracy of the original data of Crofts (1937, 1955) and Bandel (1982). Indeed, in particular Crofts' (1955) data on the patellids *Patella vulgata* and *Patina* (= *Helcion*) *pellucida* are more than doubtful: her figure 17 clearly does not show *Patella vulgata* as stated in the legend, but because of the papillate cephalic tentacles certainly figures a vetigastropod (probably *Haliotis*). Since this latter study compiled data from nearly 20 years, this strongly indicates that certain data were confused. The original data on vetigastropods (*Haliotis* and Trochidae) have also been questioned recently. By applying fluorescence dyes and confocal laser scanning microscopy, Degnan et al. (1997) showed that the larval musculature of *Haliotis rufescens* is much more complicated than previously stated. Moreover, the larval retractor muscle of *Haliotis* is not continued by the (posttorsionally left) adult shell muscle as formerly stated. Similar results on *Haliotis kamtschatkana* were provided by Page (1997a) based on TEM-studies.

During the last decade most authorities have accepted that the Patellogastropoda (formerly Docoglossa) - and not the zeugobranch Vetigastropoda as formerly believed - are the earliest extant offshoot of the Gastropoda (Golikov and Starobogatov 1975; Haszprunar 1988; Ponder and Lindberg 1997). Aside from the classic ontogenetic studies by Patten (1886), Boutan (1899), and Smith (1935), only scarce data were provided by Dodd (1955), Fretter and Graham (1962, 1976), Kessel (1964), Anderson (1965), and Rao (1975) on various patellogastropod species. However, there is no recent detailed account on the morphogenesis

of any patellogastropod limpet, whereas the early development and cell-lineage of *Patella vulgata* have been investigated in detail by applying modern methods (e.g., van den Biggelaar 1977; Serras and Speksnijder 1991, Damen and Dictus 1994a, b, 1996; Dictus and Damen 1997). Haszprunar (1988) stated that shell and muscular features of patellogastropods might be primitive for Gastropoda, whereas Ponder and Lindberg (1997) regarded them as derived. Therefore, and because of the clear evidence for erroneous data provided by Crofts (1955), *Patella* is a preferred aim to reinvestigate muscular ontogenesis. The present contribution aims to present original data on the muscular development in patellid limpets based on new and more powerful methodologies in order to provide a more accurate data-basis for discussion. The process of ontogenetic torsion in *Patella* will be described in detail elsewhere (Wanninger et al. in prep.).

MATERIALS AND METHODS

Culture and breeding of *Patella*

Because the breeding season of the protandric hermaphrodite *Patella vulgata* extends from mid October to mid April, living *Patella vulgata* were collected in October 1992 and 1996 on the rocky shore near Roscoff (Bretagne, France) and transferred to the University of Utrecht. There, the animals were kept alive in large tanks with natural seawater at 15 to 16°C until April of the following year. Larvae were cultured at different temperatures from 13 to 18°C.

The breeding season of *Patella caerulea* extends from mid December to at least September (personal observations of spontaneous spawning), artificial fertilization is possible throughout the year. Living animals were collected in March, May and July 1997 at intertidal rocks in the Northern Adriatic Sea (near Trieste or Rovinj) and transferred to the Zoological State Collection Munich, where they were kept alive over several months with artificial seawater at 21°C ± 1°C.

All fertilization and culture procedures were carried out in Millipore-filtered sea water (MPFSW) (pore size 1.2 µm in *P. vulgata,* 0.45 µm in *P. caerulea*). Following van den Biggelaar (1977), the gonads of ripe animals were dissected for artificial insemination. Eggs were treated with alkaline sea water (pH = 8.9 by addition of drops of NH_4OH) for 1 to 7 minutes before fertilization to induce the egg ripening process, followed by 1 hour of stirring. Sperm of two or three males was diluted in MPFSW until the suspension became fully clear, the agility of sperm cells was confirmed under the microscope before insemination. For

fertilization 10 to 20 drops of the sperm suspension were used per liter MPFSW containing eggs. Stirring the eggs before and after insemination proved very helpful.

Larval cultures were kept in MPFSW with 50 mg streptomycin and 60 mg penicillin per liter MPFSW to minimize microbial or fungal infection. 800 ml beakers provided with the airlift-droplet stirrer system (Strathmann 1987) and flat bowls (diameter: 19 cm) with a magnetic stirrer at slow speed were alternatively used as culture vessels. For live observations larvae were either studied under a stereo microscope or, mounted on hollow grinding slides, in a compound microscope, which enabled more detailed observations.

Some larvae of *Patella caerulea* underwent spontaneous metamorphosis from 170 hours post fertilization (hpf) onwards. To increase the metamorphic rate, substratum from the aquarium of the adults or adult animals themselves were added to the cultures. Larvae settled on the walls of the culture vessels, where the metamorphosed juveniles fed on developing algal films growing on the culture vessel wall. In *Patella vulgata* two specimens underwent spontaneous metamorphosis.

Scanning electron microscopy (SEM)

For SEM, larvae were fixed in 4% glutaraldehyde in 0.2M sodium cacodylate buffer with 0.1M NaCl and 0.35M sucrose added for osmolarity. Posttorsional stages were relaxed by adding drops of 7.14% (0.75M) $MgCl_2$ prior to fixation. Fixed larvae were treated with 1% OsO_4 in 0.2M (3.2%) sodium cacodylate buffer with 0.3M NaCl for 2 hours, dehydrated in an acetone series, critical point dried, sputter coated, and observed with a Philips XL 20 SEM.

Actin staining and examination

For actin staining procedures larvae were relaxed as described above. Fixation was done by 4% paraformaldehyde in 0.1M (1.07% Na_2HP0_4 + 0.28% NaH_2PO_4; pH 7.3) phosphate-buffer solution (PBS), 10% sucrose was added for osmolarity. Alternatively, a fixation of 2% paraformaldehyde in 0.085M PBS with 10% sucrose containing 15% saturated picric acid solution (modified from Stefanini et al. (1967)) was applied with equal results. Because of the gradual transformation of F-actin into G-actin, larvae cannot be stored in these fixations longer than approximately four weeks. If necessary the mineralized shell was decalcified in a 2% EDTA solution (Romeis 1989) for 1-2 hours or over night, which did not effect the further staining procedure.

Phalloidin is known to bind to F-actin in fixed material (Wulf et al. 1979) and can be coupled with fluorescent dyes to make muscle structures visible. Here we followed mainly the

protocols of Serras and Speksnijder (1991) and Rieger et al. (1994): The fixative was removed by rinsing with 0.05M PBS with 10% sucrose (3 steps of 10 minutes each), then the specimens were treated in 0.01M buffer with 0.2% Triton X-100 (PBS-T) for 60 minutes to make tissues permeable to staining. Staining was done using Rhodamine phalloidin (Molecular Probes, R-415), BODIPY R6G phalloidin (Molecular Probes, B-7491), or Oregon Green 514 phalloidin (Molecular Probes, O-7465). Larvae were incubated in the dark for 50-60 minutes in one unit of the dyes dissolved in 200 µl PBS-T. This was followed by three times 10 minutes rinsing in 0.01M PBS. Finally, larvae were mounted on regular glass slides in Vectashield mounting medium (Vector, H-1000) and sealed. Samples could be stored up to 6 months in a deep freezer without bleaching. The preparations were observed using epifluorescence or confocal laser scanning microscopy (CLSM). Epifluorescence was carried out on a Reichert Polyvar microscope where photographs were made. CLSM proved to be necessary for later developmental stages because of the high complexity of the muscle system. It was performed with a Leica TCS NT system mounted on a Leica DM IRBE inverse microscope. Laser light with wave lengths of 518 nm (BODIPY) and, with better results, 488 nm (Oregon green) was used. Optical sections with a distance of 1 µm (Z-series) were generated and digitally processed (Leica TCS NT software) to so-called "average projections" and stereo pairs of whole specimens.

RESULTS

General remarks

The prototroch of *Patella* and other primitive gastropods corresponds ontogenetically and phylogenetically to the velum of higher Gastropoda. Therefore we use "velar ring" instead of the more puristic "prototrochal ring" for the respective musculature.

Shell terminology follows Haszprunar et al. (1995): Primitive ("archaeo-") gastropod taxa (Patellogastropoda, Vetigastropoda, Cocculinida, Neomphalida) have an embryonic shell (protoconch I: more or less simultaneously calcified by the epithelium of the visceral hump only) which is directly followed by the adult shell (teleoconch: successively produced by the pleurally innervated mantle margin). Caenogastropoda (formerly meso- and neogastropods) and Heterobranchia (certain former mesogastropods plus Euthyneura) show in addition to the embryonic shell an often distinctly structured larval shell (protoconch II: successively built by the pleurally innervated mantle margin).

Herein we describe development from the morphological (dorsal-ventral) point of view. The anterior-posterior axis is defined by the apical organ and the tip of the protoconch opposite to the apical organ. The dorso-ventral axis is perpendicular to the anterior-posterior axis and runs through the tip of the foot.

Outlines of development

Throughout larval development, when actively swimming, the larvae move in spiral lines by means of the compound cilia of the prototroch. The orientation of the swimming larva is with the apical tuft upside (Figs. 1-3A). Larvae do not feed. Larval development of *Patella vulgata* and *Patella caerulea* is very similar. The timing of development strongly depends on temperature; relevant data given below refer to hours post fertilization (hpf) in *Patella caerulea* (unless otherwise indicated), as we cultured this species at a single constant temperature. Larvae start swimming at 8 to 9 hpf. This trochophore-like larva differentiates into an early veliger forming a foot with operculum and an embryonic shell (i.e. protoconch I, see above for terminology). The shell is formed between 15 and 20 hpf; the operculum first becomes visible 33 hpf at the posterior foot surface. Torsion (the clockwise turn of the visceral portion relative to the head-foot portion) takes place between 36 and 41 hpf. Between torsion and the phase of metamorphic competence, several larval (e.g., epipodial tentacles) and adult (e.g., creeping sole, mantle cavity, buccal apparatus, cephalic tentacles) structures are differentiated, part of which are prerequisites for post-metamorphic juvenile life. By the end of the larval phase animals are capable of both swimming and creeping. Metamorphosis is defined by the morphological event of reduction (shedding) of the velum. We found the first post-metamorphic animals at the age of 170 hpf. Metamorphic competence, however, may have preceded that timing. Because metamorphosis is rather linked to a stimulus than to timing, we could only estimate the post-metamorphic "age" of individual specimens. In *Patella* the beginning of post-metamorphic development is characterized by the loss of the operculum and epipodial tentacles, the forward growth of the mantle margin and the production of the limpet-shaped teleoconch. This leads to a permanent covering of the head and a strongly widened pallial cavity.

Pretorsional development of the muscular system

Both species show close similarities so that a common description of the ontogeny is provided. Differences will be mentioned where they occur.

In whole mounts of both *Patella* species the main larval retractor becomes visible first (Figs. 1A, 2A). It is represented by several fine muscle fibres which run along the dorsal side. In addition, the anlage of the velar (muscle) ring is represented by two bright spots at the left and right side in the apical area.

A few hours later the trochophore-like larva has further differentiated the prototroch which consists of a single row of cells, the borders of which are well visible through the actin-staining (see Serras and Speksnijder (1991) for more details). In *Patella vulgata* 4 spindle-like muscle cells (6 in *P. caerulea*) of the main retractor muscle, each with several muscle fibres, are clearly visible (Figs. 1B, 2B).

Again slightly later (Fig. 1C) the foot (ft) is formed as a bulge at the ventral (by definition, see above) side of the larva. Dorsally on the left side 5 to 6 muscle cells of the main larval retractor are visible. Apically the velar ring becomes fully developed, consisting now of several spindle-like muscle cells. The cell borders of the prototroch cells are still clearly stained (omitted in Fig. 1C). In the next stage (Figs. 1D, 2C) the foot has become more prominent and the mantle fold can be detected behind the foot. Now the main larval retractor consists of two portions: the dorsal and more central portion runs as a quite dense bundle into the apical area, whereas the smaller ventral and lateral portions run into the pedal region. The velar muscle system is a prominent ring. Two further muscular systems can be detected for the first time: the accessory larval retractor, starting with 3 longitudinal fine muscle cells in a (morphologically) ventro-terminal position (upper terminal in life position), and the pedal muscle plexus, consisting of a weak and irregular muscular grid (Fig. 1D). During the next 14 to 15 hours all four muscular systems increase in volume and prominence. Prior to torsion (Figs. 1E, 2D) the main larval retractor shows a distinct insertion area at the embryonic shell slightly to the right of the visceral hump of the larva. In *P. vulgata* 6 muscle cells (Fig. 1E: I-VI) are clearly visible: The first bundle of peripheral fibres to the left bends towards the pedal region but does not reach it, the 4 more central fibre bundles run and spread into the apical area, the far right fibres again bend downwards and run into the foot. The accessory larval retractor shows a small but distinct insertion area slightly posterior to that of the main larval retractor and consists of 4 cells, 3 of them running along the dorsal mantle and spreading into the mantle margin, plus an additional one reaching the pedal region anteriorly. The main larval retractor of *P. caerulea* consists of 9 separate muscle fibres, with 7 projecting into the velar region while the most dorsal pair runs into the foot. The accessory retractor of this species is formed by 6 myocytes, 4 of which reach into the mantle, while 2 fibres run towards the velar ring, one at its dorsal, one at its ventral pole.

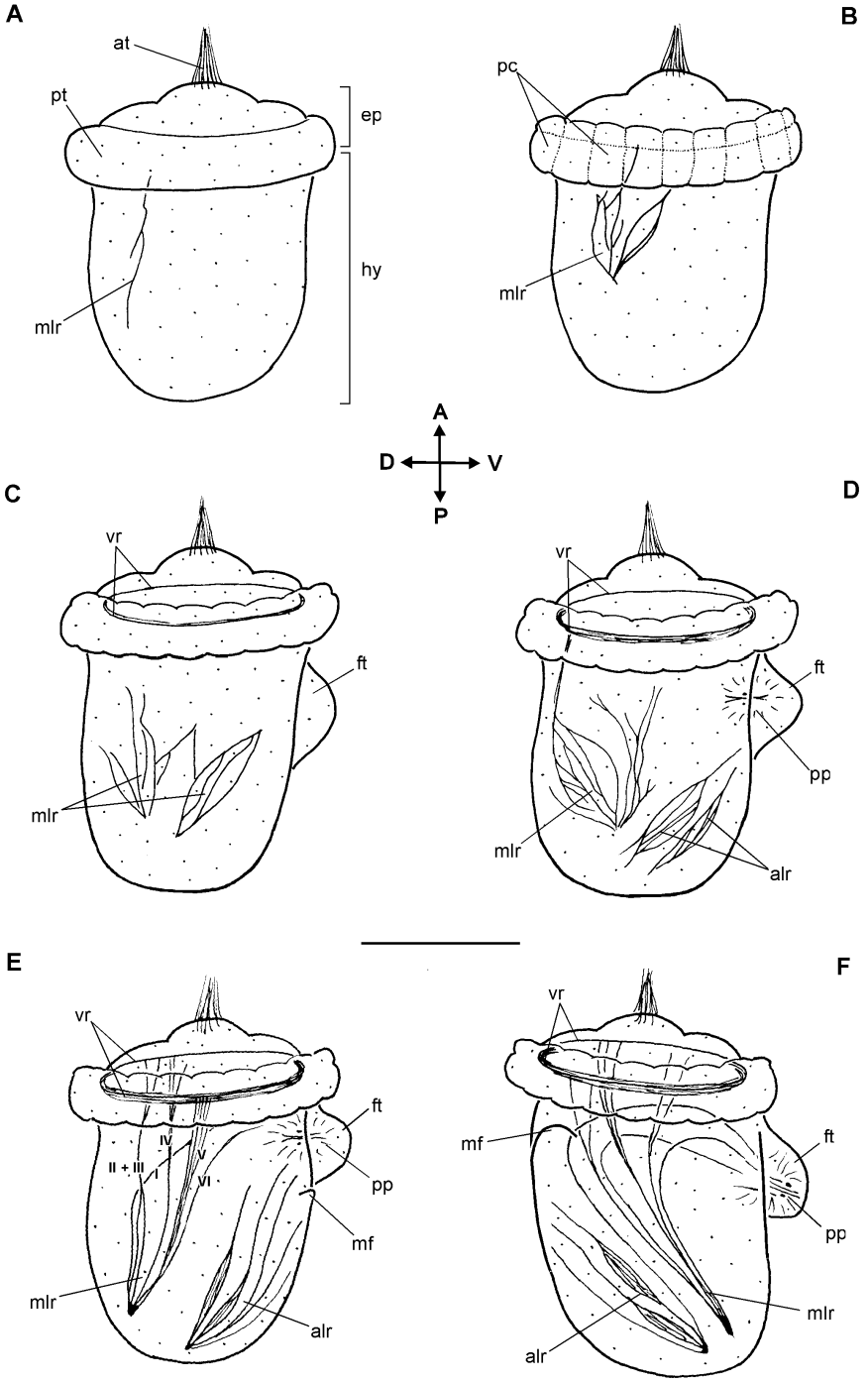


Fig. 1. *Patella vulgata*, semi-diagrammatic, myogenesis in the early larval phase. A-E. lateral view from the right. A.-B. Early trochophore-like larva. C. Trochophore-like larva with anlage of foot. D. Early veliger. E. Veliger at the onset of torsion. F. Veliger at the end of torsion (dorso-lateral view). Abbreviations: I-VI - muscle fibres of main larval retractor; A - anterior, at - apical (ciliary) tuft, alr - accessory larval retractor, D - dorsal, ep - episphere, ft - foot, hy - hyposphere, mf - mantle fold, mlr - main larval retractor, P - posterior, pc - prototroch cells, pp - pedal plexus, pt - prototroch, V - ventral, vr - velum (muscle) ring. Scale bar = 100 µm.

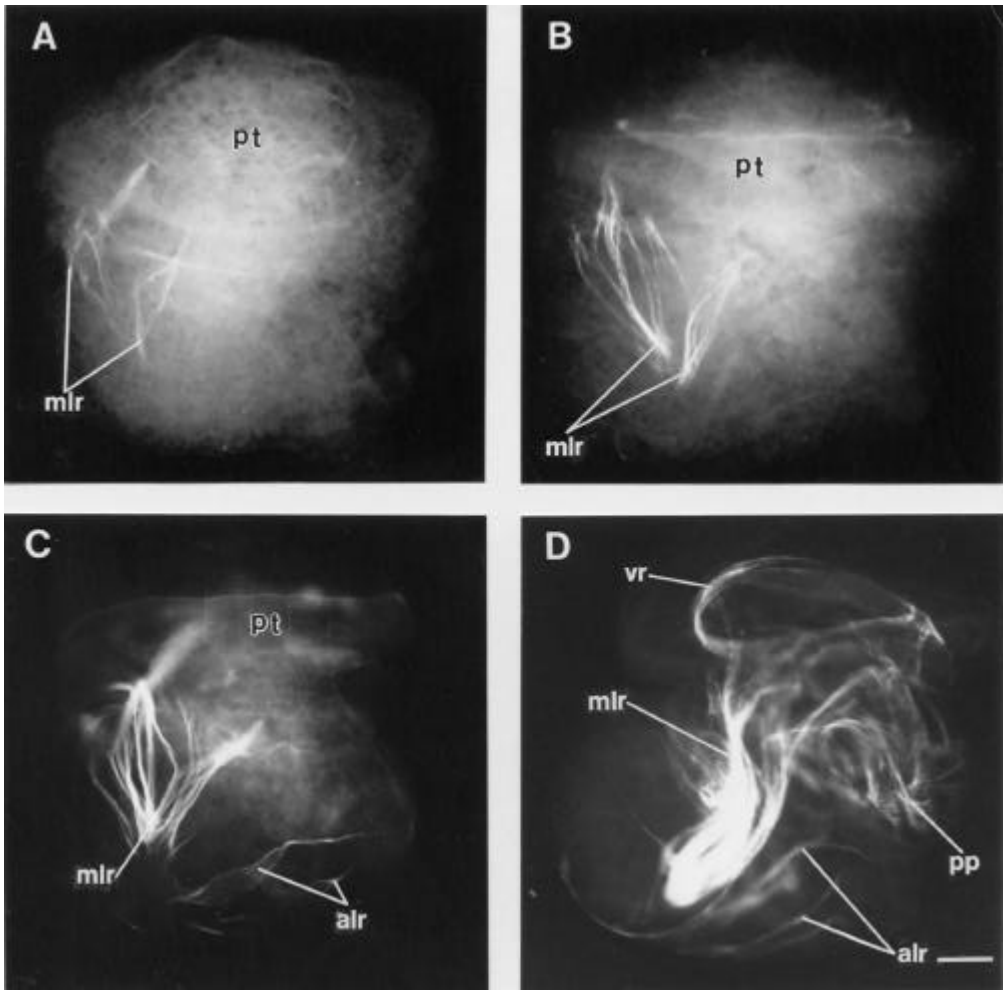


Fig. 2. *Patella vulgata*, epifluorescence micrographs, pretorsional larval stages, culture temperature: 13°C. A.-B. Trochophore-like larva, 48 / 51 hpf. C. Early veliger, 54 hpf. D. Veliger shortly before the onset of torsion, 73 hpf. Abbreviations: alr - accessory larval retractor, mlr - main larval retractor, pp - pedal plexus, pt - prototroch, vr - velum (muscle) ring.Scale bar = 25 µm.

In both species the velar ring is still prominent, the pedal plexus has become significantly stronger and shows two bilaterally symmetrical centres of muscle fibres. Until this stage, larvae are unable to retract into the shell. If disturbed they close the compound cilia of the prototroch, stop swimming and sink to the ground.

Posttorsional development of musculature in *Patella vulgata*

After torsion is completed (Fig. 1F) the muscle conditions are as follows: The whole system of the main larval retractor is twisted within itself. Whereas the insertion area of the main larval retractor is placed to the upper left of that of the accessory larval retractor, the fibres of the main larval retractor seem to be situated mainly on the right side (morphological and physiological) of the larval body. The very right fibre of the main larval retractor runs ventrally into the foot. The arrangement of muscle fibres of the accessory larval retractor, the velar ring and the pedal plexus have not been affected by torsion.

When torsion is completed the operculum is present at the dorso-posterior surface of the foot - its formation already starts prior to torsion. It is associated with two thin, symmetrical muscle fibres curving upwards from the posterior end of the foot in a half-circle into the ventral region of the larva (see *P. caerulea* in Fig. 6A). These laterally positioned muscle fibres are the anlagen of the left and right shell muscles. Later on, when the fibres of the main larval retractor arrange in pairs (see below), the number of these fibres significantly increases and the two symmetrical muscles ("left and right shell muscle" of Smith 1935) form distinct, laterally placed insertion areas at the shell (see *P. caerulea* in Figs. 3B-D, 4A-D, 6B).

During late larval development the conditions of the larval musculature change as follows: The 6 myocytes of the main larval retractor arrange in pairs forming 3 muscle bundles. 2 of these lead from the insertion area in the right posterior part of the larva straight into the head/velar region, the third, very right one forms a connection with the pedal plexus. However, the latter plexus does not directly insert at the operculum.

The accessory larval retractor ("ventral retractor muscle" of Smith 1935) consists of 4 muscle cells. 3 of these reach the mantle fold, the fourth terminates in the anterior part of the pedal muscle plexus, where it contacts the muscular grid. During posttorsional larval life 2 new, separate muscle fibres, which run dorsally of the accessory larval retractor, become visible. Towards metamorphosis the fibres of the accessory larval retractor degenerate and its insertion area at the protoconch is lost, whereas two new, circular and transversal muscle fibres occur at the apical mantle margin (compare *P. caerulea* in Figs. 4D, 5A-D, 6B-C). The pedal muscle plexus remains quite constant throughout late larval development, as does the velar ring. The future musculature of the cephalic tentacles arises as an independent system. It consists of two longitudinal, cross-bridged fibres and resembles a rope-ladder.

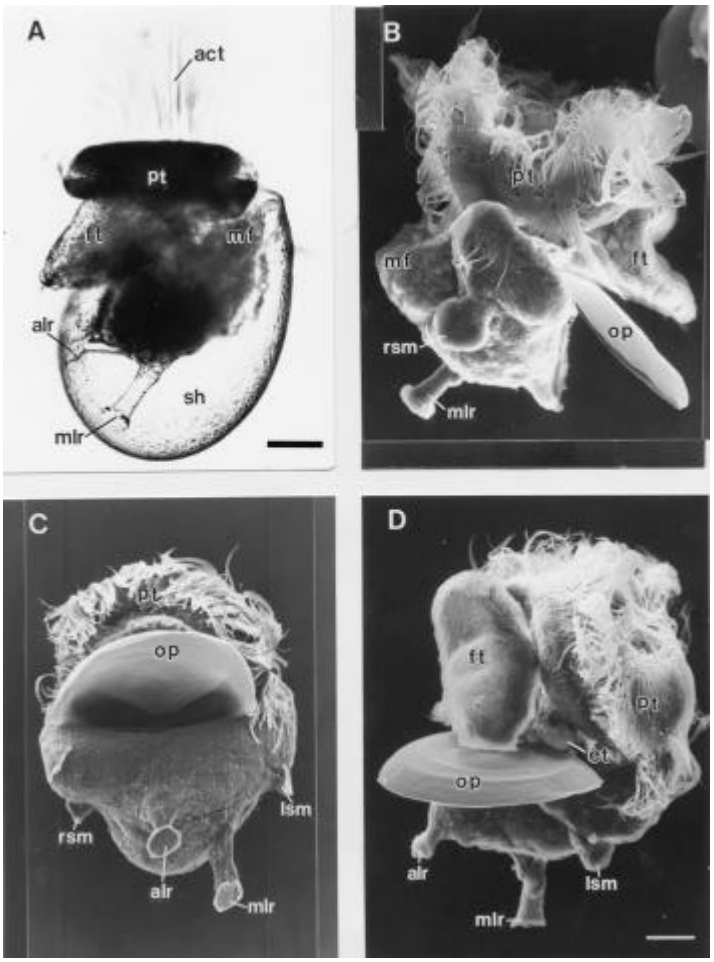


Fig. 3. *Patella caerulea*, A. light micrograph of a living larva after completion of torsion (39 hpf). Scale bar = 25 µm. B.-D. SEM micrographs of posttorsional larvae (93 hpf). Scale bar = 25 µm. B. lateral view from the right, C. ventro-lateral view from posterior end, D. ventro-lateral view from the left. Abbreviations: act - apical ciliary tuft, alr - accessory larval retractor, et - epipodial tentacle, ft - foot, lsm - left shell muscle, mf - mantle fold, mlr - main larval retractor, op - operculum, pt - prototroch, rsm - right shell muscle, sh - shell.

Development of musculature in *Patella caerulea* from torsion to metamorphic competence

The two shell muscles rapidly increase in size and volume, both originally consisting of two main bundles (Figs. 4A, 6A).

Contrary to *Patella vulgata*, the main larval retractor of *P. caerulea* consists of 4 pairs of muscle bundles plus one additional single muscle fibre. Three of these paired bundles insert at the velar ring. The fourth, most ventral bundle, reaches the apical part of the foot with one of its fibres also connecting to the ventral part of the velar muscle ring (Fig. 6A-B). The accessory larval retractor of *Patella caerulea* consists of 6 muscle cells (instead of 4 in *P. vulgata*). Four of these reach the mantle fold region, the fifth runs dorsally of the main larval retractor, the sixth terminates ventrally in the velar region. There is no connection with

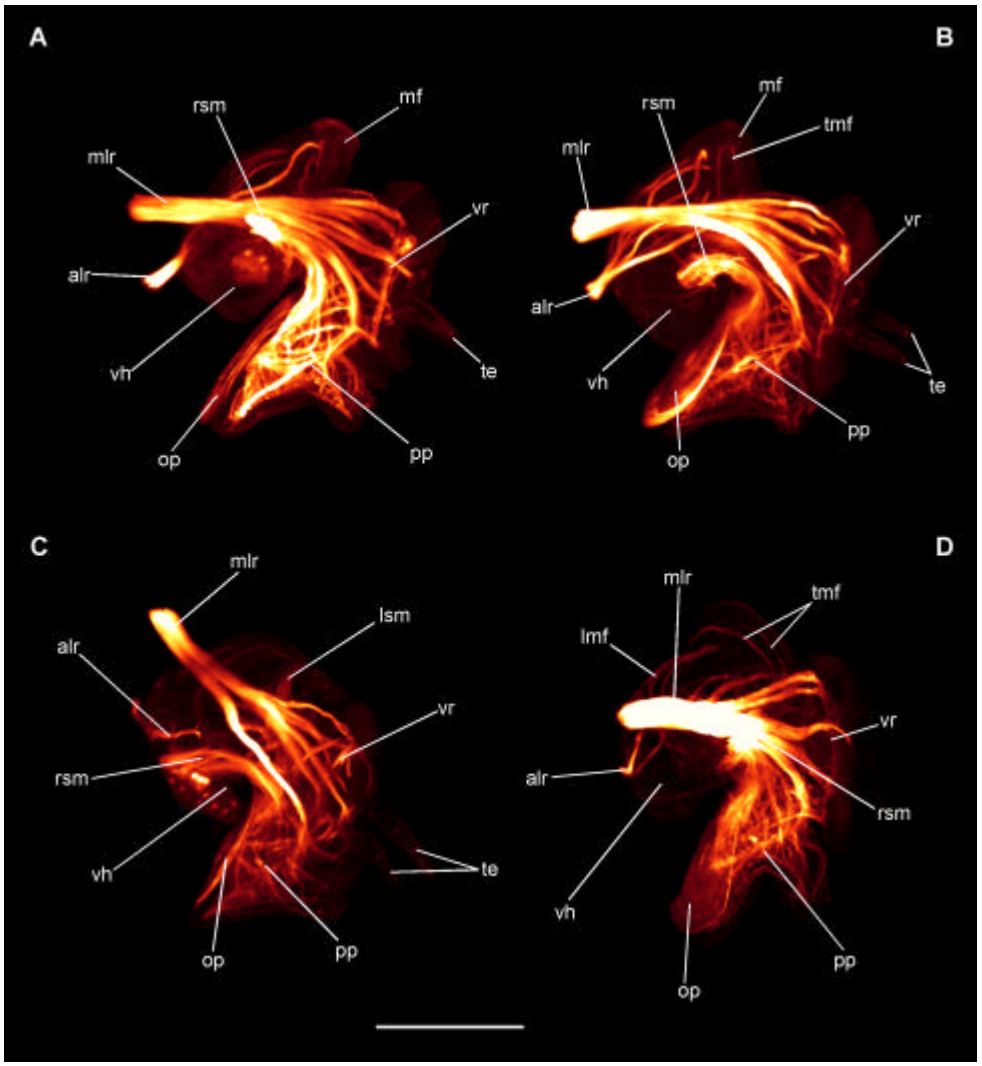


Fig. 4. *Patella caerulea*, myogenesis, CLSM, posttorsional larval stages. A. 75 hpf, B. 80,5 hpf, C. 101,5 hpf, D. 123,5 hpf. Abbreviations: alr - accessory larval retractor, lmf - longitudinal mantle fibers, lsm - left shell muscle, mf - mantle fold, mlr - main larval retractor, op - operculum, pp - pedal plexus, rsm - right shell muscle, te - cephalic tentacle, tmf - transversal mantle fibers, vh - visceral hump, vr - velum (muscle) ring. Scale bar = 100 µm.

the foot. Slightly later, there are again several (i.e. more than 2 as in *P. vulgata*) separate longitudinal muscle fibres dorsal to the accessory larval retractor, and also the 2 above-mentioned transversal fibres do occur (Figs. 4D, 5A, 6B).

Both shell muscles are thicker now and interconnect increasingly with the fibres of the pedal plexus. This significantly raises the ability of larvae to promptly retract into the protoconch if disturbed.

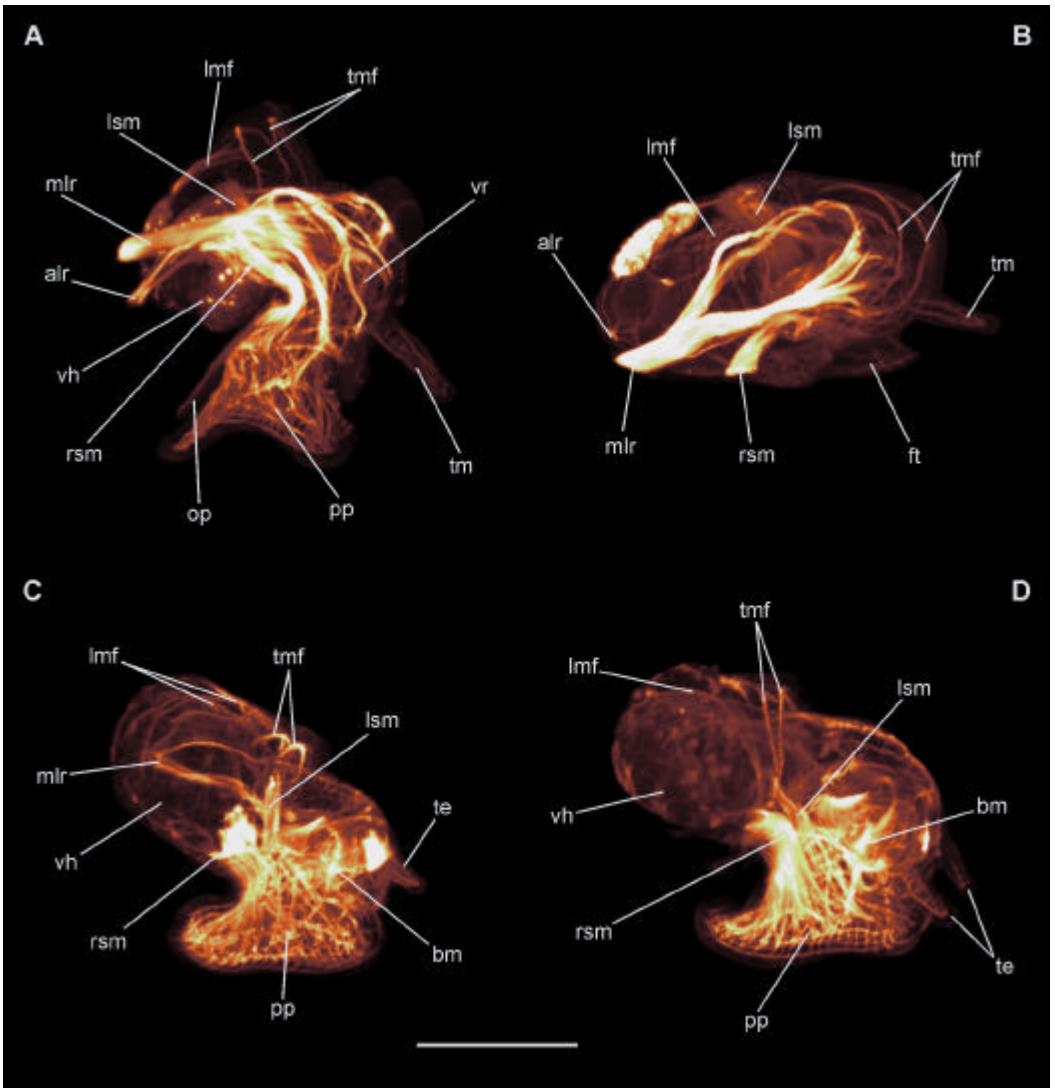


Fig. 5. *Patella caerulea*, myogenesis, CLSM, near metamorphic competence to juvenile. A. Late veliger, 147 hpf. B.-D. Juvenile with increasingly differentiated muscle system, approximately one to five days after metamorphosis. A, C, D. Lateral view from the right side. B. dorso-lateral view from slightly left (therefore alr to the left of mlr). Abbreviations: alr - accessory larval retractor, bm - buccal musculature, ft - foot, lmf - longitudinal mantle fibers, lsm - left shell muscle, mlr - main larval retractor, op - operculum, pp - pedal plexus, rsm - right shell muscle, te - cephalic tentacle, tm - musculature of cephalic tentacle, tmf - transversal mantle fibers, vh - visceral hump, vr - velum (muscle) ring. Scale bar = 100 µm.

Muscular development towards and after metamorphosis in *Patella caerulea*

Towards metamorphosis the main larval retractor remains quite prominent, whereas the accessory larval retractor degenerates (the fluorescence staining becomes relatively weaker) and its fibres become more and more inhomogeneous (Figs. 5A-B, 6B-C). Finally, the latter system loses its insertion area at the posterior protoconch so that it is no longer able to retract the animal. By contrast, the paired shell muscles become predominant (Figs. 5C-D), each showing a large insertion area at the lateral shell. Continuously newly formed fibres of the left and right shell muscle as well as several strands of the pedal muscle plexus run into the

juvenile's apical region, interconnecting the pedal and anterior muscle systems (Figs. 5C-D). The fibres of the pedal plexus also stain more and more intensively and keep growing dorsally. The fibres of the tentacular musculature likewise connect to the pedal plexus (omitted in Fig. 6C).

The velum ring disappears simultaneously with the reduction of the prototroch at metamorphosis (Figs. 5A-B, 6B-C).

The longitudinal and transversal mantle fibres remain after metamorphosis as does the main larval retractor in early postmetamorphic stages, whereas the accessory larval retractor becomes reduced. A first anlage of the future buccal musculature becomes visible between the bases of the tentacular muscle systems (Fig. 5C). Eventually, the main larval retractor loses its insertion area at the shell and is finally completely resorbed, while the buccal apparatus forms the most prominent anterior muscle system of the juvenile animal (Fig. 5D).

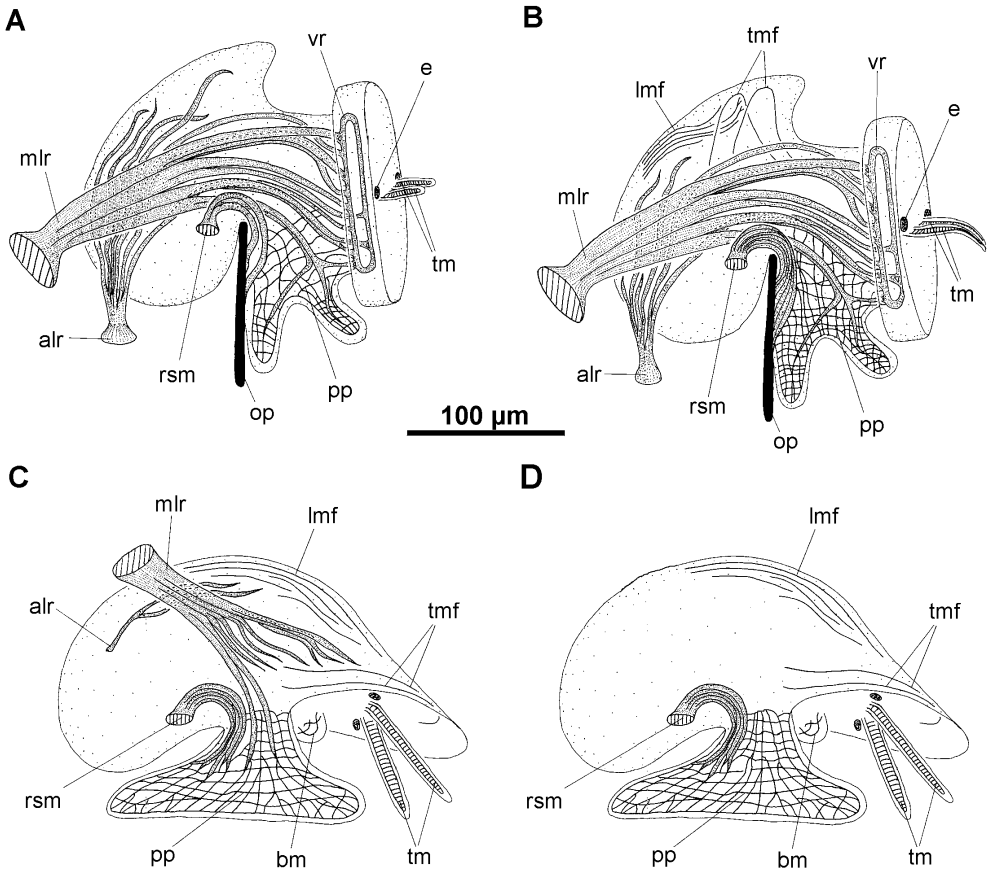


Fig. 6. *Patella caerulea*, semi-diagrammatic, myogenesis from posttorsional veliger to juvenile. A. Posttorsional veliger, 75 hpf. B. Late veliger, 145 hpf. C. Postmetamorphic juvenile, approximately one day after metamorphosis. D. Postmetamorphic juvenile, approximately five days after metamorphosis. Abbreviations: alr - accessory larval retractor, bm - buccal musculature, e - eye, lmf - longitudinal mantle fibers, mlr - main larval retractor, op - operculum, pp - pedal plexus, rsm - right shell muscle, tm - musculature of cephalic tentacle, tmf - transversal mantle fibers, vr - velum (muscle) ring. Scale bar = 100 µm.

DISCUSSION

General remarks

The combination of SEM, semithin sectioning, TEM, and specific F-actin staining by fluorescence-labelled phalloidin allows more accurate and detailed data on muscle development than classic light microscopy of living animals or of paraffin sections could provide. Rather than to blame earlier authors, who in many respects did marvellous work indeed, we want to point out the main inconsistencies and errors in earlier descriptions which could be cleared up based on combined approaches and available better methodology (see Table 1, Fig. 8).

Patellogastropoda

Concerning Patellogastropoda the data provided by Smith (1935) on *Patella vulgata* and also (much less detailed) by Anderson (1965) on three species of Lottiidae are much more accurate and correct than those by Crofts (1955) on *Patella vulgata* and *Patina pellucida*. The latter author failed to detect the second main larval shell muscle with an insertion area proper, the accessory larval retractor, and also obviously confused later stages with those of a vetigastropod (probably *Haliotis*), resulting in erroneous statements on the origin of the adult shell muscles. Unfortunately, the latter view - and not Smith's (1935) correct data on the independent origin of the adult shell muscles - was used by Fretter and Graham (1962, 1994) in their classic work on British prosobranch molluscs and so have become the standard version.

The present study provides clear evidence of the independent origin of the adult shell muscles in two respects: (1) The insertion areas of the adult shell muscles are entirely independent from those of the main and accessory larval muscles. (2) As in *Haliotis kamtschatkana* (cf. Page 1997a: figs. 11, 17, 19) the larval shell muscles are obliquely striated (not cross striated as erroneously stated by Page), whereas the adult shell muscles are smooth (Wanninger et al. 1999).

It is difficult to evaluate the significance of the differences in muscle development between *Patella vulgata* and *Patella caerulea*, because detailed data on other patellogastropod species are missing. It seems likely, however, that the general pattern, i.e., four distinct larval muscle systems and independent adult shell muscles, is characteristic for all patellogastropods, and that the exact numbers of muscle cells differ between species.

Vetigastropoda

Whereas the earlier reports by Crofts (1937, 1955) and Bandel (1982) are inaccurate (see above), recent investigations by means of electron microscopy and specific staining procedures have provided new insights and proved both earlier authors to be wrong. Page (1997a) clearly demonstrated two larval shell muscles, Degnan et al. (1997) show the velar and pedal muscle systems in their figures. Both studies provide evidence that, in contrast to Crofts (1937, 1955) and Bandel (1982), the adult shell muscles occur independently as shown by Smith (1935) and herein for *Patella*. However, according to Page (1997a), the position of the insertion area of the adult left shell muscle is close to that of the main larval retractor. It remains to be shown whether or not this last feature occurs generally in Eogastropoda (all gastropods except Patellogastropoda; cf. Ponder and Lindberg 1997), or is a vetigastropod (haliotid) synapomorphy.

As in *Patella* species, there are differences in the number of myocytes between species of *Haliotis*: whereas both *Haliotis tuberculata* (Crofts 1937) and *Haliotis rufescens* (Degnan et al. 1997) show 6 myocytes in the main larval retractor, this muscle consists of 8 myocytes in *Haliotis kamtschatkana* (Page 1997a). Similar differences might occur among trochid species.

Higher gastropods

The innervation pattern of the adult shell muscles provides clear evidence that the original left shell muscle becomes (accompanied by multiple reduction of the right shell muscle) the sole spindle muscle in gastropod evolution (Haszprunar 1985, 1988). The relationship to the spindle muscle of the veliger larva of higher gastropods is less clear, however. Two conditions are possible: (1) The larval spindle muscle of caenogastropod or heterobranch larvae is the remaining main larval retractor, or (2) the larval spindle muscle is the preformed adult shell muscle. Because of the obliquely striated nature of the larval shell muscle in nudibranchs (Page 1995) the first version is to be preferred for Nudibranchia, whereas caenogastropod conditions are still equivocal, since the fine structure of the larval spindle muscle is unknown.

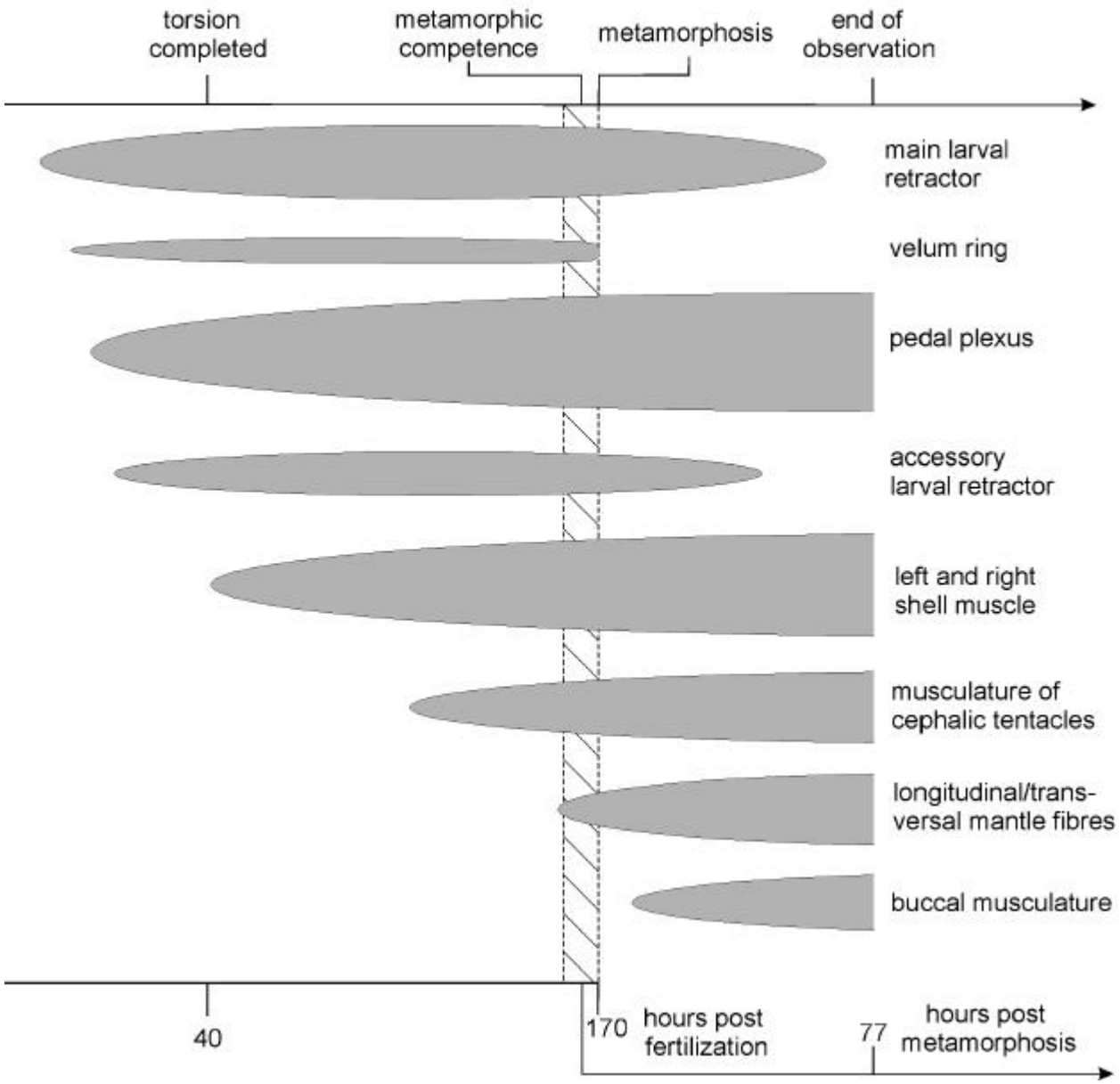


Fig. 7. *Patella caerulea*, diagrammatic sequence of myogenesis. The thickness of shaded areas indicates the relative prominence of individual muscle systems. Metamorphic competence and hours post metamorphosis are estimated.

Table 1. Comparison of descriptions of muscle development in patello- and vetigastropods (chronological arrangement)

Species / Family (Reference)	Larval muscles	Adult shell muscles
PATELLOGASTROPODA:		
Patella vulgata (Smith 1935)	"dorsal retractor muscle" (*mlr*) "ventral retractor muscle" (*alr*)	both shell muscles (*lsm, rsm*) originate independently from all larval muscles
Patella vulgata, Helcion pellucida (Crofts 1955)	"larval retractor" (*mlr*) "ventral portion of larval retractor" (*alr*?)	*mlr* + *alr* become left shell muscle, right shell muscle (*rsm*) is newly formed
3 Lottiidae (Anderson 1965)	"two columella muscles" (*mlr* + *alr*)	no data
Patella vulgata, Patella caerulea (this paper)	main larval retractor (*mlr*), accessory larval retractor (*alr*), velar muscle system (*vr*), pedal muscle plexus (*pp*)	both shell muscles (*lsm, rsm*) originate independently from all larval muscles
VETIGASTROPODA:		
Haliotis tuberculata (Crofts 1937)	"larval retractor" (*mlr*)	*mlr* becomes left shell muscle; right shell muscle (*rsm*) is newly formed
Calliostoma zizyphinum (Crofts 1955)	"larval retractor" (*mlr*)	*mlr* degenerates; "right"/spindle muscle (*rsm*) is newly formed
4 Trochidae (Bandel 1982)	"right retractor muscle" (*mlr*) "left retractor muscle" (*alr*)	*mlr* becomes the left/spindle muscle; *alr* degenerates
Haliotis kamtschatkana (Page 1997a)	"larval retractor" (*mlr*) "accessory larval retractor" (*alr*)	adult shell muscles (*lsm, rsm*) originate independently from all larval muscles
Haliotis rufescens (Degnan et al. 1997)	"dorsal and ventral portion of larval retractor" (*mlr; alr*?) not named (*vr*) not named (*pp*)	right shell muscle (*rsm*) is newly formed

CONCLUSIONS

Recent investigations based on various new methodologies and approaches have revealed the following general pattern of muscle ontogenesis in primitive Gastropoda (Patello- and Vetigastropoda) (Fig. 8):

(1) There are four different larval muscle systems, the anlagen of which occur already in the pretorsional larva: the main larval retractor, the accessory retractor muscle, the velar muscle system, and the pedal muscle plexus. Both the main and accessory larval retractors have distinct insertion areas at the embryonic shell.

(2) The accessory larval retractor and the velar ring degenerate with metamorphosis, the main larval retractor becomes reduced and finally lost during early juvenile life, whereas the pedal plexus continues into the adult animal. In *Patella* and *Haliotis* the adult shell muscles originate entirely independently from the larval musculature.

(3) At least parts of the (adult) transversal and longitudinal mantle musculature occur prior to metamorphosis and are continued and elaborated during juvenile and adult life.

(4) The musculature of the cephalic tentacles originates independently from all other muscles prior to metamorphosis, the buccal muscle system occurs after metamorphosis.

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APPENDIX VI

Muscle development and ontogenetic torsion in the limpet *Patella* (Mollusca, Patellogastropoda)

Published as parts of the article "The development of the musculature in the limpet *Patella* with implications on its role in the process of ontogenetic torsion" in: *Invertebrate Reproduction and Development* 36 (1999): 211-215.

Abstract. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) after using a fluorescent dye for filamenteous actin revealed 4 distinct muscle systems in the pretorsional larvae of *Patella*, i.e. the velum ring, the pedal plexus and the main and accessory larval retractors. After torsion, 2 adult (i.e. left and right) shell muscles arise independently from all larval muscles. In addition, tentacular as well as adult mantle and buccal musculature are formed during subsequent development. Both larval retractors and the velum ring are lost during or shortly after metamorphosis, while the pedal plexus, left and right (adult) shell muscles, tentacular, mantle and the buccal musculature continue in the adult animal. These findings, together with observations of living larvae, strongly support the theory that muscular and hydraulic activity are primarily responsible for the process of ontogenetic torsion. Shell formation in *Patella caerulea* includes an unsculptured, symmetrical embryonic shell (protoconch I) as well as the successively mineralized juvenile/adult limpet-shaped teleoconch. Considering the Patellogastropoda as the earliest offshoot of the class Gastropoda (see below), we regard the following 3 conditions as basal for gastropods: (1) the adult shell muscles arise independently from the larval shell musculature, (2) ontogenetic torsion is a primarily larval process, (3) the embryonic shell is symmetrically shaped - asymmetrical/helicoid conditions of the adult shell in higher gastropods are independent from the ontogenetic torsion process.

INTRODUCTION

Torsion, a counter-clockwise rotation of the visceropallium relatively to the cephalopodium, is the major apomorphy of the class Gastropoda and thus defines the group as a whole (e.g., Haszprunar 1988, Falniowski 1993). Its ontogenetic process has been a matter of debate for over a century (see recent review by Falniowski 1993). Although some earlier works (e.g., Smith 1935, Crofts 1937, 1955) consider larval shell muscles as being primarily responsible for this dramatic morphological twist during larval ontogeny, they strongly contradict each other concerning the genesis of both larval and adult musculature (cf. Wanninger et al. 1998). Due to this fact and because of much more powerful methodologies available today, the main aim of this paper is to present new data on the myogenesis in the limpet *Patella*, a member of the Patellogastropoda, which today is considered the most basal gastropod clade (Haszprunar 1988, Ponder and Lindberg 1997). In addition, observations on the torsion process in *Patella caerulea* are discussed, with special reference to the identified larval and adult muscles and

their putative role in this process. For detailed features on both topics, see elsewhere (Wanninger et al. 1998, in prep.).

MATERIALS AND METHODS

Animal cultures and breeding

For a detailed description, see Strathmann (1987) and Wanninger et al. (1998). The addition of a diluted sperm suspension to mature eggs marks the point of fertilization, to which the age (hours post fertilization, hpf) of larvae is referred.

SEM

Preparation of larvae and juveniles was done according to routine protocols (Bouin`s or glutaraldehyde fixative, cf. Wanninger et al. 1998 for details). To prepare shells for SEM, animals were relaxed, killed with distilled water and subsequently macerated in saturated NaClO. After that, shells were transferred into 70% and 100% acetone, air dried, mounted on SEM stubs and sputter coated (for details see Hadfield and Strathmann 1990). All SEM observations were done with a Philips XL 20 SEM.

Actin staining and confocal laser scanning microscopy (CLSM)

Preparations followed the detailed descriptions of Serras and Speksnijder (1991), Rieger et al. (1994) and Wanninger et al. (1998). Briefly, animals were relaxed and fixed in 0.1M phosphate-buffered 4% paraformaldehyde (PFA) with 10% sucrose added, or, alternatively, in 0.1M phosphate buffered 2% PFA with 15% saturated picric acid and 10% sucrose. If necessary, preparations were decalcified in 2% EDTA. Next, specimens were treated with 0.2% Triton X-100 in 0.01M phosphate buffer ("PBS-T") to permeabilize tissues. Finally, Oregon Green 514 phalloidin (1 unit in 200 µl PBS-T; Molecular Probes) was applied for F-actin staining. Specimens were mounted on glass slides in Vectashield medium (Vector), sealed, and stored at -20°C. Whole mounts were studied using a Reichert Polyvar epifluorescence microscope or, preferably, a confocal laser scanning microscope (Leica DM IRBE) with Leica TCS NT software.

RESULTS

General remarks

Both in *Patella vulgata* L. and *Patella caerulea* L., muscle development as well as shell formation and ontogenetic torsion in many respects follow the same developmental patterns. A study with detailed features on differences between both species, especially concerning myogenesis, is published elsewhere (Wanninger et al. 1998).

Myogenesis

The main larval retractor is the first muscle to arise in early pretorsional larvae, followed by the velar muscle ring, the pedal plexus and the accessory larval retractor (Fig. 1A, 2). Both retractors represent the larval shell musculature, which is completely resorbed some time after metamorphosis (Fig. 1B). Despite this, the pedal plexus stays functional in the juvenile/adult stage, forming a more and more complex 3D muscle grid. The velum ring is lost during metamorphosis, when the ciliated prototroch cells are shed off.

Immediately after torsion, both (i.e. left and right) adult shell muscles are formed (Fig. 1B, 3). They insert laterally at both sides of the shell, bend forwards in a half circle and reach into the pedal region. Thus, their insertion areas are entirely different from those of the larval retractors. Moreover, both larval and adult shell muscles can be identified at the same time in (late) veligers (Fig. 1A, 3) and show obliquely striated (larval shell muscles) versus smooth (adult shell muscles) conditions (Wanninger et al. 1998). The left and right shell muscles form the U-shaped muscle of the adult animal. Well after torsion the cephalic tentacles appear (Fig. 1A, 3), together with their rope-ladder like musculature (Fig. 1B). Both structures remain present in the adult animal.

In metamorphic competent larvae the first fibres of the adult mantle musculature arise (longitudinal and transversal mantle fibres). It is not until metamorphosis is completed, that the first anlage of the adult buccal musculature can be identified (Fig. 1B).

The process of ontogenetic torsion

The exact timing of the beginning of ontogenetic torsion strongly depends on the temperature in the culture vessels (herein 20-22°C during cultivation; however, during live observations under the microscope, the temperature might have increased for about 2 hours). In this paper only a few aspects on ontogenetic torsion in relation to muscular activity are given, rather than a quantitative analysis of the torsion process itself. A paper dealing with this special problem is in preparation.

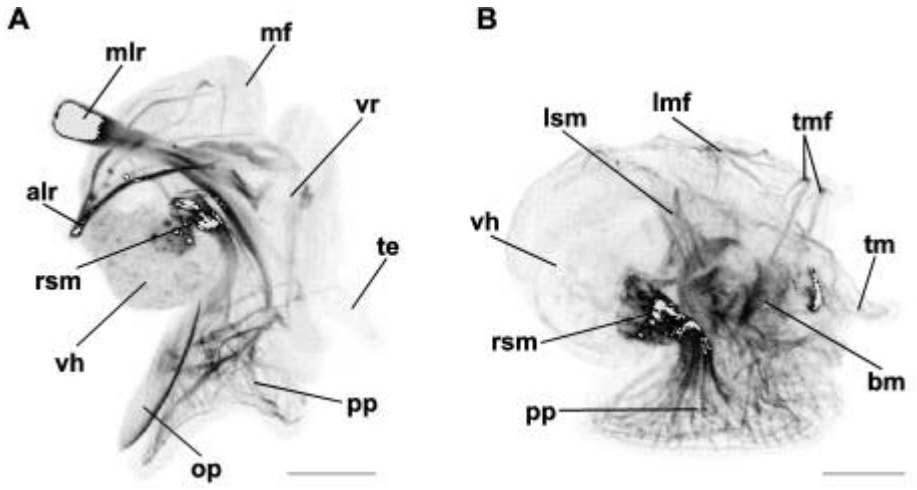

Fig. 1. *Patella caerulea*, myogenesis in posttorsional larval and juvenile stages, all CLSM. A - Posttorsional veliger at 80.5 hpf, lateral view. B - Juvenile with larval shell muscles completely lost, approx. 5 days after metamorphosis, lateral view. Abbreviations: alr - accessory larval retractor, bm - buccal musculature, lmf - longitudinal mantle fibers, lsm - left shell muscle, mf - mantle fold, mlr - main larval retractor, op - operculum, pp - pedal plexus, rsm - right shell muscle, te - cephalic tentacle, tm - musculature of cephalic tentacle, tmf - transversal mantle fibres, vh - visceral hump, vr - velum (muscle) ring. Scale bars: 50 µm.

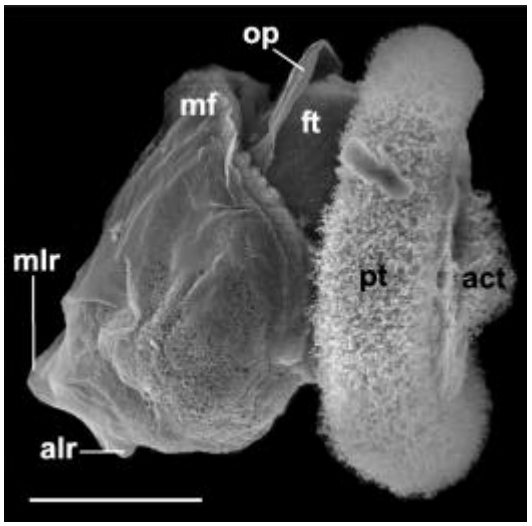

Fig. 2. *Patella caerulea*, posttorsional veliger (32.25 hpf), lateral view, SEM. Abbreviations: act - apical ciliary tuft, alr - accessory larval retractor, ft - anlage of foot, mf - mantle fold, mlr - main larval retractor, op - operculum, pt - prototroch. Scale bar: 50 µm.

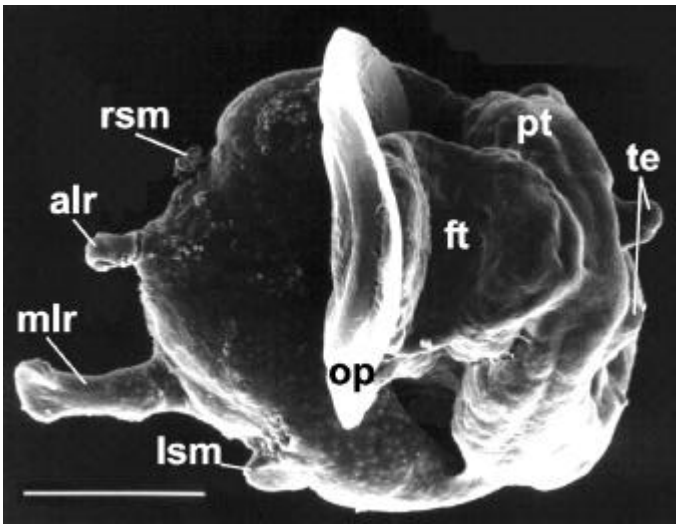

Fig. 3. *Patella caerulea*, veliger at the end of torsion (80.5 hpf), ventral view, SEM. Abbreviations: alr - accessory larval retractor, ft - foot, lsm - left shell muscle, mlr - main larval retractor, op operculum, pt - prototroch, rsm - right shell muscle, te - cephalic tentacles. Scale bar: 50 µm.

Individuals observed alive started torsion at about 36-39 hpf. By this time the operculum is already well developed (Fig. 2). In *Patella caerulea* the full 180° rotation only takes about 2 hours and is carried out in one single phase with a constant speed (i.e. there is no "slow" and "fast" phase, see Discussion). Thus, every 45° twist takes about 30 minutes. During the whole time both larval retractors contract cramp-like every 30 seconds, followed by peristaltic

movements ("pumping") of the foot. Despite this, animals are not able to retract fully into the shell until torsion is completed.

Shell formation

In *Patella caerulea* the embryonic shell (protoconch I) is formed at about 15-20 hpf. It is symmetrical, unsculptured (Fig. 4) and shows simultaneous calcification by the epithelium of the visceral hump (Bandel 1982). In primitive gastropod groups (including Patellogastropoda) the embryonic shell is directly followed by the adult teleoconch, which is not formed at a continuous speed, and therefore shows areas of different intensity of calcification (Fig. 4). Lateral clefts on both sides of the embryonic shell, as described for Vetigastropoda (Bandel 1982, Page 1997a), are lacking. Obviously, in *Patella caerulea* the right part of the juvenile teleoconch undergoes faster growth than its left counterpart (Fig. 4).

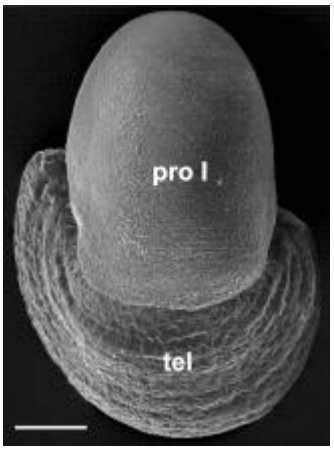

Fig. 4. *Patella caerulea*, embryonic shell (protoconch I) followed by adult shell (teleoconch), approx. 1.5 days after metamorphosis, antero-dorsal view, SEM. Abbreviations: pro I - protoconch I (i.e. embryonic shell), tel - teleoconch (i.e. adult shell). Scale bar: 50 µm.

DISCUSSION

The present study supports Smith (1935), who already described the independent origin of larval and adult shell muscles in *Patella vulgata*, although he identified the accessory larval retractor ("ventral retractor muscle") not until torsion is completed. In contrast, Crofts' (1955) statement of only one larval retractor, which continues in the adult animal where it forms the left shell muscle, has to be rejected. Recent data on other gastropod taxa - especially the slightly higher evolved *Haliotis* (Vetigastropoda) (Degnan et al. 1997, Page 1997b) - show similar conditions concerning the indepence of larval and adult shell muscles, which are regarded as a basal condition in gastropods. In *Haliotis*, the main larval retractor degenerates

too, but its insertion area is replaced by that of the left adult shell muscle. This may mark a trend among higher gastropods, where the adult shell muscle(s) might successively replace their larval precursors. However, data on the myogenesis in higher gastropods are scarce, so further conclusions in this field remain hypothetic.

Earlier descriptions of the ontogenetic torsion process of various gastropod families (Patellidae, Acmaeidae, Haliotidae, Trochidae) indicate, that the duration of the 180° rotation might range from 3 minutes (Boutan 1899) to 200 hours (Crofts 1937, 1955). Nevertheless, nearly all authors found a different time span for both 90° phases: whereas Crofts (1937, 1955) and Underwood (1972) observed a quick first and a slow second 90° twist, Smith (1935) describes a slow first and a quick second phase. According to these authors, the quick phase is caused by muscular activity of the larval retractors, while the slow phase is due to differential cell growth. However, the results presented here (continuous contraction during a constantly quick rotation process) indicate, that hydraulic as well as muscular activity of both larval shell muscles are the sole inducers of ontogenetic torsion at least in patellid limpets. Thus, the condition of ontogenetic torsion as a primarily larval process - with no major adult (muscle) structures being involved - can be regarded as basal for the class Gastropoda.

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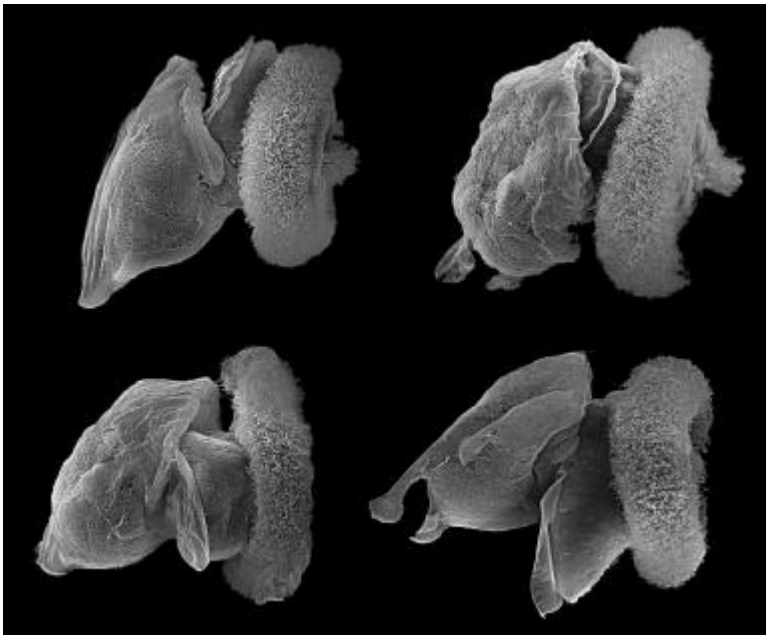
APPENDIX VII

The ontogeny, timing, and mechanisms of the torsion process in *Patella caerulea* (Mollusca, Patellogastropoda)

Published as parts of the article "Torsion in *Patella caerulea* (Mollusca, Patellogastropoda): ontogenetic process, timing, and mechanisms" in: *Invertebrate Biology* 119 (2000): 177-187.



"On ne peut actuellement affirmer que cette torsion a lieu dans tous les cas avec la même rapidité ... "

["One can actually not propose that this torsion occurs with equal speed in every case ... "]

Louis Boutan (1902: 243).

Abstract. Torsion is a process in gastropod ontogenesis where the visceral body portion rotates by 180° relative to the head/foot region. We investigated this process in the limpet *Patella caerulea* by using light microscopy of living larvae, as well as scanning electron microscopy (SEM) of larvae fixed during the torsion process. The completion of the 180° twist takes considerably less time in larvae of *Patella caerulea* than previously described for other basal gastropod species. At a rearing temperature of 20--22°C, individuals complete ontogenetic torsion within 2 hours. Furthermore, the whole process is monophasic, i.e. carried out at a constant speed, without any evidence of distinct "fast" or "slow" phases. Both larval shell muscles --- the main and the accessory larval retractor --- are already fully contractile before the onset of torsion. During the torsion process both retractors perform cramp-like contractions approximately every 30 seconds, which are followed by hydraulic movements of the foot. However, retraction into the embryonic shell occurs only after torsion is completed. The formation of the larval operculum is entirely independent from ontogenetic torsion and starts before the onset of rotation, as does the mineralization of the embryonic shell. The reported variability regarding the timing (mono- versus biphasic; duration) of torsion in basal gastropod species precludes any attempt to interpret these data phylogenetically.

The present findings indicate that the torsion process in *Patella caerulea*, and probably generally in basal gastropods, is primarily caused by contraction of the larval shell muscles in combination with hydraulic activities. In contrast, the adult shell musculature, which is independently formed after torsion is completed, does not contribute to ontogenetic torsion in any way. Thus, fossil data relying on muscle scars of adult shell muscles alone appear inappropriate to prove torted or untorted conditions in early Paleozoic univalved molluscs. Therefore, we argue that paleontological studies dealing with gastropod phylogeny require data other than those based on fossilized attachment sites of adult shell muscles.

INTRODUCTION

Although the ontogeny of gastropod torsion --- a counter-clockwise 180° rotation of the visceropallium relative to the cephalopodium and the key apomorphy of the class --- has been studied for over a century (e.g., Amaudrut 1898; Boutan 1899; Drummond 1902; Robert 1902; Smith 1935; Crofts 1937, 1955; Régondaud 1961; Underwood 1972; Bandel 1982; Voltzow 1987, 1996; Page 1997b), remarkably few detailed investigations about its exact timing as well as its possible (biomechanical) cause(s) exist until today. Nevertheless, there have been extensive discussions about its phylogenetic significance throughout the past

decades (e.g., Lang 1891; Pelseneer 1892; Grobben 1899; Boutan 1902; Naef 1911; Garstang 1928; Crofts 1937, 1955; Morton 1959; Ghiselin 1966; Underwood 1972; Giusti 1981; Pennington and Chia 1985; Edlinger 1988a; Haszprunar 1988, 1989; Falniowski 1993). Moreover, paleontological studies in malacology have tried to reveal whether adult shell muscle attachment sites, represented by "muscle scars" on fossilized adult molluscan shells or steinkerns, once belonged to already torted gastropod molluscs, or whether these animals were yet untorted and either possible gastropod ancestors or members of Paleozoic gastropod sister clades (Wenz 1940; Knight 1947; Rollins and Batten 1968; Runnegar and Pojeta 1974; Runnegar 1981; Yochelson and Gil Cid 1984). Recently, new light was shed on this subject by Page (1997a, b) and Wanninger et al. (1999a, b), who showed that the larval shell muscles, which are fully contractile before torsion, are completely resorbed towards or shortly after metamorphosis (i.e. a long time after torsion has already been completed) in basal Patello- and Vetigastropoda. In contrast, the adult shell musculature arises *de novo* after the completion of torsion. Therefore, muscle scars on fossilized adult shells or steinkerns are inadequate to identify the respective specimen as an ancient torted gastropod mollusc.

According to literature data, the time span covered by the torsion process ranges very widely, namely from 2--3 minutes in both a patello- and a vetigastropod (Boutan 1899) to as much as 200 hours in a vetigastropod (Crofts 1937, 1955) (Table 1). The hypotheses held by the various authors about the main cause(s) of this ontogenetic twist show similar variability. The most popular ones are differential growth, often used to explain a slow torsion process, and muscular activity, held responsible for quick rotational phases (cf. Table 1). Because of the new data on the myogenesis in *Patella vulgata* and *Patella caerulea* (Wanninger et al. 1999b), as well as to evaluate the significance of the torsion process for the paleontological record, its ontogeny was studied in veligers of the limpet *Patella caerulea* Linnaeus 1758, a member of the most basal gastropod taxon, the Patellogastropoda (see, e.g., Haszprunar 1988; Ponder and Lindberg 1997).

MATERIALS AND METHODS

Animal culture and breeding

Procedures were described in detail by Wanninger et al. (1999b). Presumably adult specimens of *Patella caerulea* with shell diameters of 30 mm and more were collected from intertidal rocks in the Northern Adriatic Sea near Trieste or Rovinj and transferred to the Zoological States Collections Munich (ZSM), where they were kept alive in artificial seawater.

Table 1. The process of ontogenetic torsion in Patello- and Vetigastropoda. Note (1) the great variation in time (due to different species and/or author) described as required to perform the 180° twist, (2) the different observations concerning a biphasic torsion process, and (3) the different hypotheses given by the authors to explain its biomechanical cause(s).

Species/TAXON [Reference]	Time span for the whole 180° twist	2 distinct phases? (0°--90°/90°--180°)	Proposed (main) cause(s)	Temperature
PATELLOGASTROPODA				
Patella caerulea [this paper]	2 hrs	no; torsion is a monophasic process within 2 hours	the asymmetrical larval retractors plus hydraulic activity of the foot	20°--22°C
Patella vulgata [Smith 1935]	40--50 hrs	0°--90°: 30--40 hrs 90°--180°: about 10 hrs ("next few hours")	not exactly determined, but differential growth and activity of larval retractors are involved	no data
Patella vulgata [Crofts 1955]	36--45 hrs	0°--90°: 10--15 hrs 90°--180°: 26--30 hrs	activity of larval retractor; mainly differential growth; pedal musculature assists torsion	no data
Patella vulgata [Dodd 1955]	48 hrs	no data	no data, but "retractors of larval shell are present" at the onset of torsion	12.5°+/-0.5°C
Patina (=Helcion) pellucida [Crofts 1955]	no data (similar to *Patella vulgata*?)	0°--90°: no data (similar to *Patella vulgata*?) 90°--180°: 26--30 hrs (as in *Patella vulgata*)	as in *Patella vulgata* as in *Patella vulgata*	no data
Cellana radiata [Rao 1975]	18 hrs	no data	no data	26°+/-1°C
Acmaea virginea [Boutan 1899]	2--3 min	no data	antagonism between foot and visceral hump	no data
Acmaea testudinalis [Kessel 1964]	”less than one hour”	no data	no data	12.1°+/-0.5°C
Notoacmaea petterdi [Anderson 1965]	≤17 hrs (cf. Figs. 3--4)	no data	no data, but "neither ... nor muscular activity... are observed during this time"	20°C

Table 1 (continued)

Patelloida alticostata [Anderson 1965]	"towards the end of the second day"	no data	no data	20°C
VETIGASTROPODA				
Haliotis tuberculata [Boutan 1899]	2--3 min	no data	antagonism between foot and visceral hump	no data
Haliotis tuberculata [Crofts 1937, 1955]	about 200 hrs	0°--90°: 3--6 hrs 90°--180°: about 200 hrs	contraction of larval retractor; operculum might mechanically support the beginning of torsion; mainly differential growth; pedal musculature assists the progress of torsion	no data
Haliotis kamtschatkana [Voltzow 1987]	no data (about 24 hrs?)	0°--90°: no data (3--6 hrs as in Crofts?) 90°--180°: 18 hrs	no data, but "larval retractor ... appears fully functional before, during, and after the torsion process"	12°C
Haliotis kamtschatkana [Page 1997b]	20 hrs	no; each phase takes 10 hrs	probably differential growth, but "a possible role for muscles ... can not be excluded"	12°--13°C
Margarites helicinus [Holyoak 1988a]	0.5--1.5 days	no data	no data	7°--9°C
Trochus striatus (plus 5 other Trochidae) [Robert 1902]	6--8 hrs	no data	no data	no data
Trochus niloticus [Heslinga 1981]	no data	0°--90°: 4 hrs 90°--180°: no data	no data no data	27°--30°C
Gibbula cineraria [Underwood 1972]	48 hrs	0°--90°: 8 hrs 90°--180°: 40 hrs	"contraction of the larval retractor"; "differential growth of different parts of the shell"	12°C
Calliostoma zizyphinum [Crofts 1955]	36 hrs	0°>90°: 4 hrs >90°--180°: 32 hrs	activity of larval retractor; mainly differential growth; pedal	no data

Table 1 (continued)

			musculature assists torsion	
Calliostoma ligatum [Holyoak 1988b]	1.5 days	no data	no data	7°--9°C
4 Trochidae [Bandel 1982]	no data	no data	differential growth of the epithelium of the visceral hump; no muscular activity involved	no data

The gonads of ripe animals were obtained by dissection, all further fertilization as well as culture and breeding procedures were carried out in Millipore-filtered artificial seawater. Both larvae and adults were kept at a temperature of 20--22°C, i.e., within the range of the ambient seawater temperature in the field (15--25°C) during the reproductive season of *Patella caerulea*.

Live observations

Frequent fertilizations were carried out throughout 1997 and during spring 1998. The earliest onset of torsion occurred at 32 hours post fertilization (hpf). Accordingly, specimens were observed individually from 32 hpf onwards, either under a stereo microscope or --- for exact determination of their torsion state --- on a depression slide under a compound microscope (Leica DM RBE). The whole 180° rotation was studied individually in about 60 specimens.

Scanning electron microscopy (SEM)

All specimens were relaxed by adding drops of 7.14% $MgCl_2$-solution prior to fixation. Larvae were fixed either in 4% glutaraldehyde in 0.2M sodium cacodylate buffer with 0.1M NaCl and 0.35M sucrose and postfixed with 1% OsO_4 in 0.2M sodium cacodylate buffer with 0.3M NaCl for 2 hours (see Wanninger et al. 1999b) or, preferably, in hot (30--60°C) Bouin's fluid. The latter fixative causes homogeneous shrinking of the prototrochal cilia, which prevents major parts of the animal (e.g. foot, operculum, mantle fold) from being covered with them during preparation. This provided a better overview of the general morphology of larvae at the cost of detailed epithelial structure which was better preserved by the glutaraldehyde-osmium fixation. Approximately 50 to 120 individuals were fixed in intervals of 15 minutes between 32 hpf and 40.5 hpf. Fixed specimens were dehydrated in an acetone series (Bouin's fixed specimens: 70% to 100%, glutaraldehyde-osmium fixed specimens: 30% to 100%), critical point dried, mounted on SEM stubs and sputter coated. Observations were carried out using a Philips XL 20 SEM.

RESULTS

General notes

(1) All orientations refer to Wanninger et al. (1999b), with the foot defining the ventral side. This implies that the positions of the mantle fold and the mantle cavity change from the pretorsional ventral to the posttorsional dorsal side due to the 180° rotation of the head/foot

relative to the visceral portion. In the following, posttorsional orientation is given unless otherwise indicated.

(2) For definition of torsion stages, see Fig. 1A3--E3. For better understanding, the whole 180° twist is divided into steps of 45° movements. This results in rotation stages of 0° (pretorsional), 45°, 90°, 135° and 180° (posttorsional). *In vivo*, however, torsion occured as a monophasic, gradual process in *Patella caerulea*, not as single "pulses" of rotation (see below). The torsion angle (α) is defined by 2 imaginary projection lines (Y, Z): line Y runs through the anterior tip of the mantle fold (arrow) and the most posterior point of the larva's visceral hump (X). Line Z marks the connection of the axis through the operculum with X, the point where both lines (Y and Z) meet, thus forming the torsion angle α.

The torsion process in *Patella caerulea*

In the lecithotrophic larvae of *Patella caerulea*, ontogenetic torsion started between 32 and 39 hours post fertilization (hpf) (T=20--22°C). Individuals which remained completely untorted beyond 40 hpf seemed to be misdeveloped and most of them died during subsequent development. However, certain untorted, free swimming veligers, which had obviously retarded development, could be found in the culture dishes even several days after the first individuals had already metamorphosed. All specimens which underwent torsion proved healthy at least until they reached metamorphic competence (i.e. at around 170 hpf, see Wanninger et al. 1999b). In the pretorsional larva the foot lies on the same side as the mantle fold and is situated between the prototroch and the opening of the mantle cavity (Fig. 1A1-A3).

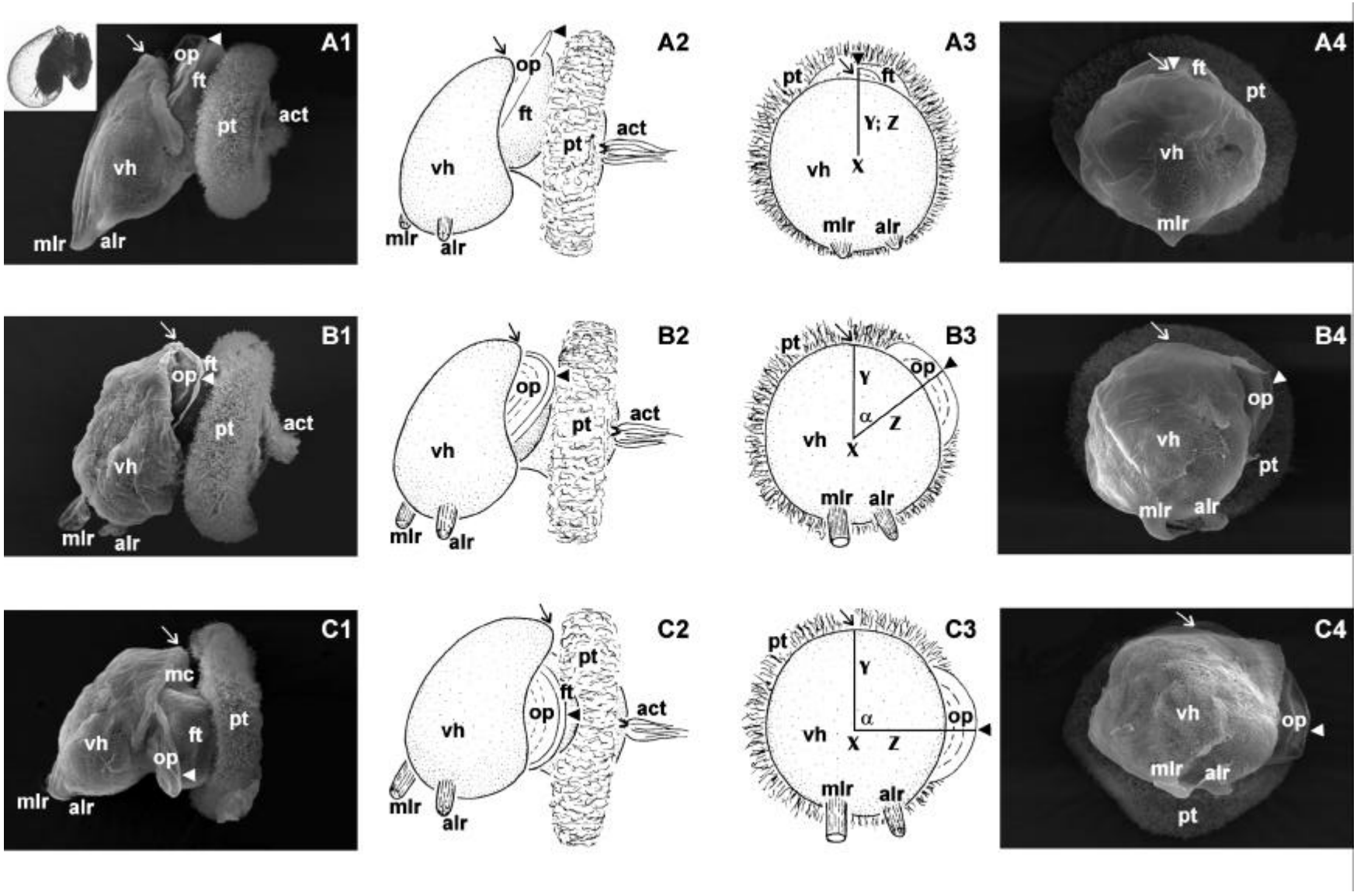
At the onset of torsion the operculum and the calcified embryonic shell are already well developed and both larval shell muscles are fully contractile. Their attachment sites on the embryonic shell (protoconch I, see Haszprunar et al. 1995, Wanninger et al. 1999b for definition) are asymmetrical, with the main larval retractor inserting posterior of the visceral hump while the accessory larval retractor meets the shell at the posttorsional ventral side of the larva (Fig. 1A1, E1 insets). Seen from a postero-dorsal angle, the accessory larval retractor is situated on the lower right side of the main larval retractor (Fig. 1A3, B3--B4, C3--C4, D3--D4, E3--E4). Both muscles perform simultaneous contractions approximately every 30 seconds. These occur cramp-like, starting with 1 powerful contracting movement which is usually followed by several less powerful contractions. Due to the fact that the foot still lies between the mantle fold and the prototroch, retraction of the cephalopedal region of the larval body into the embryonic shell is not yet possible. Instead, the embryonic shell seems to act

antagonistically against the activity of the larval shell muscles, resulting in a clockwise movement of the head/foot region relative to the visceral portion. In morphological terms (ventral side defined by the foot remains constant) the visceral hump rotates counter-clockwise to the head/foot. The muscular contractions are followed by slow, gradual "pumping" movements of the larval foot. Light microscopical observations of larvae *in vivo* show, that body fluid is peristaltically transported from the visceral part to the pedal region of the animal, which causes the foot to swell and become elongated. After 30 seconds the next series of muscular contractions occurs, followed by hydraulic movements and so on.

30 minutes after the onset of torsion, an angle of 45° exists between the Y- and Z-Lines (Fig. 1A1--A4; note that in B3--E3 and B4--E4 the foot is represented by the position of the operculum which is attached to the posterior part of the foot). Due to its circular structure the region of the prototroch misleadingly seems to be unaffected by torsion, while, seen from the larva's posterior end, the foot performs a clockwise twist relative to the visceropallium (Fig. 1). Another 30 minutes later, the axis of the operculum (Z-Line) runs perpendicular to the Y-Line through the mantle tip (Fig. 1C1--C4). Thus, the posttorsional dorsal opening of the mantle cavity is visible for the first time (Fig. 1C1). However, retraction into the embryonic shell remains impossible until completion of the full 180° twist. A further half hour later, 135° of rotation is achieved (Fig. 1D1--D4). About 2 hours after the onset of torsion all specimens have completed the 180° twist. The foot with its attached operculum lies on the opposite side of the mantle fold, which is now located on the dorsal side of the larva (Fig. 1E1--E4).

The larval operculum and shell muscles during torsion

Besides the dramatic morphological change caused by the 180° twist itself, other developmental progresses can be recognized during the torsion process (Fig. 1). The foot grows steadily and is prominent after the completion of torsion. The formation and growth of the larval operculum occurs independently of the ontogenetic torsion process, since opercular formation starts in late pretorsional larval stages (Fig. 1A1--A3). Moreover, opercula equal in size to those of regularly torted specimens are formed by (misdeveloped?) larvae even if they remain untorted for several days, i.e. when regular specimens have already reached metamorphic competence.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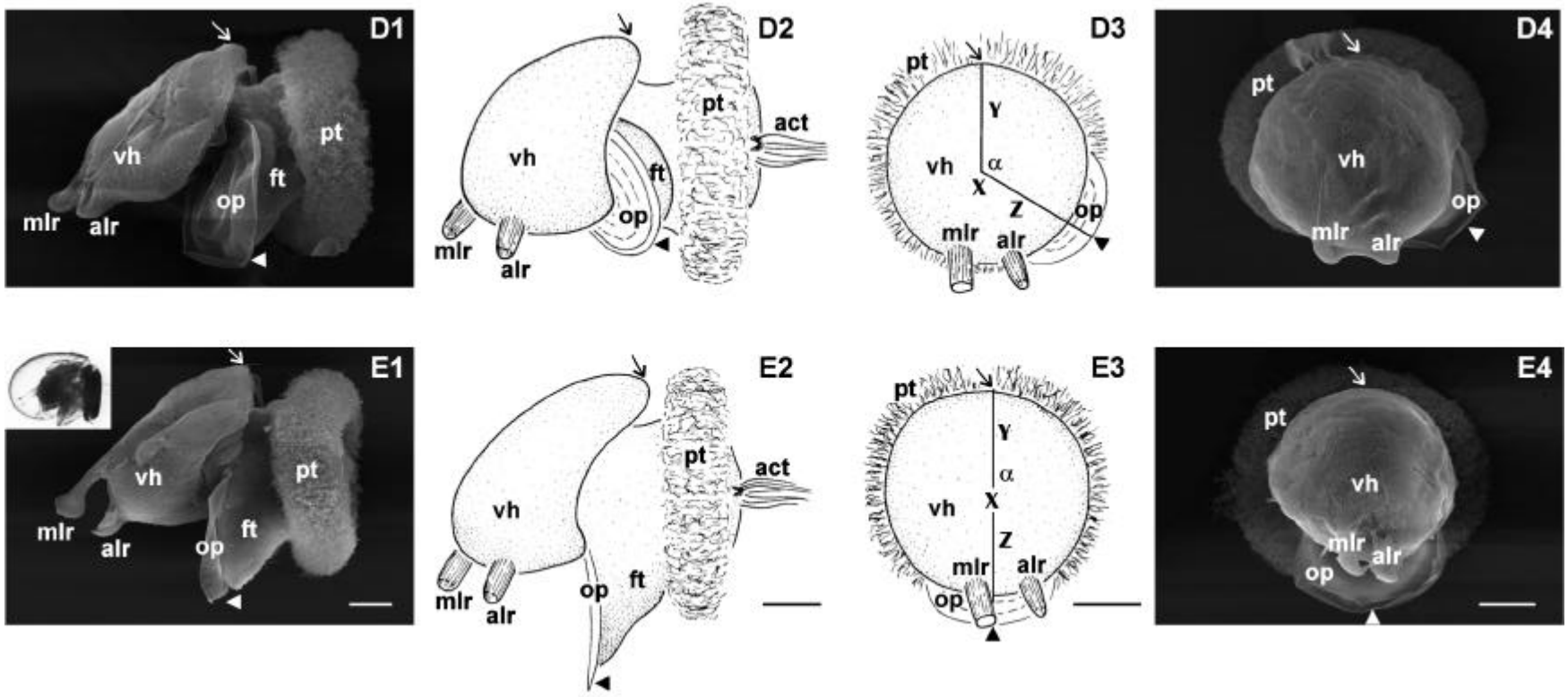

Fig. 1. Ontogeny of the torsion process in *Patella caerulea*. Far left (1) and far right (4) columns present SEM images of the 180° twist of the cephalopodium relative to the visceropallium. The inner columns (2, 3) provide semidiagrammatic drawings according to the adjacent SEM pictures. The view in columns (1) and (2) is lateral from the right, in columns (3) and (4) postero-dorsal (with respect to the posttorsional morphological orientation). Torsion is described as the subsequent establishment of a 180° angle between 2 imaginary projected lines (Y; Z), which both meet at the most posterior pole (X) of the larva's visceral hump (vh). This gives rise to the torsion angle α, which develops from 0° in the pretorsional state (A1--A4) to 180° at the completion of torsion (E1--E4). Note that both larval shell muscles, the main (mlr) and accessory larval retractor (alr), are well developed and functional at the onset of torsion (A1--A3; alr not yet formed in A4). **A1--A4.** Pretorsional larvae at the onset of torsion (A1--A3); larva in A4 has slightly retarded development compared to the specimens in A1--A3, thus lacking the operculum (op). Instead, the foot (ft) is visible (age: A1. 36.5 hpf., A4. 33 hpf). **B1--B4.** Larvae at 45° of torsion (age: B1. 32.75 hpf., B4. 36 hpf.). **C1--C4.** Half torted veligers at 90° of torsion (age: C1. 35.25 hpf., C4. 35 hpf.). **D1--D4.** 135° of torsion achieved (age: D1. 38.25 hpf., D4. 36.25 hpf). **E1--E4.** Torsion completed, foot (ft) and operculum (op; arrowhead marks the outer point of its median axis) are situated opposite the mantle fold (arrow) (age: E1. 38 hpf., E4. 35.5 hpf). Insets in A1 and E1 show light microscopy photographs of pre- (A1) and posttorsional (E1) larvae *in vivo*. Note the asymmetrical attachment sites of both larval retractors. Further abbreviations: act - apical ciliary tuft, mc - mantle cavity, pt - prototroch. Scale bars equal 25 µm for each column.

Both larval shell muscles, the main larval retractor and the accessory larval retractor, insert at the embryonic shell and penetrate the visceral hump. Prior to torsion, the trunk of the main larval retractor is a short, relatively thin muscle (Fig. 1A1--A4). Yet, the accessory larval retractor is hardly recognizable in SEM examinations and its shell attachment site is only represented by a small bulge emerging from the epithelium of the visceral hump (compare Figs. 1A1 and 1A3: the latter specimen is slightly younger and has not yet reached the stage where torsion starts. Thus, the operculum and the accessory larval retractor are not as clearly detectable by SEM in Fig. 1A3 as they are in Fig. 1A1). However, at the onset of torsion, both larval retractors are fully contractile and insert at the embryonic shell (see Fig. 1A1 (inset) and Wanninger et al. 1999b). During torsion both muscles grow in length and diameter. When the total 180° rotation is completed, both larval shell muscles have reached their maximum size, ready to retract the animal into the shell, which can now be closed with the larval operculum.

DISCUSSION

General notes

Developmental timing strongly depends on the temperature under which organisms are maintained. In the present study we document ontogenetic torsion in *Patella caerulea* under controlled laboratory conditions. Our results provide evidence about possible ontogenetic mechanisms for that process in basal gastropods. Comparing these findings with previous studies (see Table 1), it is important to note (a) the different temperatures under which the larvae were cultured, and (b) the various gastropod species investigated, showing major differences concerning their general development, e.g. intracapsular development or brooding in trochids (see Hickman 1992) versus a free swimming larval stage in patellogastropods (Wanninger et al. 1999b). Those kinds of factors strongly influence larval ontogeny as a whole, including the process of ontogenetic torsion.

Causes of ontogenetic torsion: old and new data

A brief overview of major accounts on the torsion process in several patello- and vetigastropod species and its hypothetical cause(s) is given in Table 1. Some of the more detailed studies divide the 180° twist into 2 distinct phases (0°--90° and 90°--180°) of which either the first 90° proceed quicker than the second 90° (e.g., Crofts 1937, 1955; Underwood 1972), or vice versa (e.g., Smith 1935). Other publications lack information about a mono- or

biphasic torsion process and provide only general information about the time taken for the entire 180° rotation (e.g., Boutan 1902; Robert 1902; Dodd 1955; Rao 1975). Only a few cases of a clearly monophasic torsion process have thus far been reported (Page 1997b; this study). Moreover, the total amount of time taken for the complete 180° rotation differs remarkably among species and even within a single species: while Boutan (1899) observed an extremely quick 180° twist in individuals of *Haliotis tuberculata* (2--3 minutes), Crofts (1937, 1955) reports it as lasting 200 hours in the same species. Furthermore, the latter author states a biphasic process with a short first phase and a long second phase of approximately 200 hours. According to Voltzow (1987), who only refers to the second phase of an obviously observed biphasic process in larvae of *Haliotis kamtschatkana*, this second half is completed in no more than 18 hours. In contrast, Page (1997b) describes a monophasic torsion process in the same species which takes only 20 hours *in toto*, although the culturing temperature (12°--13°C) was similar to that in Voltzow's (1987) investigation (12°C). Equally contradictory data are given for *Patella*: Smith (1935) observed a slow first and a rapid second phase in specimens of *Patella vulgata*, while the reverse is stated in Crofts' (1955) work on the same species. However, both authors widely agree with Dodd (1955) regarding the total time of about 40 hours taken for the completion of the whole 180° twist (see Table 1 for details). This is in striking contrast to the results presented here, demonstrating a much more rapid completion of torsion in *Patella caerulea* (2 hours), which is carried out monophasically (i.e. at a constant speed). It is difficult to evaluate the differences between these data, since many of the earlier studies lack information on the rearing temperature of the larvae. Thus, the very different values on the timing of torsion reported by Dodd (1955) for *Patella vulgata* (T=12.5°+/-0.5°C) and herein for *Patella caerulea* (T=20°--22°C) might be due to the different temperatures of maintenance and/or the different species of both investigations. Results concerning the onset of torsion in both papers also support this idea: while Dodd (1955) marks the start of torsion at 72 hpf in larvae of *Patella vulgata*, the present data reveal its onset at 32-39 hpf. However, there is a remarkable discrepancy between the 2 species concerning the relation of the onset of torsion to its duration (72 hpf : 40 hrs in *Patella vulgata* versus 32 hpf : 2 hrs in *Patella caerulea*).

Despite the high variability of data on the duration of the torsion process, the hypotheses on its main causes closely resemble each other: muscular activity (of the larval shell muscles) is said to be responsible for quick rotational phases, while slower movements are caused by differential growth. The exception is Boutan (1902), who stated that the extraordinarily quick torsion process in *Acmaea virginea* and *Haliotis tuberculata* (2--3 minutes) is the result of an

antagonism of the foot and the visceral hump. Due to the new data based on live observations as well as SEM investigations, we regard the following mechanisms as driving forces of the ontogenetic torsion process in *Patella caerulea*:

(1) The activities of both larval shell muscles (main and accessory larval retractor) primarily cause the rotation of the cephalopodium relative to the visceropallium. This is mainly achieved by short but intense contractions of these 2, asymmetrically crossing muscles. The recent detailed study of Wanninger et al. (1999b) on the myogenesis in *Patella vulgata* and *Patella caerulea* supports this view by showing that most myofibrils running into the cephalopedal region of the larva belong to the main larval retractor, while the majority of the fibers of the accessory larval retractor terminate in the mantle fold. Thus, in combination with their asymmetrically situated attachment sites, both larval retractors form a powerful, antagonistic muscular system. Due to their resorption during or shortly after metamorphosis (Wanninger et al. 1999b), their main functions are (a) to cause torsion and (b) to retract posttorsional individuals into the embryonic shell.

(2) Both juvenile/adult shell muscles arise after the completion of torsion and are entirely independent of the larval retractors (see Wanninger et al. 1999b). Thus, they do not contribute to the ontogeny of torsion, and all hypotheses based on this assumption (e.g., Edlinger 1988a, b; Edlinger and Gutmann 1997) should be abandoned. Accordingly, the torsion process is regarded as a primarily larval feature (see Wanninger et al. 1999a).

(3) Hydraulic activities presumably play a second major role in the ontogeny of torsion. This is achieved by the active pumping of body fluid into the anterior part of the foot, which apparently results in an increased hydrostatic pressure in this body region. Thus, the movement of the foot relative to the mantle fold --- initiated by the muscular contractions --- is supported.

(4) The already calcified embryonic shell probably serves as an antagonist against the contracting movements of the larval retractors and thus supports the torsion process.

(5) Differential growth seems to play a role only insofar as it apparently fixes the newly gained relative position of the head/foot to the visceral hump.

Timing of ontogenetic torsion and its phylogenetic significance

Considering the great differences in the timing of torsion among various gastropod taxa, the highly heterogeneous character of the ontogeny of torsion becomes obvious (Table 1). Thus, we regard phylogenetic conclusions about any hypothetical general pattern of the timing of torsion as impossible. Instead, it can be concluded that ontogenetic torsion has been highly modified within various gastropod clades since its first (phylogenetic) occurrence. However, the various features of the torted veliger appear to be highly constant between the different taxa of basal gastropods (Patellogastropoda, Neritaemorphi, Vetigastropoda). In contrast, the actual process and in particular the timing of torsion appear highly variable among gastropod taxa, "building similar animals in different ways" (Raff 1996: 211). To gain further insight in this developmental phenomenon, future investigations should focus on the genetic basis of ontogenetic torsion in basal gastropods.

Shell muscle data and the fossil record

It is widely accepted today that the Patellogastropoda (formerly Docoglossa) are the most basal clade of the Gastropoda (see Golikov and Starobogatov 1975; Haszprunar 1988; Ponder and Lindberg 1997). Recent data presented by Page (1997a, b) for the basal vetigastropod *Haliotis kamtschatkana*, and by Wanninger et al. (1999a, b) for *Patella vulgata* and *Patella caerulea*, agree that the 2 larval retractors are (at least partly, see Page 1997a) resorbed prior to or shortly after metamorphosis, while the adult shell musculature arises *de novo* after the completion of torsion.

The arrangement of fossilized (adult) muscle scars on molluscan shells or steinkerns is commonly used to infer whether or not an early univalved mollusc was torted (see, e.g., Wenz 1940; Knight 1947; Rollins and Batten 1968; Runnegar and Pojeta 1974; Dzik 1981; Runnegar 1981; Yochelson and Gil Cid 1984; Peel 1991a, b; among others). However, these attempts have also been severely criticized by Yochelson (1978). Other authors tried to solve this problem by taking asymmetrically coiled adult shell forms as a major proof for torsion (but see Linsley and Kier 1984). Since torsion is the key apomorphy of the Gastropoda (see Haszprunar 1988; Falniowski 1993), these features are crucial for the understanding of both early conchiferan phylogeny and gastropod origin. The present study as well as Wanninger et al. (1999a, b) show that ontogenetic torsion is neither related to adult shell shape nor to the activity or arrangement of adult shell muscles. In contrast, similarly shaped adult shells and adult musculature can house animals of very different bauplans (compare, e.g., the serial shell musculature of adult tryblidiidans with the multiple bundles of the U-shaped adult shell

muscle of patellogastropods). Moreover, different shells and muscles may house very similar animals, e.g. Lepeta-Propilidium, Clypeosectus-Pseudorimula, Pyramidellidae-Amathinidae.

Thus, as far as the fossil record is concerned, the present paper supports Yochelson (1978: 178), who concluded: "Let me say once again that it is impossible to determine whether an extinct form has undergone torsion." This is at least true for fossils that provide no more information than fossilized attachment sites of adult shell muscles.

The monophyly of torsion

Recent papers by Page (1997a) on *Haliotis kamtschatkana* and Wanninger et al. (1999b) on *Patella caerulea* and *Patella vulgata* show striking similarities in the larval shell musculature of basal veti- and patellogastropods. This is especially true for the asymmetrically situated attachment sites of both larval retractors as well as for ultrastructural data: in all 3 species the larval shell muscles are obliquely striated while the adult shell musculature shows smooth conditions. Furthermore, the latter muscles arise independent of the larval retractors. These findings in combination with the identical anatomical consequences caused by the torsion process (see Haszprunar 1988) strongly suggest monophyly of torsion in gastropods (see also Ponder and Lindberg 1997). However, due to the heterogeneity of its ontogenetic process (timing) among various taxa (see Table 1), phylogenetic conclusions of torsion based on ontogenetic data alone remain problematic.

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ZUSAMMENFASSUNG

Die vorliegende Arbeit beinhaltet detaillierte Studien über die Ontogenese larvaler Muskelsysteme bei Mollusken mittels Rasterelektronenmikroskopie, Transmissionselektronenmikroskopie, Fluoreszenzfärbungen in Kombination mit konfokaler Laserscanningmikroskopie, sowie Analysen histologischer Semidünnschnitt-Serien in Verbindung mit Rekonstruktionenstechniken. Es wurden diverse Vertreter der Polyplacophora, Bivalvia, Scaphopoda und Gastropoda bearbeitet, sowie die Adultmuskulatur der basalsten Mollusken, der Solenogastres, neu untersucht. Zur Klärung der Verwandtschaftsverhältnisse der Scaphopoda wurden zusätzlich die Schalenentwicklung und die Expression des Homeoboxgens *engrailed* in *Antalis entalis* analysiert. Die gewonnenen Erkenntnisse ermöglichen bedeutende Schlußfolgerungen bezüglich der Evolution und Phylogenie der Mollusca.

Solenogastres

Adulte Solenogastres besitzen eine aus äußeren Ring-, mittleren Diagonal- und inneren Längsmuskeln bestehende Körperwandmuskulatur, die derjenigen anderer wurmförmiger Taxa ähnelt. Die für Mollusken charakteristische, sich ventral überkreuzende Dorsoventralmuskulatur zeigt eine multiple, serielle Anordnung entlang der Körperlängsachse.

Polyplacophora

Polyplacophoren (Chitonen) weisen in späten Larvalstadien eine Serialität der Dorsoventralmuskulatur auf, welche an die Situation in adulten Solenogastres erinnert. Die Konzentration dieser Muskeln auf sieben und später acht Schalenplatten-Muskelpaare ist sekundärer Natur und erfolgt erst nach der Metamorphose. Theorien, welche von einer annelidenartigen Segmentierung der Polyplacophora ausgehen, können deshalb nicht aufrecht erhalten werden. Darüber hinaus findet sich im prätrochalen Körperbereich der Chitonenlarven ein der Körperwandmuskulatur adulter Aplacophoren (Solenogastres + Caudofoveata) ähnliches Muskelgitter, welches bei den Polyplacophoren während der Metamorphose verloren geht. Dieses larvale Muskelgitter sowie die Serialität der Dorsoventralmuskulatur werden als Rekapitulation des ursprünglichen (und in adulten Solenogastres weitgehend vorherrschenden) Molluskenbauplanes in der Ontogenese der

Polyplacophora interpretiert. Daraus ergibt sich das Postulat eines wurmförmigen und unsegmentierten Körpers an der Basis der Mollusken-Phylogenie.

Wie bei den Bivalvia und den Gastropoden (siehe unten), so findet sich auch in Polyplacophorenlarven ein Prototroch-Muskelring, welcher als homolog für diese Taxa angesehen wird.

Bivalvia

Neben etlichen larvalen Retraktormuskeln besitzen die Veligerlarven autobrancher Muscheln (d.h. aller Bivalvia außer der Protobranchia, welche eine "Hüllglockenlarve" aufweisen) den bereits erwähnten Prototroch-Muskelring. Sowohl die Larvalretraktoren als auch der Prototrochring sind rein larvale Systeme, welche im Zuge der Metamorphose vollständig reduziert werden.

Scaphopoda

Die Ontogenese und die Myogenese des Dentaliiden *Antalis entalis* laufen wesentlich direkter ab als bei Polyplacophoren oder Gastropoden und eigenständige larvale Muskelsysteme fehlen völlig. Die einzige Ausnahme bilden den Prototroch retrahierende Muskelfasern, welche aber keine eigene Schalenansatzstelle bilden, sondern mit den jeweils paarigen Kopf- und Fußretraktoren assoziiert sind. Die Existenz eines Kopfretraktorsystems kann als Synapomorphie für ein Taxon Scaphopoda + (Gastropoda + Cephalopoda) angesehen werden. Dies wird unterstützt durch Analysen des Genexpressionsmusters von *engrailed*, welches eine entscheidende Rolle bei der Schalenbildung von Mollusken spielt: In frühen Stadien von Bivalvialarven wird *engrailed* in den beiden Anlagen der zweiklappigen Embryonalschale exprimiert, wohingegen es bei *Antalis* in Zellen, die das einteilig angelegte embryonale Schalenfeld umgeben, nachgewiesen werden kann. Diese Unterschiede widersprechen fundamental einem direkten Schwestergruppenverhältnis der Bivalvia und Scaphopoda.

Gastropoda

Ursprüngliche Gastropoden, wie die hier untersuchten Vertreter der Patellogastropoda, *Patella vulgata* und *Patella caerulea*, besitzen ein Paar asymmetrischer larvaler Schalenmuskeln mit jeweils eigener Schalenansatzstelle, sowie einen Prototroch-Muskelring. All diese Muskelsysteme werden unmittelbar vor, während, oder kurz nach der Metamorphose resorbiert. Sowohl die adulte Mantel- als auch die Tentakelmuskulatur werden

zumindest teilweise vor der Metamorphose angelegt, während die gesamte Buccalmuskulatur erst in frühen Juvenilstadien nachzuweisen ist.

Der Torsionsprozeß wird bei basalen Gastropoden in erster Linie durch Muskelaktivität der larvalen Schalenmuskeln verursacht. Im Gegensatz dazu wird die adulte Schalenmuskulatur erst nach Vollendung der Torsion gebildet. Dementsprechend kann der ontogenetische Ablauf der Torsion als rein larvaler Prozeß angesehen werden. Daraus ergibt sich, daß die Anordnung der adulten Schalenmuskulatur - welche oft als Abdruck auf fossilen Schalen erhalten ist - nicht zur Klärung der Frage herangezogen werden kann, ob ein bestimmter einschaliger, paläozoischer Mollusk tortiert war, und damit der Klasse der Gastropoda zuzurechnen ist.

LIST OF PUBLICATIONS

(1) Wanninger A, Ruthensteiner B, Lobenwein S, Salvenmoser W, Dictus WJAG, Haszprunar G. Development of the musculature in the limpet *Patella* (Mollusca, Patellogastropoda). *Development Genes and Evolution* (1999a) 209: 226-238.

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(3) Wanninger A, Ruthensteiner B, Haszprunar G. Torsion in *Patella caerulea* (Mollusca, Patellogastropoda): ontogenetic process, timing, and mechanisms. *Invertebrate Biology* (2000) 119: 177-187.

(4) Haszprunar G, Wanninger A. Molluscan muscle systems in development and evolution. *Journal of Zoological Systematics and Evolutionary Research* (2000) 38: 157-163.

(5) Wanninger A, Haszprunar G. Chiton Myogenesis: Perspectives for the development and evolution of larval and adult muscle systems in molluscs. *Journal of Morphology* (in press).

(6) Wanninger A, Haszprunar G. The role of *engrailed* in larval development and shell formation of the tusk-shell, *Antalis entalis* (Mollusca, Scaphopoda). *Evolution and Development* (in press).

(7) Wanninger A, Haszprunar G. Muscle development in *Antalis entalis* (Mollusca, Scaphopoda) and its significance for scaphopod relationships. *Journal of Morphology* (in review).

(8) Haszprunar G, Friedrich S, Wanninger A, Ruthensteiner B. Fine structure and immuno-cytochemistry of a new chemosensory system in the chiton larva (Mollusca: Polyplacophora). *Journal of Morphology* (in press).

(9) Ruthensteiner B, Wanninger A, Haszprunar G. The protonephridial system of the tusk shell, *Antalis entalis* (Mollusca, Scaphopoda). *Zoomorphology* (in press).

CURRICULUM VITAE

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School

1976-1980	Elementary school in Schleching
1980-1989	High School ("Gymnasium") Landschulheim Marquartstein
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Military service

June 1989-April 1990	Fernmeldeausbildungskompanie Daun/Eifel and Peilzentrale Heer V Traunstein/Obb (wireless radio stations)

University studies

Nov. 1991-March 1998	Student of Biology at the Ludwig-Maximilians-Universität München (LMU) Major subject: Zoology; additional subjects: Ecology, Botany, Fishery Biology
March 1998	Studies completed with scientific Diploma. Mark: 1.1
March-Nov. 1998	Diploma thesis in Zoology Supervisor: Prof. Dr. G. Haszprunar, LMU Mark: 1.0

Ph.D. studies

since April 1998	Ph.D. thesis "Micromorphology and gene expression in muscle and shell development of the Mollusca" Supervisor: Prof. Dr. G. Haszprunar, LMU

Congress contributions

July 1998	World Congress of Malacology (Washington/USA) (poster)
Aug. 1998	International Congress "Invertebrate Reproduction and Development" (Amsterdam/The Netherlands) (poster and lecture)
May 1999	Annual meeting of the German Zoological Society (DFG) (Innsbruck/Austria) (poster)
July 1999	Meeting of the American Malacological Society (Pittsburgh/USA) (poster)
Nov. 1999	Phylogenetic Symposium (Vienna/Austria)
Jan. 2000	Invited guest lecture at the University of Mainz
June 2000	Annual meeting of the German Zoological Society (DFG)(Bonn) (poster and lecture)
July 2000	Meeting of the American Malacological Society (San Francisco/USA) (poster and lecture)