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ORIGINAL ARTICLE

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Differentiation of the body wall musculature in *Macrostomum hystricinum marinum* and *Hoploplana inquilina* (Plathelminthes), as models for muscle development in lower Spiralia

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Abstract Recent studies on the differentiation of the body wall musculature in a medicinal leech and in the free-living plathelminth *Macrostomum hystricinum marinum*, Beklemischev 1950 provide the first evidence of a complex developmental signalling pattern, possibly involving stem cells and the nervous system, in the organization of the muscle grid formed by developing myocytes. To enhance further our understanding of the ontogenetic and phylogenetic origin of such muscle grids, which consist of circular, longitudinal and diagonal muscle fibres, we have undertaken a study of muscle development in the polyclad flatworm *Hoploplana inquilina* Wheeler 1894 in collaboration with the Marine Biological Laboratory, Woods Hole. We have also continued our examination of the development of the body wall musculature in *M. hystricinum*. Both species were studied using rhodamine-phalloidin staining and transmission electron microscopy. Additional visualization of the fluorescent whole mount preparations was performed with confocal laser microscopy and digital image processing. The results of our investigation suggest that: (1) the mechanism of muscle development in *H. inquilina* supports the deeply rooted concept of bilateral symmetry (right and left longitudinal founder muscle), and (2) a first circular muscle in this species develops on the border between an anterior body unit and the main body; a caudalmost region is less obvious. The presence of a spiral muscle functioning as a circular muscle system of the "head region" points to a separate developmental mechanism for this region and the trunk. In contrast to *H. inquilina*, where the larval stage forces an intermediate restructuring of the musculature of the body wall before the adult body shape is finally developed, the formation of the body wall musculature of *M. hystricinum* already seems constrained by the adult body shape.

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Introduction

Turbellarian morphology is still thought to hold the key to understanding metazoan evolution (Ax 1995). Most authors suggest a microscopic organism similar to primitive turbellarians, such as certain members of the Acoelomorpha, Catenulida or Macrostomorpha, as a model for the stem species of the Bilateria (see references in Ax 1995), and it is now a common phylogenetic assumption that the musculature of the body wall, organized in the form of outer circular and inner longitudinal muscles such as characterizes the turbellarians, represents an ancestral trait of all Bilateria. Therefore, it is most surprising that neither a developmental hypothesis nor any studies on the embryonic origin of such a muscle grid exist for acoelomate flatworms. This kind of investigation has only recently been carried out on the body wall musculature of a coelomate spiralian, the leech (Jellies and Kristan 1988; Jellies 1990, 1994a, b), which demonstrates a complex signalling pattern controlling differentiation of the body wall musculature that involves interactions of myogenic and neurogenic components (see Jellies 1994b, for review).

Results on the microturbellarian *Macrostomum hystricinum marinum* Rieger 1977 demonstrate a close relationship between differentiating myoblasts and nerve cells during postembryonic growth (Rieger et al. 1994), suggesting a similar developmental mechanism to that found in the leech. These observations stimulated us to examine muscle pattern formation in the body wall of flatworms during late embryogenesis (Rieger et al. 1995). The present study compares development of the body wall musculature of macrostomids and polyclads, which are particularly suitable for comparative analysis because both are presently assigned to the ancestral "Archoophora" but have differing patterns of development and life histories (Baguna and Boyer 1990, Ehlers 1995). The cleavage pattern of the Macrostomorpha is

of the ancestral quartet spiral type and development is direct; both of these features are probably primitive, wether a monophasic or biphasic life cycle is seen as primitive in the Bilateria. (Rieger 1994a, b; Ax 1995). The polyclads, on the other hand, are turbellarians with the ancestral characteristics of quartet spiral cleavage and indirect development to a lobed larva (Muller's larva), making them an especially suitable group in which to investigate the development of musculature during indirect development.

Materials and methods

Macrostomum hystricinum marinum Rieger 1977 (Turbellaria, Macrostomida) was kept in laboratory cultures (see Rieger et al. 1988; Gehlen 1989; Gehlen and Lochs 1990). Adults carrying mature eggs were isolated in small petri dishes and embryos were cultured until the appropriate developmental stage (see Rieger et al. 1991a). Adult specimens of the polyclad *Hoploplana inquilina* Wheeler 1894 were extracted from the mantle cavity of the gastropod *Busycon canaliculatum* and maintained in finger bowls of sea water. Isolation and fertilization of the eggs were done according to Boyer (1987).

Rhodamine-phalloidin staining and fluorescence microscopy

The egg shells of embryos of *M.h. marinum* were punctured with tungsten needles (Tyler 1981) and the embryos were fixed for 1 h with either 4% paralformaldehyde in 0.1 M phosphate buffer (PBS; pH 7.2) containing 10% sucrose, or with Stefanini's fluid (Stefanini 1967). Embryos and larvae of *H. inquilina* at the appropriate developmental stages were fixed without pretreatment with the PFA solution described above. For actin staining, we used a whole-mount fluorescence technique with rhodamine-phalloidin (previously described in Rieger et al. 1994). Preparations were observed with a Reichert polyvar epifluorescence microscope.

Electron microscopy

Adults and larvae of *H. inquilina* and adults of *M.h. marinum* were relaxed with 7% $MgCl_2$ and fixed according to Eisenman and Alfert (1982) using the "weak cocktail" method. The egg shells of embryos of *M.h. marinum* were punctured with a tungsten needle either immediately before or immediately after primary fixation with the cocktail. Embryos of *H. inquilina* were fixed either with the above fixative or with a solution of 4% formaldehyde in PBS at pH 7.2, 2.5% glutaraldehyde and 1% tannic acid in 0.1 M cacodylate buffer containing 10% sucrose, and postfixed with 1% OsO_4 in cacodylate buffer. After dehydration in a standard ethanol or acetone series, specimens were embedded in Spurr's low viscosity resin. Semithin and ultrathin sections were cut on a Reichert Ultracut E microtome, stained with uranyl acetate and lead citrate, and examined with a Zeiss EM 902 transmission electron microscope.

Confocal microscopy and digital image processing

Specimens were examined with a Biorad MRC 500 confocal scanning microscope. Optical sections were taken along the *z*-axis of the microscope with a step size of 0.6–1.0 µm. This value was estimated, depending on the theoretical resolution along the *z*-axis at a given numerical aperture, the optical depth of field and the diameter of the confocal pinhole (Brakenhoff et al. 1990; Pawley 1995). Depending on the low intensity of some of the fluorescing muscle fibres, the confocal pinhole of the MRC-500 was set to a reasonable minimum to avoid oversampling. Further image procesing was performed with an Apple Macintosh Quadra 950 computer using the public domain image processing program "NIH Image" (written by Wayne Rasband at the National Institutes of Health and available via Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disc from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, part number PB93-504868). Stereopairs were generated with mean value projection mode of the data volume with a stereo angle of 6°.

Results

To compare development of the musculature of *H. inquilina* and *M.h. marinum*, the time from egg laying (*M.h. marinum*) or fertilization (*H. inquilina*) until hatching is used to represent a relative developmental time of 100%. This corresponds to an actual time of about 100–120 h in *M.h. marinum* (Fig. 6) and 90–110 h in *H. inquilina*. Because *H. inquilina* undergoes further development of the Mueller's larva, involving major morphological changes after hatching, further comparison of the two species will require a separate study.

The muscle grid in mature animals and hatchlings

Underneath the epidermis of *H. inquilina* is a thick basal matrix (for terminology, see Fransen 1982; Pedersen 1991) consisting of a thin basal lamina and a lamina fibroreticularis, followed by an outermost layer of longitudinal muscle fibres on the ventral body surface, with circular, diagonal, and further longitudinal fibres beneath (Fig. 1; for review literature on polyclad body wall organization, see Prudhoe 1985). Longitudinal and diagonal muscles consist of bands of up to five individual muscle fibres.

Three distinct muscle layers, each consisting of regularly oriented fibres, constitute the body wall musculature in *M.h. marinum* (see Rieger et al. 1991a, b, 1994; Rieger and Salvenmoser 1991). The outermost layer of the musculature consists of circular muscle fibres, followed medially by layers of diagonal and longitudinal fibres (Fig. 2). The basal matrix consists of a thin, irregular net-like arrangement, not subdivided into basal lamina and lamina fibroreticularis (Fig. 2, arrows; see also Rieger et al. 1991a).

The mature larva of *H. inquilina* shows a complex three-dimensional structure of body wall musculature, which will be described in a separate paper. This work, focuses on the major features of the body wall muscles of the Mueller's larva shortly after hatching (Figs. 3, 4). There are clearly fewer diagonal muscle fibres than in hatchlings of *M.h. marinum*. The longitudinal and circular muscle fibres are of a different thickness, and there are more longitudinal muscle fibres, laterally spanning the whole length of the body (Fig. 3, 4). One of these fibres is thicker than the rest and is the first muscle fibre visible in the embryo. It is therefore referred to as the primary longitudinal muscle. A primary circular muscle

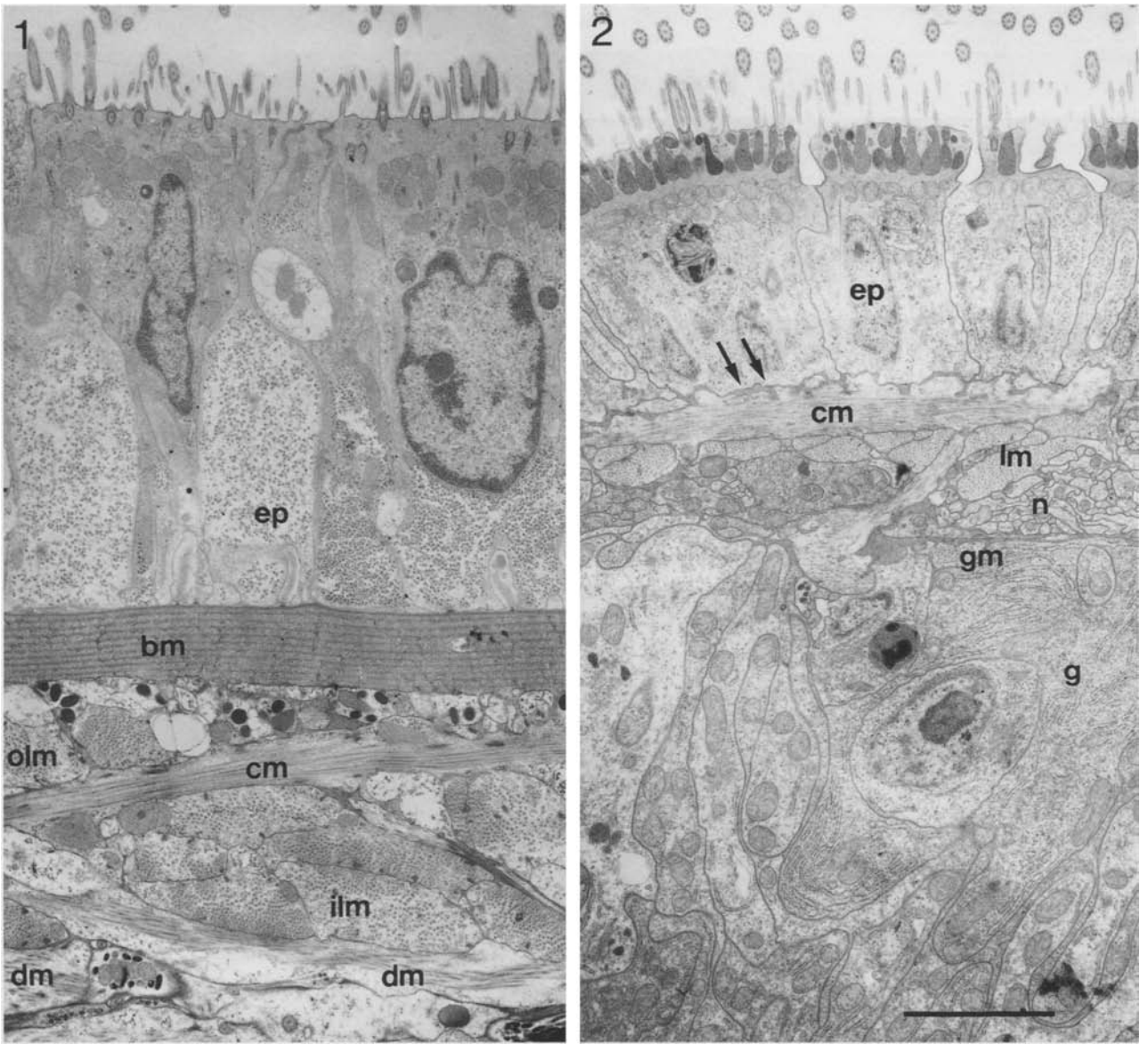


Fig. 1 Cross-section through body wall and musculature in juvenile *Hoploplana inquilina* (*ep* Epidermis, *bm* basal matrix, *ilm* inner (*olm* outer longitudinal muscle layers, *cm* circular, *dm* diagonal muscles, *bar* 3 μm)

Fig. 2 Cross-section through body wall and musculature of adult *Macrostomum hystricinum marinum*. Notice parts of the extracellular matrix (ECM) network (*arrows*) beneath the epidermis (*ep*), circular (*cm*) and longitudinal (*lm*) muscle fibres, gut longitudinal muscles (*gm*), the gut (*g*) and axonal cross-sections of a longitudinal nerve (*n*) (*bar* 3 μm)

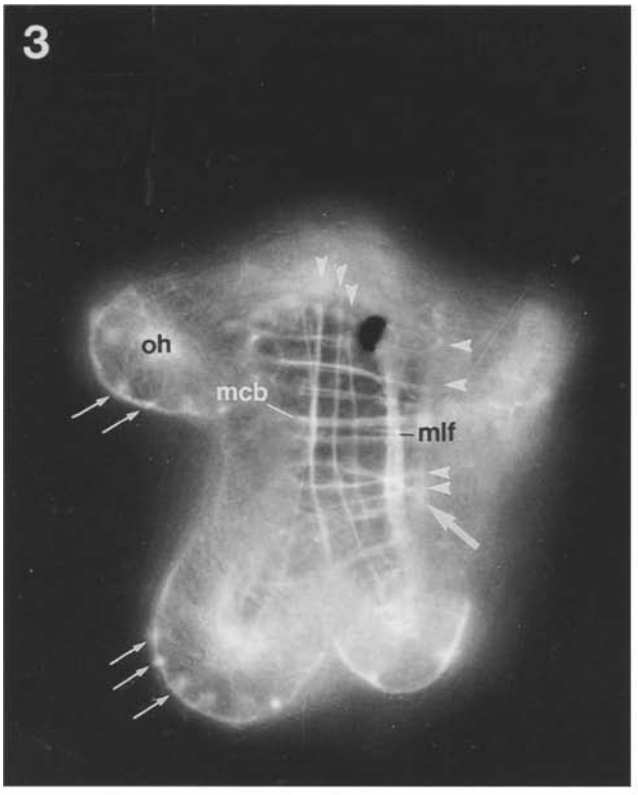
fibre is also present (mcb, Figs. 3, 4), spanning the body laterally from dorsal to ventral side in the region of the oral hood (oh, Figs. 3, 4).

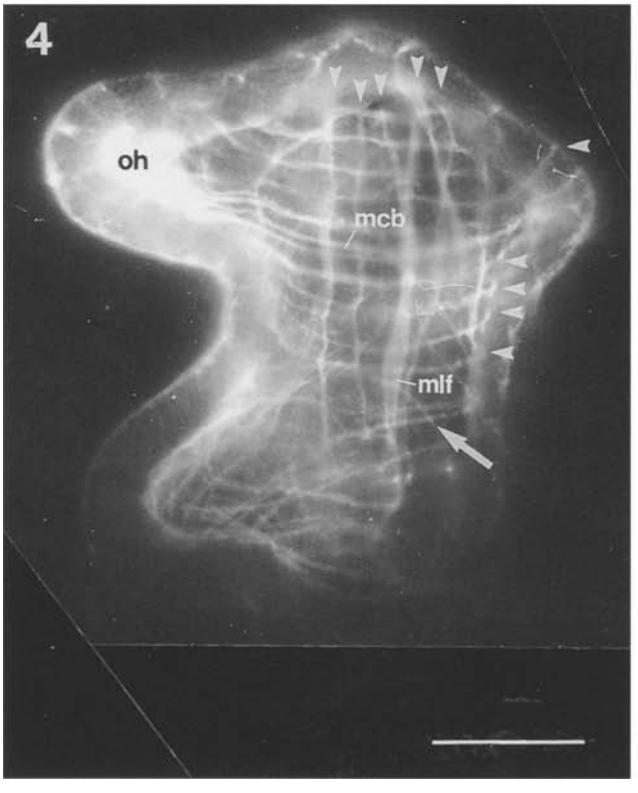
The density of the muscle fibres in the hatchling of *M.h. marinum* is distinctly higher than that in the young larva of *H. inquilina*, and no "primary" muscle fibres can be observed during the early phase of differentiation. Most of the circular muscle fibres as well as the longitudinal fibres are grouped into bands, each consisting of more than two individual muscle fibres (black arrows, Fig. 5; see also Rieger et al. 1994 for details). Diagonal fibres are also present, building a layer of widely spaced diamond-like lattice over most parts of the trunk (for details, see Rieger et al. 1994).

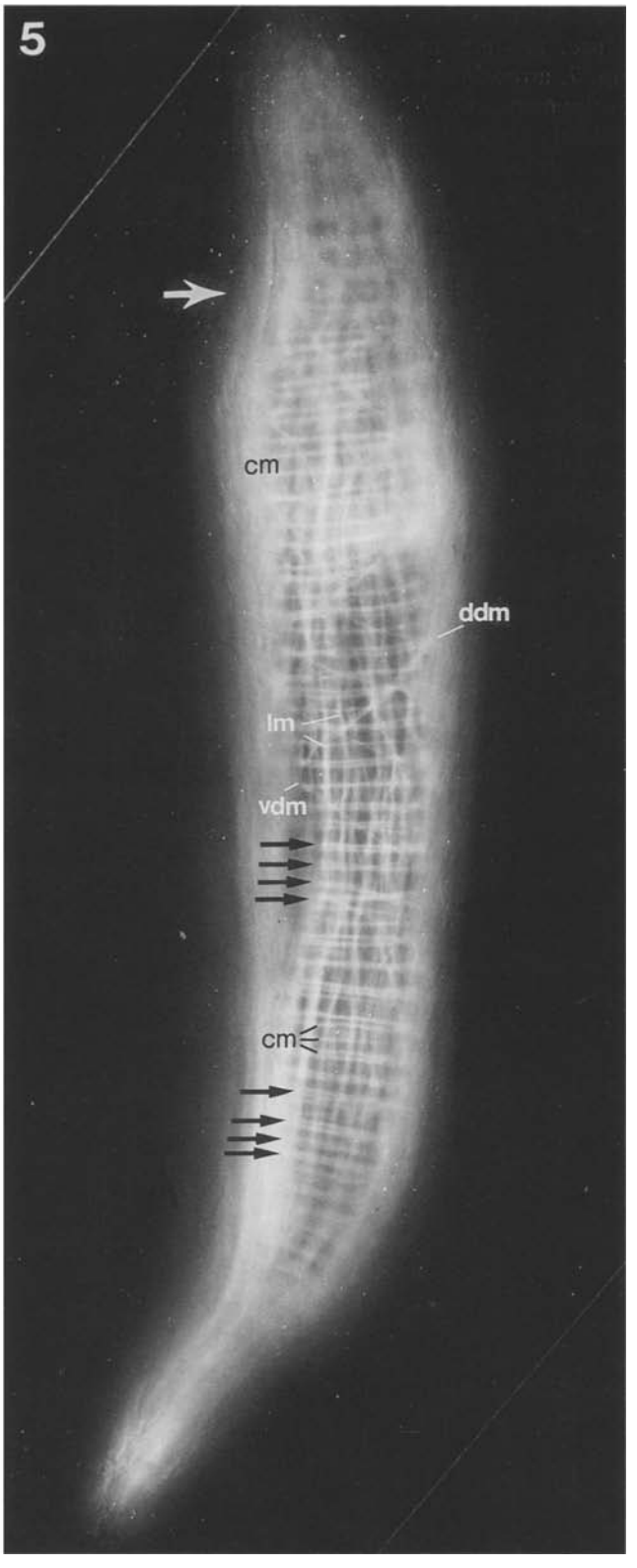
Differentiation in Macrostomum

From our transmission electron microscopy (TEM) data or early embryonic stages of *M.h. marinum*, we know that myofilament bundles may be laid down irregularly within early myoblasts, and thus obscure rhodamine-phalloidin preparations of very early stages of fibre differentiation before 60% development (Fig. 6).

Muscle fibre differentiation starts no earlier than about 55% development. Soon after, the first arrangements of muscle fibres can be seen with the rhodamine-







For legend of Figs. 3–5 see page 414

phalloidin method (Figs. 7, 8). The overall pattern is a diffuse, net-like arrangement of longitudinal, diagonal (Fig. 7, arrowheads) and circular muscle fibres. At 60% development, the body wall musculature consists of remarkably more circular than longitudinal muscle fibres, which are concentrated in distinctly visible, "lattice-like" clusters. The clusters consist of up to ten short, circular muscle fibres, equidistantly and perpendicularly oriented to a longitudinal muscle fibre (Fig. 7, asterisk; Fig. 8). These clusters are henceforth refered to a "muscle lattices". From the orientation of the embryo in TEM preparations (horizontal rootlets of epidermal cilia always point anteriorly), we know that the arms of the muscle lattices represent longitudinal muscles, and the perpendicular elements are formed by circular muscles. The muscle lattices are obviously multicellular in *M. hystricinum*, and superficially resemble the early differentiating "c cell" of the leech (Jellies and Kristan 1988). At this stage of differentiation the main orientation of the future orthogonal body wall grid is already visible (Fig. 7, arrows), as well as first signs of diagonal muscle fibres (Fig. 7, arrowheads). Forking of muscle fibres is also visible as (Fig. 8).

The orthogonal character of the muscle grid becomes more distinct during further development (see Rieger et al. 1991a). Circular muscle fibres span the entire circumference of the embryo (Figs. 9, 10), longitudinal muscle fibres run almost from the anterior body tip (which is muscle free as seen in Fig. 9, arrow) posteriorly, and single, diagonally oriented fibres run transversely along the apical body tip. Rieger et al. (1994) have shown that in *M.h. marinum* longitudinal muscle fibres can reach a length of at least 140 µm in hatchlings to 350 µm in adults.

Changes in the body shape of the embryos characterize the developmental stages from about 60% development to the prehatchling. Elongation along the anterior-posterior axis and indentation of the mouth opening occurs during this period. This in turn leads to changes in the orientation of fibres in the body wall musculature, so that the mouth opening, the pharyngeal muscle holding

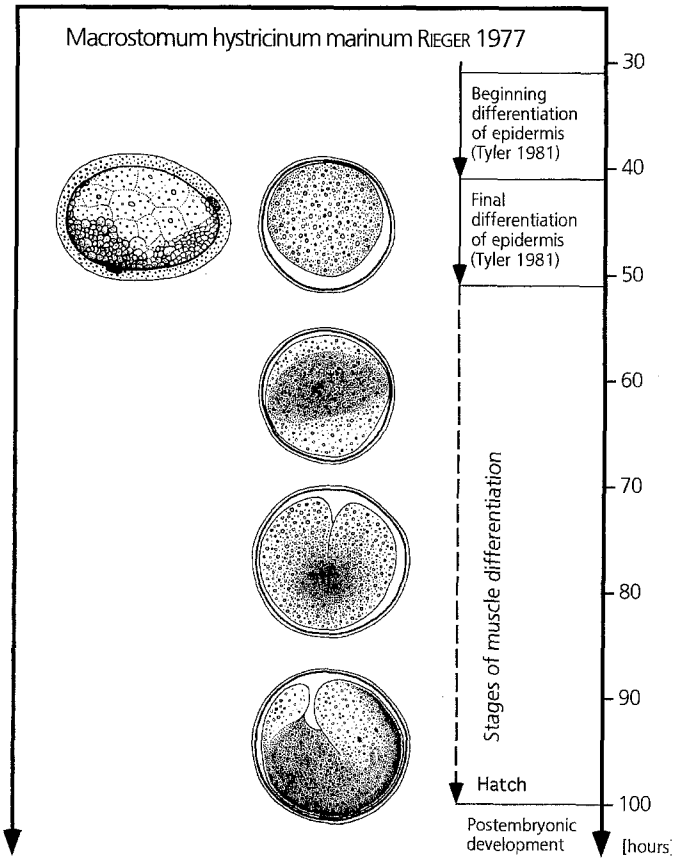


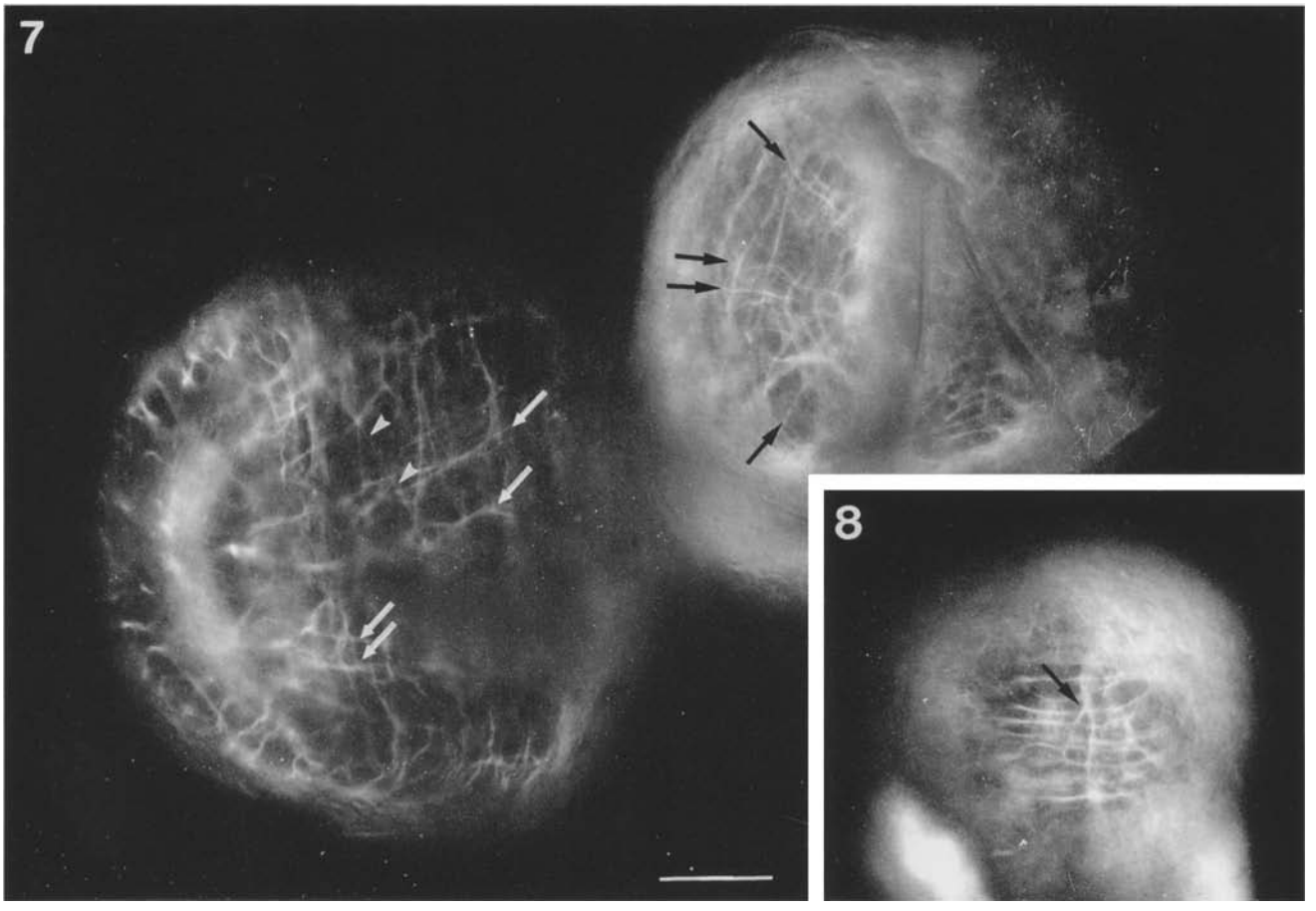
Fig. 6 Drawings of embryonic stages of *M.h. marinum* to illustrate the timing of the first stages of muscle fibre differentiation. 100% of developmental time corresponds to 100 h from egg laying until hatching. The embryo on the *upper left* is drawn after Seilern-Aspang (1957). The species Seilern-Aspang worked with is closly related to *M.h. marinum*

apparatus, and several dorsoventral fibre tracks in the head and tail region can be formed. Ultrastructural observations of embryos at 64% development provide first evidence of a close spatial relationship between the differentiating myocytes, neurons and neoblasts (for postembryonic muscle differentiation, see also Rieger et al. 1994). Along the anlage of the anterior region of the ventrolateral main nerve cords (Fig. 11, vln), neurons with axonal projections are observed (Fig. 11, n1) leading to a young circular muscle cell (Fig. 11, cm), situated between two longitudinal fibre myoblasts (Fig. 11, m1, and m2). Such spatial clustering of young neurons and myocytes is still seen in embryos at 80% development.

At about 95% development (Figs. 9, 10), still more circular than longitudinal muscles are present in *M. hystricinum*. However, the cluster-like muscle-lattices are no longer visible and the embryo is covered by a homogeneous, reticulate muscle grid. A head-on view to the anterior end of an embryo shows individual longitudinal muscle fibres converging radially at the apex (Fig. 9). Forking of muscle fibres is increasingly common during

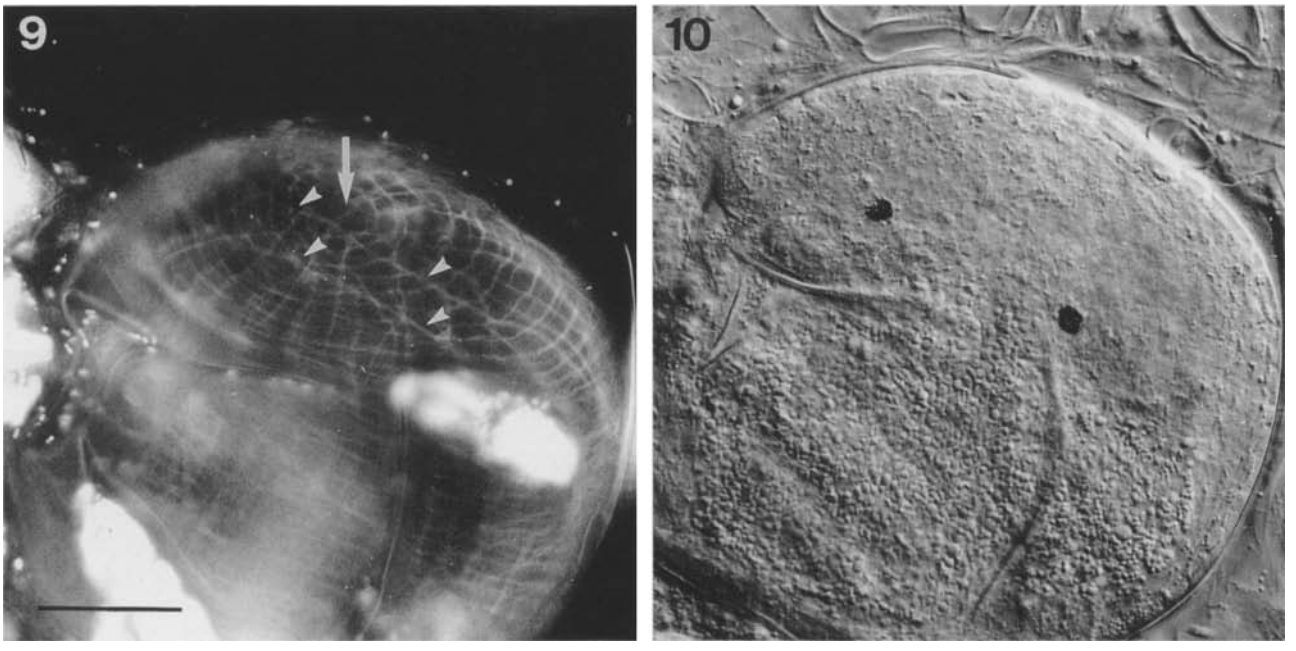
Figs. 3, 4 Phalloidin/rhodamine staining of two specimens of the Mueller's larva (at normal hatching time of about 110 h of *H. inquilina*, seen from the left side. Focus on upper left muscle grid (**Fig. 3**) and on lower right muscle grid (**Fig. 4**) with primary longitudinal muscle fibre (*mlf*) and additional longitudinal fibres ventral and dorsal to the primary muscle (*vertical arrowheads*). Notice also the primary circular muscle fibre band (*mcb*) accompanied by anterior and posterior circular muscle fibres (*horizontal arrowheads*), the first dorsal diagonal arc fibre (*arrow*), and stained actin microfilaments of the zonulae adhaerentes in between epidermal cells (*thin arrows*) of the oral hood (*oh*) (*bar* 40 µm)

Fig. 5 Lateral view of phalloidin/rhodamine stained whole mount of *M.h. marinum* (hatchling) showing the body wall musculature. Notice circular (*cm*), dorsal diagonal (*ddm*), ventral diagonal (*vdm*) and longitudinal (*lm*) fibres, and the groupings of circular fibres into bands (*thin arrows*). The mouth opening is at the left side (*white arrow*) (*bar* 40 µm)



Figs. 7, 8 Lattice-like muscle modules of circular and longitudinal muscle fibres during early differentiation in *M.h. marinum*; 60% developmental time until hatching. Two embryos seen from different directions, longitudinal muscle fibres marked by *white arrows*, diagonal fibres by *arrowheads*. Notice forking of individual or attachment of additional longitudinal muscle fibres (**Fig. 8**, *black arrows*) (*bar* 30 μm)

Figs. 9, 10 Oblique head-on view of one prehatchling (95–98% developmental time until hatching) of *M.h. marinum*, seen with rhodamine-phalloidin (Fig. 9) and with Nomarski differential interference contrast (**Fig. 10**). Observe two muscle fibres (*arrowheads*) running just transverse to the apex, which is muscle-free at the very tip of the animal (*arrow*). Observe arrangement of longitudinal and circular fibres around the apex (*bar* 30 μm)



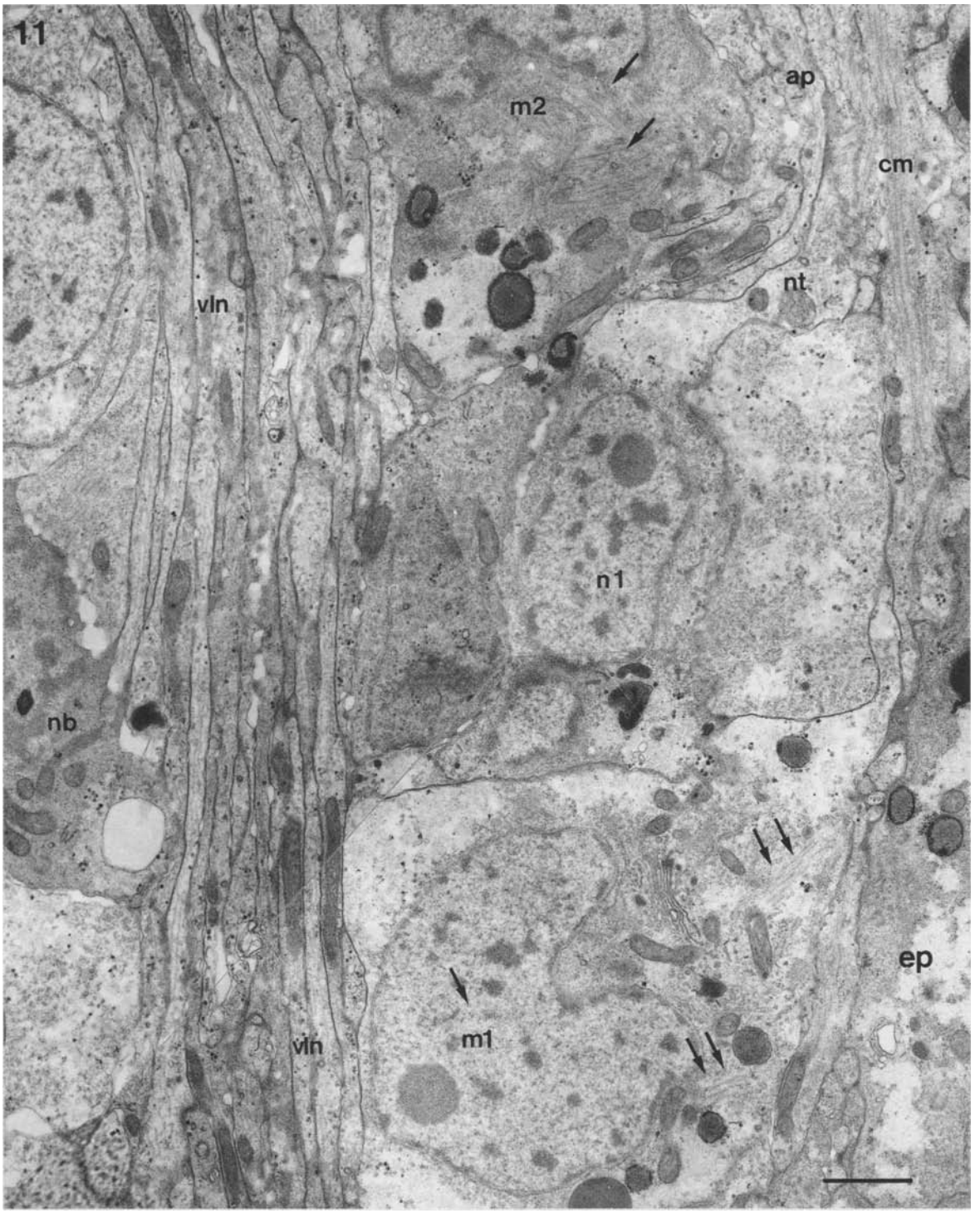


Fig. 11 Myoblast formation and the development of longitudinal nerve cords in *M.h. marinum* (64% developmental time until hatching). Observe anlage of ventrolateral main nerve cord (*vln*) and adjacent differentiating longitudinal myocytes (*m1*, *m2*) and a neuron (*n1*). Observe axons containing neurotubuli (*nt*) in n1, and groups of differentiating muscle myofilaments (*small arrows*) in m2. Note also a circular muscle fibre (*cm*) outside the differentiating myocytes and neurons, and axonal projections of nerve cells (*ap*) in between n1 and m2. The dark cell partly seen on the left side is a neoblast (*nb*) (*ep* epidermis, *bar* 1 μm)

later stages of embryonic development and in the hatchling.

Differentiation in *Hoploplana*

The muscle development of *H. inquilina* is characterized by primary formation of an orthogonal muscle fibre framework. It may serve as an orientational cue, gradual-

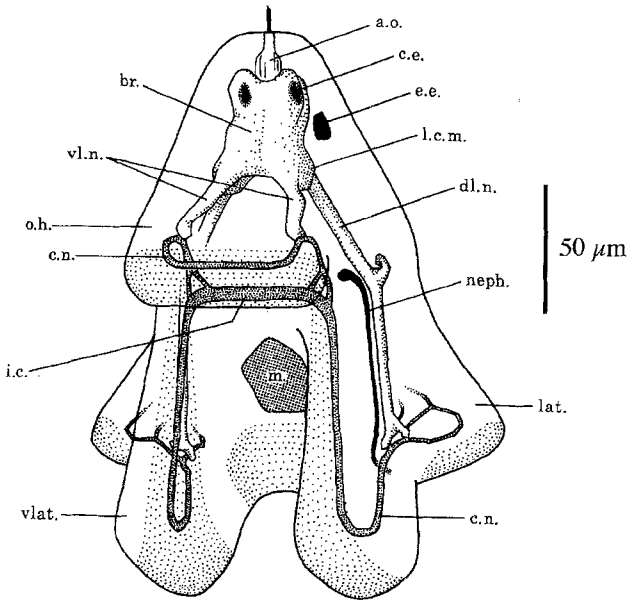


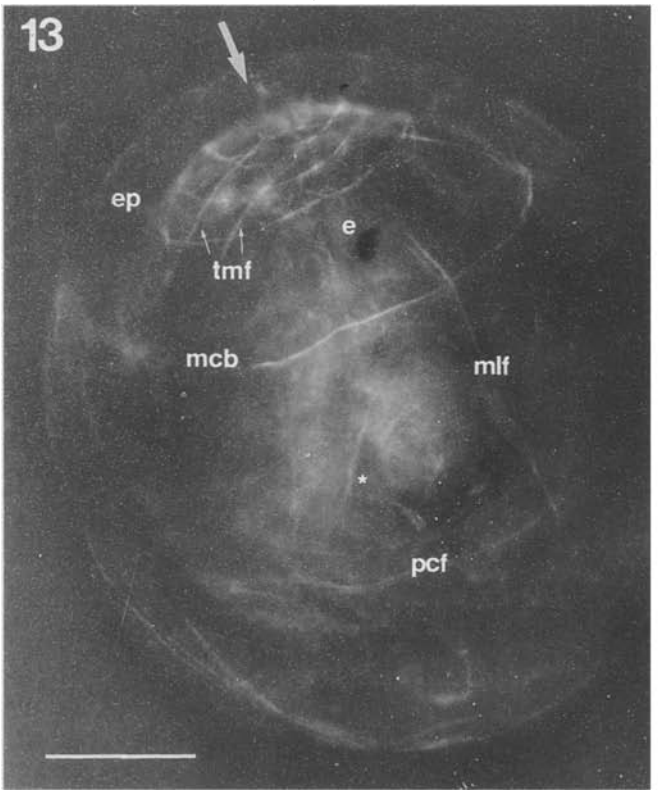
Fig. 12 Schematic drawing of the nervous system of a Mueller's larva of *Pseudoceros canadensis* (after Lacalli 1983) for comparison with our findings. Our preliminary TEM reconstructions confirm the existence of epidermal and subepidermal longitudinal nerve tracts in *H. inquilina* (*bar* 50 μm). Abbreviations: apical organ (a.o.), brain (br.), cerebral eyes (c.e.), lateral cell masses (l.c.m.), ventrolateral nerve cords (vl.n.), dorsolateral nerve cords (dl.n., one partially hidden in this view), ciliary nerves (c.n.), ventrolateral (vlat.) and lateral (lat.) lobes, oral hood (o.h.), single epidermal eye (e.e.), mouth (m.), left protonephridium (neph.).

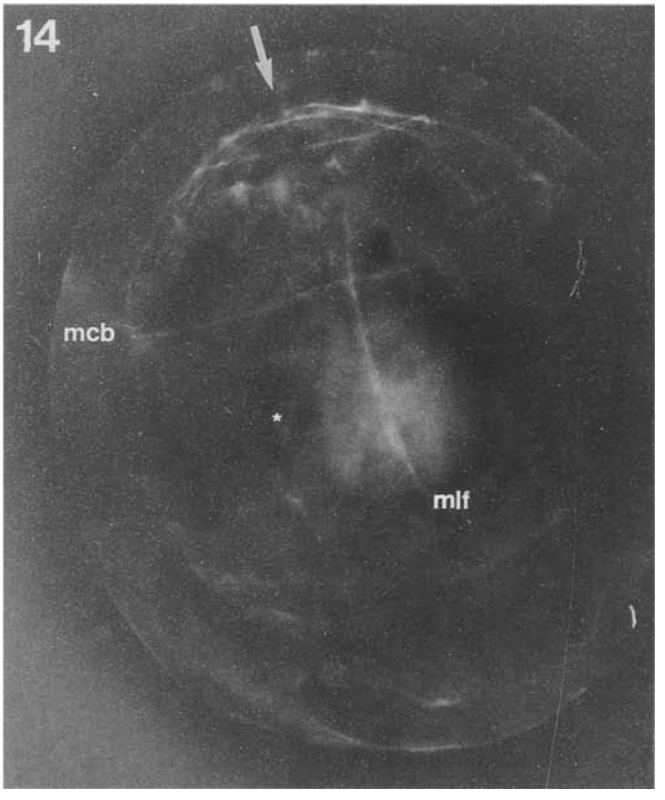
ly becoming more dense by formation of further muscle fibres during development. In the body of the embryo, a head and trunk region, separated by individual muscle fibres, can be distinguished (compare with nervous system of *Pseudoceros canadensis*, Fig. 12).

We observed the first, faintly stained regularly disposed muscle fibres in the embryos of *H. inquilina* at 80% development (Figs. 13, 14). The primary muscle grid is formed by three strictly orthogonal muscle fibres: a lateral longitudinal, an anterior and a posterior circular muscle fibre. They may guide the orientation of further developing muscle fibres, and both the lateral longitudinal and anterior circular muscle fibres are clearly distinguishable by their greater diameter compared to other longitudinal and circular fibres which are formed in later stages.

The primary longitudinal fibres are bilaterally symmetrical, running from the anterior third to the posterior third of the body (Fig. 13, mlf). The anterior circular

Figs. 13, 14 First stage of oriented muscle fibre differentiation of *H. inquilina* as seen with rhodamine-phalloidin staining; embryo at 80% development with focus on the left side (**Fig. 13**) and on the right side (**Fig. 14**). The epidermis (*ep*) is clearly visible, primary muscle fibres of the body-wall are seen underneath. Notice the apical spiral muscle complex (*arrow*) with two fibres transversing the apex in a dorsoventral direction (*tmf*), the primary longitudinal muscle fibre (*mlf*), the primary circular muscle fibre band (*mcb*) and one posterior circular fibre (*pcf*). See also, lying deeper, obliquely oriented fibres (*asterisk*), the eye (*e*) and an unspecific fluorescence halo in the caudal part of the pharynx (*bar* 40 μm)





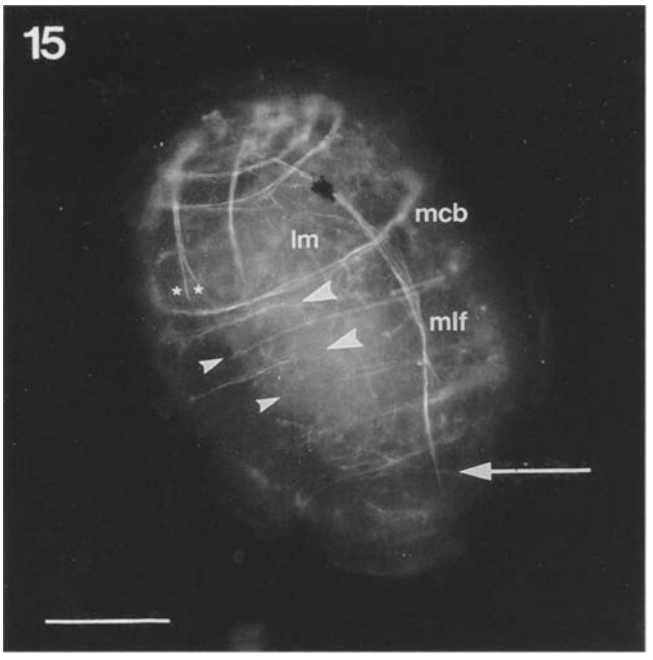
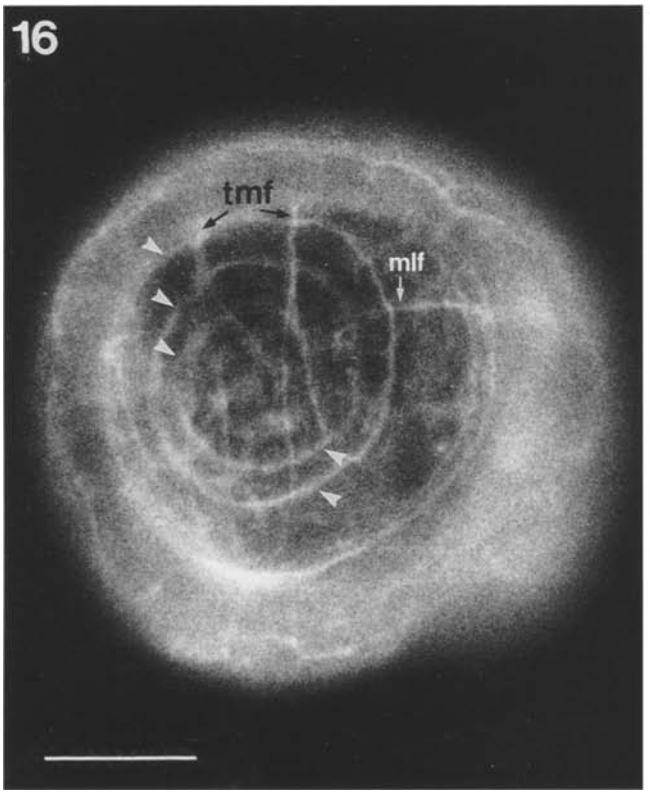


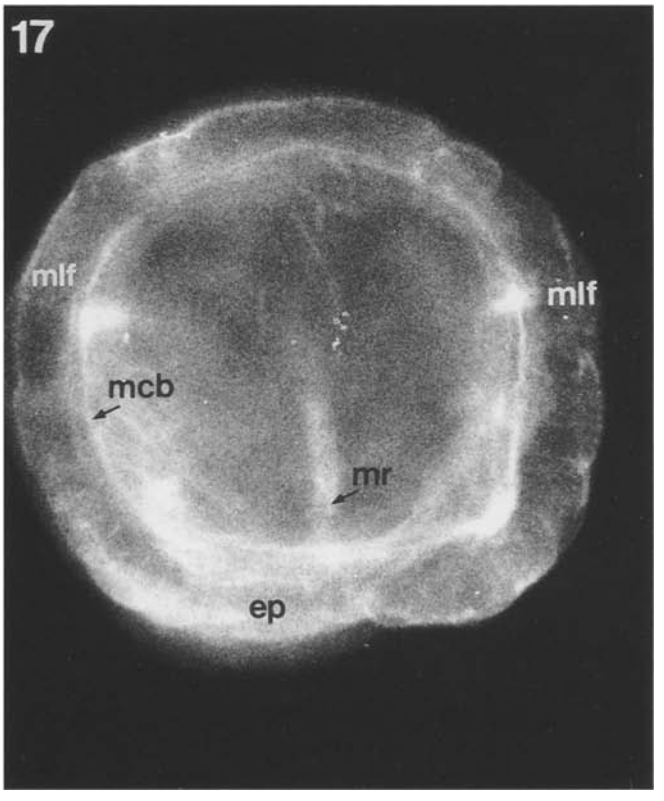
Fig. 15 Embryo of *H. inquilina* at 92% development, seen from the left side. The animal shows the apical muscle complex as seen in Fig. 13, differentiation of the primary longitudinal muscle fibre (*mlf*), the primary circular muscle fibre band of the body wall (*mcb*), and two sets of interdigitating circular fibres below (*small and large horizontal arrowheads*). Notice a diagonal muscle fibre towards the caudal end (*thin arrow*; compare also Fig. 17) and, faintly stained, a second longitudinal muscle fibre (*lm*) ventrolateral to the main fibre, as well as forking ends of or attachment of further longitudinal fibres (*asterisks*; *bar* 40 μm)

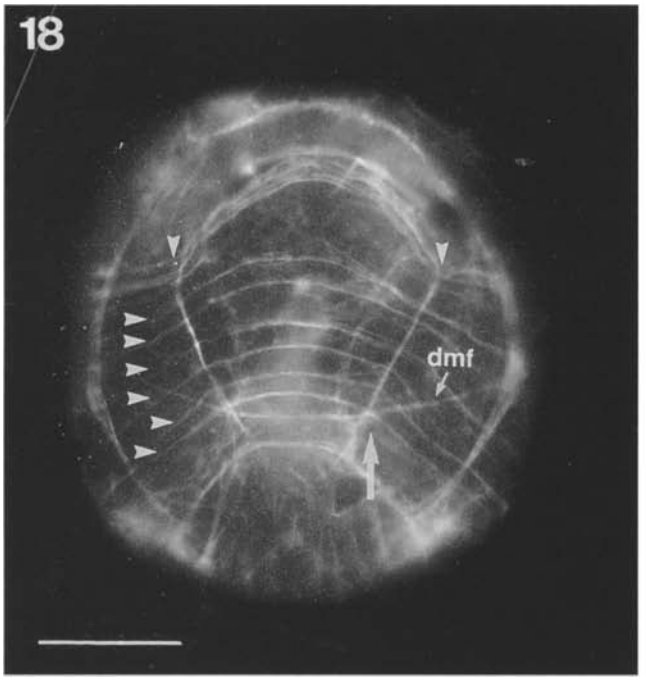
muscle fibre (Fig. 13, mcb), at this stage obviously consisting of a single fibre, is seen to run at a level where the oral hood is formed later in development. At its posterior end, the primary longitudinal muscle fibre crosses the posterior circular muscle fibre (Fig. 13, pcf) at the level of the future ventrolateral lobes. Thus, an anterior-posterior compartmentalization of the embryo is recognizable: the anterior and posterior circular muscles visually separate two zones of different muscle develpment, which eventually give rise to a head and a trunk region. A special caudal region is apparently only visible in the larva, and not in the adult. Two "transverse" muscles (Fig. 13, tmf) run peripherally over the apical tip of the body from dorsal to ventral sides, terminating at the anterior circular muscle band. Lying deeper, individual diagonal muscle fibres are seen (Fig. 13, asterisk).

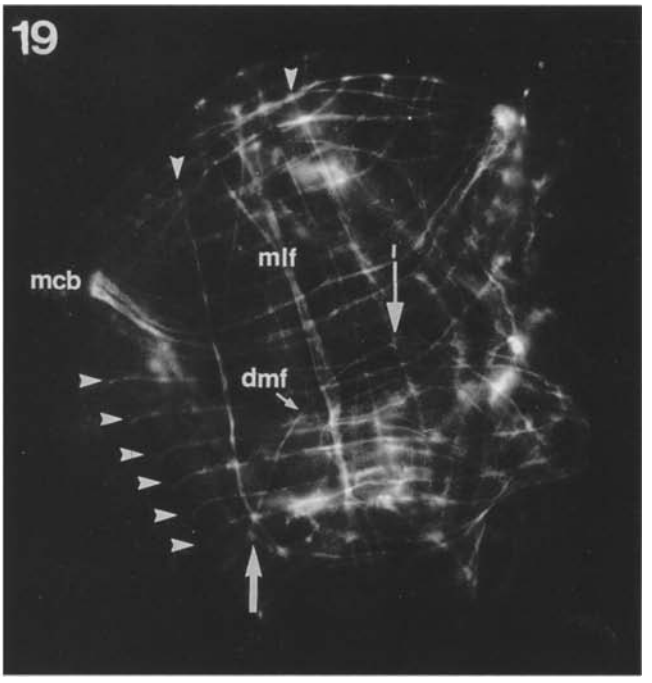
At 92% development, a spirally arranged muscle fibre complex ("apical spiral complex") forms at least three loops at the apical body tip (Figs. 15, 16) of the head region. An eye (Fig. 15, e) is located distal to the longitu-

Figs. 16, 17 Apical view of *H. inquilina* embryo at 92% development. Ventral side of larva is recognizable because of differentiating ventrolateral lobes and oral hood (*thick arrows*). **Fig. 16** Focus on the apical spiral muscle complex; **Fig. 17** an optical section at about the level of the primary circular muscle fibre band. In **Fig. 16**, notice the spiral nature of the apical 'circular' muscles (*arrowheads*), transversing muscle fibres (*tmf*) and anterior end of left primary longitudinal muscle fibre (*mlf*). In **Fig. 17** observe the primary circular muscle fibre band (*mcb*) running below the epidermis (*ep*), and also the retractor muscle for the anterior mouth margin (*mr*, also visible in Fig. 20); *bar* 40 μm)









Figs. 18, 19 Embryo of *H. inquilina* at 96% of developmental time until hatching, seen from the dorsal side, anterior up (**Fig. 18**), and from the left side (**Fig. 19**). Notice a further pair of longitudinal muscle fibres (*vertical arrowheads*) and six circular muscle fibres (*horizontal arrowheads*) behind the primary circular muscle fibre band (*mcb*). Observe a muscle fibre running diagonal on each dorsolateral side (*dmf*), and a triple-cross-like fibre arrangement (*arrow*). The diagonal muscle terminates at the second ventral longitudinal muscle fibre (**Fig. 19**, *thin arrow*), running from the triple cross at the posterior end of the second dorsal longitudinal muscle fibre (*arrow*) across the primary longitudinal muscle fibre band (*mlf*, *bar* 40 μm)

dinal muscle fibre. Additional circular muscle fibres appears between the anterior and posterior primary circular muscle fibres (Fig. 15, horizontal arrowheads). They do not span the whole periphery of the larva as seen in later stages, but form two sets of fibres interdigitating at the ventral side of the body. Each set of fibres is perpendicularly oriented to the corresponding primary lateral longitudinal muscle, resembling the primary lattice-like muscle molecules of *M.h. marinum* at a larger scale. The circular fibres of *H. inquilina* elongate and encircle the periphery during further development. A second longitudinal fibre appears ventral to the longitudinal primary muscle cell (Fig. 15, lm), terminating below the apical spiral loop. A first ventral diagonal muscle emerges laterally at the most posterior part of the embryo at an angle of about 45° (Fig. 15, thin arrow).

Focusing at the most apical surface of the body wall musculature, an anterior view reveals the fibre arrangement of the apical spiral muscle complex (Fig. 16). Between 80% and about 92% development, the spiral muscle forms at least three or four concentric loops, and the primary lateral longitudinal muscle (Fig. 16, mlf) forms a connection with the outer most loops. Both dorsoventrally transversing fibres run parallel, left and right of

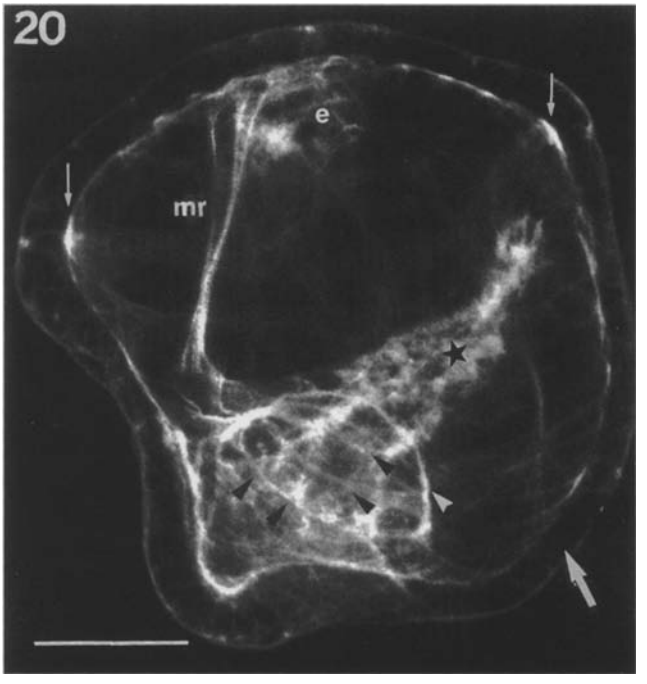


Fig. 20 Contrast enhanced average projection through confocal sections of the larva (4 h after mean hatching time) of *H. inquilina*. The image shows a 10-section slice out of a stack of 30 sections, slightly tilted toward the left side (see Fig. 21 for orientation). Observe fibres of the anterior mouth retractor muscle (*mr*), beginning of differentiating circular, longitudinal and forking radial foregut musculature (*arrowheads*), and unspecific staining leading into the gut area (*asterisk*). See also the primary circular muscle fibre band (*thin arrows*), and forking posterior part of left longitudinal muscle fibre (*arrow*). Parts of the animal's left body wall grid are visible in a flat projection due to the tilt of the specimen's left lateral surface into the sampled data slice. Notice the eye (*e*) next to the unspecifically stained area below the apical plate (*bar*=40 μm)

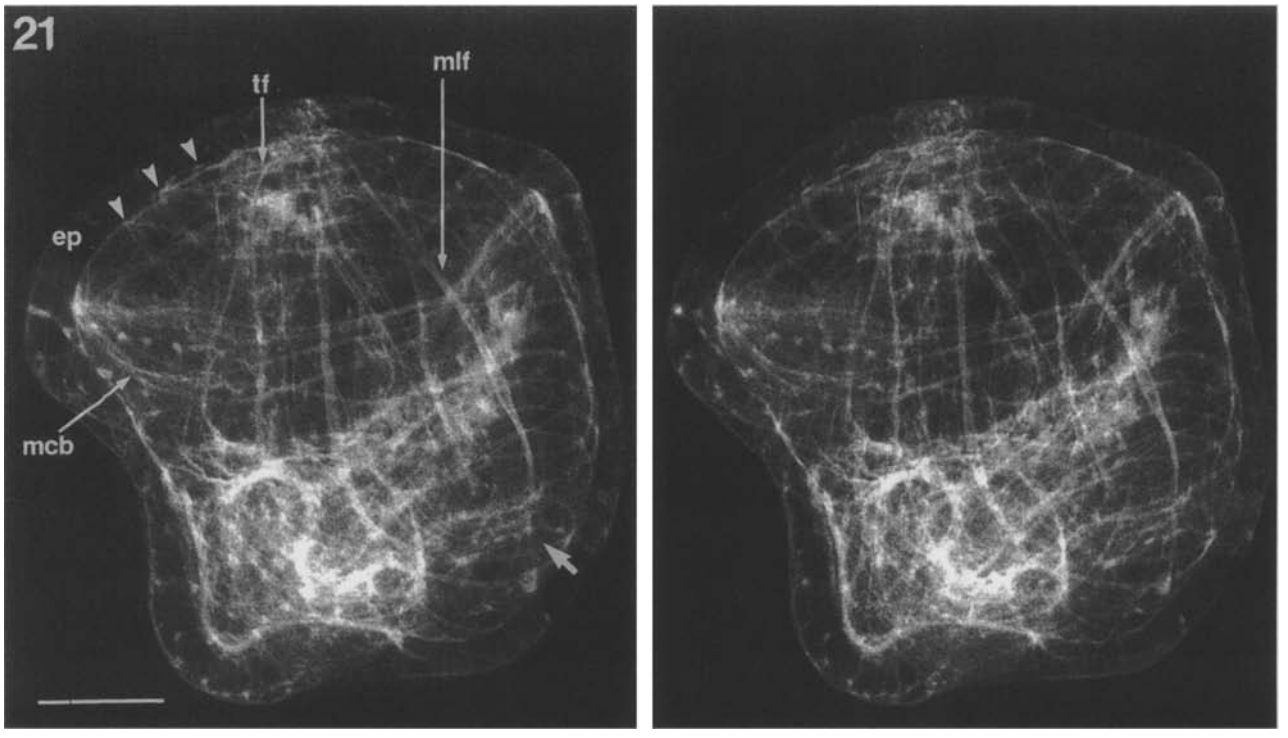


Fig. 21 Stereopair of same animal as in Fig. 19; contrast enhanced average projection of the whole sample (30 optical slices). Left lateroventral side of the animal is to the front. Observe left and right primary longitudinal muscle fibres (*mlf*), apically transversing muscle fibres (*tf*), the primary circular muscle fibre band (*mcb*) running below the epidermis (*ep*) along the oral hood (*thin arrow*), three fibres of the anterior mouth retractor muscle (*mr*) drawing from caudal part of the mouth region to under the apical spiral muscle fibres (*arrowheads*). Notice the complex foregut muscle fibre system (*pmf*), as seen in the whole data set. Also notice the spatial lining of the body wall musculature along the inner epidermis, clearly visible in the stereo view, and the fan-like branching at the posterior end of the primary longitudinal muscle fibre (*double arrow*; *bar* 40 μm)

the apex (Fig. 16, tmf). Focusing at the level of the anterior circular muscle band shows the layer of body wall musculature directly underneath the epidermis (Fig. 17, ep). A ventral mouth retractor muscle running within the embryo from anterior to posterior can be seen (Fig. 17, mr).

At about 96% development, six closed circular muscle fibres were in counted in between the primary anterior and posterior circular muscle fibres (Fig. 18, horizontal arrowheads), a number consistently found in three different animals. A second pair of longitudinal fibres emerges dorsally and ventrally of the primary longitudinal muscle cell, connecting the transverse muscle fibres with the primary posterior circular muscle fibre (Fig. 19, vertical arrowheads). The prominent diagonal muscle fibre starts at the dorsomedian line of the animal, between the last two circular fibres, runs ventrolaterally at an angle of about 45° to the orthogonal grid (Fig. 18, dmf, Fig. 19, thick arrow), and terminates abruptly at the second ventral longitudinal fibre at the mid-body (Fig. 19, thin arrow). The diagonal muscle fibre, the second dorsal longitudinal muscle fibre and the second to last posterior circular fibre meet at a site-constant triple-cross in all observed animals (Fig. 18, arrow). Longitudinal and transverse fibres are forked at their ends (Fig. 15, asterisks; Fig. 20, arrow), suggesting an enhanced anchoring of muscle elements at anterior and posterior regions of the body.

Optical sections of the innermost part of the embryo reveal that the mouth retractor muscle (Fig. 20, mr) consists of up to three muscle, fibres, running from the anterior lip of the forming mouth opening to the apical spiral muscle complex (Fig. 20, sm). The slightly tilted optical cross-sections allows an obliquely sagittal view of the animal. Cross-sections of the primary circular muscle fibre are observed (Fig. 20, thin arrows) running ventrally along the oral hood anlage, as well as the complex radial, longitudinal and circular fibres comprising the pharynx musculature (Fig. 20, arrowheads). Clearly visible in the optical section is the body wall musculature beneath the epidermis.

Increasing deviations of fibre orientation from a relatively strict orthogonal pattern occur, since the developing embryo changes its body shape from nearly spherical to a multiple-lobed Mueller's larva (Lacalli 1982). This particularly involves rearrangement and musculature around the lobes and the mouth opening (Fig. 21). Additional longitudinal muscle fibres emerge and more circular muscle fibres (Figs. 3, 4, upper horizontal arrowheads) are formed in between the apical spiral fibre complex and the primary circular muscle band.

Discussion

It is well known that guiding mechanisms play a role in the development of muscle cells, neuroblasts, glioblasts and other cell types in the medicinal leech (Torrance and Stuart 1986). From work on arthropods and annelids it is known that cells other than neurons have a guiding function during further assembly and arrangement of myocytes (for mesodermal "muscle pioneers" scaffolding further muscle assembly in the grasshopper see Ho et al. 1983; for "founder cells" of circular and longitudinal muscles and "comb cells" preceding diagonal muscle formation in leeches see Jellies and Kristan 1988; Jellies 1990). During this time of early myogenesis, it is likely that dynamic changes in the surface cell markers reflect differentiation and pattern formation processes (Maine and Kimble 1993; for vertebrates, see Marusich and Simpson 1983; Mclennan 1993), which supposedly also occur in neurons and stem cell populations of Turbellarians (Baguña et al. 1989a, b).

In the future, immunohistochemical staining of surface membrane or other cell-type-specific markers of adult myocytes will help to trace the pathways and location of myoblasts and pre-myocytes before and during migration, and allow a better understanding of the underlying cellular guidance mechanisms.

Control mechanisms and the development of three-dimensional patterns

From the ultrastructural data on advanced embryonic stages, hatchlings and adults of *M.h. marinum* it is known that differentiating pre-myocytes as well as the cell somata of the differentiated circular muscles are located adjacent to neurons and neoblasts along the ventrolateral main nerve cords of the animal (Fig. 11; Rieger et al. 1994). Thus, migration and differentiation of pre-myocytes of circular muscles appear to be spatially restricted to the nerve cord. These observations support the concept of a central guiding function of the nerve cord at least during the development of the circular muscles. As many as four individual muscle fibres have been observed to assemble into one ring of circular muscles around the circumference of the worm body. The cell bodies of these muscle fibres are not equidistantly dispersed along the body wall circumference, but stay close to the main longitudinal nerve cords sending out the contractile part of their cells in a dorsal and ventral direction (Legniti et al. 1989; Rieger et al. 1994). Communication with the main lateral nerve cords is highly probable. It remains unknown how the perikarya of the various cell types (e.g. circular, diagonal and especially longitudinal muscle fibres) stay close to this part of the central nervous system, and what inputs are responsible for forming such a complex myoneural network during embryogenesis.

The observed muscle fibres of the body trunk of *Macrostomum* develop, finally ending up in three distinct main systems: a longitudinal, a circular and a diagonal fibre system. Additionally, complex minor systems such as the dorsoventral muscle fibres of the head or the pharynx musculature are formed (Rieger et al. 1994). We can assume that at least a portion of the myocytes are located in their final position before the first visual appearence of muscle fibres, such as the lattice-like muscle modules seen with the phalloidin method at about 80% embryonic development of *M.h. marinum*. The muscle modules might provide a high-level guiding framework for the future orthogonal orientation of other longitudinal and circular myocytes. They are visually similar to the "C cells" (comb cells, Jellies and Kristan 1988) which provide orientational cues during the development of the diagonal musculature of the leech. The lattice-like muscle modules of *M.h. marinum* are units of muscle cells, however, rather than a single muscle-related pattern-guiding cell.

The data on *H. inquilina* indicate the appearance of a pair of longitudinal muscle founder cells oriented parallel to the longitudinal nerve cords. The longitudinal, ribbon-like fibres appear first, suggesting that they are muscle founder cells, similar to those found in the longitudinal body wall musculature of leeches (Jellies 1990). The first anterior circular muscle cell may also be a founder cell, although the increased thickness of the fibre is seen only later in development. The second posterior circular muscle seems to be laid down at the border between the trunk and caudalmost body portion bearing the anus, and because of its position could also be a muscle pioneer. However, this cell does not become thicker during further development. From these findings, we conclude that the first structured element of the muscular system is a muscle cross consisting of two fibres, which demarcates the orientation of all further longitudinal and circular fibres. The primary circular fibre may delineate the anterior border of the trunk region, illustrating the primitive unsegmented spiralian body plan from which segmentation in annelids and arthropods must have been derived (Lawrence 1992).

In *M.h. marinum* in contrast, we found several longitudinal muscle cells with many circular fibres rectangularly attached. Thus, the visible signs of bilaterality as well as a demarcation of the trunk region are lacking. Later, during postembryonic development and in the adult body, the distribution and occurrence of dorsoventral muscle fibres clearly shows a division of the body into rostrum, trunk and tail plate, which is similar to the pattern seen in the *H. inquilina* larva. Our data suggest that the overall muscle grid is assembled if individual units of circular and longitudinal muscles oriented along the body axis during further development. More data are necessary to determine if this is related to the difference in the developmental pattern (direct versus indirect) between *M.h. marinum* and *H. inquilina*. These observations suggest that the assembly of a highly complex muscle grid by early myocytes is dependent on the local environment of the differentiating cells.

How little we actually know of the underlying mechanisms is apparent from recent results on the body wall

musculature of *Convoluta pulchra*. Tyler et al. (1994) found that the orientation of the longitudinal muscle fibres on the ventral side of the organism change behind the mouth pore: the same fibres that run parallel in the anterior body region bend sharply, becoming fibres with an almost circular orientation.

Conclusions

The observation of closely related ontogenetic development between neurons and muscle fibres, well documented from leeches (Jellies 1990, 1994a, b) and now from lower turbellarians, suggests co-evolution of both cell types. Stem cells with their molecular and cellular communication mechanisms have proven to be fundamental for the development of both nervous and muscle system in turbellarians (Baguña et al. 1989a, b; Reuter and Palmberg 1989; Palmberg 1990). To elucidate the ontogenetic mechanisms of the differentiation of neurons and muscle cells from stem cells will be of central importance to a better understanding of the diversification within bilaterian body plans (Raff 1994).

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References

Ax P (1995) Das System der Metazoa I. Ein Lehrbuch der phylogenetischen Systematik. Fischer, Stuttgart

Baguña J, Boyer B (1990) Descriptive and experimental embryology of the Turbellaria: present knowledge, open questions and future trends. In: Mathy HJ (ed) Experimental embryology of aquatic plants and animals. Plenum Press, New York, pp 95–128

Baguña J, Romero R (1981) Quantitative analysis of cell types during growth, regrowth and regeneration in the planarians *Dugesia (S) mediterranea* and *Dugesia (G) tigrina*. Hydrobiologia 84:181–194

Baguña J, Salo E, Romero R (1989a) Effects of activators and antagonists of the neuropeptides substance P and substance K on cell proliferation in planarians. Int J Dev Biol 33:261–264

Baguña J, Salo E, Auladell C (1989b) Regeneration and pattern formation in planarians. III. Evidence that neoblasts are totipotent stem cells and the source of blastema cells. Development 107:77–86

Boyer BC (1987) Development of in vitro fertilized embryos of the polyclad flatworm *Hoploplana inquilina* following blastomere separation and deletion. Roux's Arch Dev Biol 196: 158–164

Brakenhoff GJ, Voort HTM van der, Spronsen EA van, Nanninga N (1990) Three dimensional imaging in fluorescence by confocal scanning microscopy. J Microsc 153:151–159

Ehlers U II (1995) The basic organization of the Plathelminthes. Hydrobiologia 305:21–26

Eisenman EA, Alfert M (1982) A new fixation procedure for preserving the ultrastructure of marine invertebrate tissue. J Microsc 125:117

Fransen ME (1982) The role of ECM in the development of invertebrates: A phylogeneticist's view. In: Hawkes S, Wang JL (eds) Extracellular matrix. Academic Press, pp 177–181

Gelhen M (1989) Morphologische und populationsdynamische Untersuchungen an experimentellen und natuerlichen Populationen des *Macrostomum hystricinum marinum* Rieger (Macrostomida). Thesis, University of Innsbruck

Gelhen M, Lochs A (1990) Quantification of characters from live observations in meiobenthic Turbellaria-Macrostomida. Cah Biol Mar 31:463–472

Ho RK, Ball EE, Goodman CS (1983) Muscle poineers: large mesodermal cells that erect a scaffold for developing muscles and motoneurones in grasshopper embryos. Nature 301:66–69

Jellies J (1990) Muscle assembly in simple systems. Trends Neurosci 13:126–131

Jellies J (1994a) Cellular interactions in the development of Annelid neuromuscular systems (abstract). Am Zool 33:98A

Jellies J (1994b) Cellular interactions in the development of Annelid neuromuscular systems. Am Zool 34:554–561

Jellies J, Kristan WB Jr (1988) Embryonic assembly of a complex muscle is directed by a single identified cell in the medicinical leech. J Neurosci 8:3317–3326

Lacalli TC (1983) The brain and central nervous system of Müller's larva. Can J Zool 61:39–51

Lawrence PA (1992) The making of a fly. The genetics of animal design. Blackwell, Cambridge

Legniti A, Rieger RM, Haszprunar G (1989) Cellular components in the acoelomate body cavity in the hatchling and the adult of *Macrostomum hystricinum* (Turbellaria, Macrostomida) (abstract). Eur J Cell Biol 49 [Suppl 27]: 55

Maine EM, Kimble J (1993) Suppressors of GLP-1, a gene required for cell communication during development in *Caenorhabditis-Elegans*, define a set of interacting genes. Genetics 135:1011–1022

Mclennan IS (1993) Localization of transforming growth-factor-beta-1 in developing muscles – implications for connective-tissue and fiber-type pattern-formation. Dev Dynam 197:281–290

Marusich MF, Simpson SB Jr (1983) Changes in cell surface antigens during in vitro lizard myogenesis. Dev Biol 97:313–328

Palmberg I (1990) Stem cells in Microturbellarians. An immunocytochemical and autoradiographical study. Protoplasma 158: 109–111

Pawley J (1995) Handbook of biological confocal microscopy 2nd edn. Plenum Press, New York

Pedersen KJ (1991) Invited review: Structure and composition of basement membranes and other basal matrix systems in selected invertebrates. Acta Zool (Stockh) 72:181–201

Prudhoe S (1985) A monograph on polyclad Turbellaria. Oxford University Press, Oxford

Raff RA (1994) Developmental mechanisms in the evolution of animal form: origins and evolvability of body plans. In: Bengston S (ed) Early life on earth. (Nobel Symposium 84) Columbia University Press, New York, pp 489–500

Reuter M, Palmberg I (1989) Development and differentiation of neuronal subsets in asexually reproducing *Microstomum lineare*. Immunocytochemistry of 5-HT, RF-amide and SCPB. Histochemistry 91:123–131

Rieger RM (1994a) Evolution of the "lower" Metazoa. In: Bengston S (ed) Early life on earth. (Nobel Symposium 84) Columbia University Press, New York, pp 475–488

Rieger RM (1994b) The biphasic life cycle – a central theme of Metazoan evolution. Am Zool 34:484–491

Rieger RM, Salvenmoser W (1991) Demonstration of the muscle-system in whole mounts of *Macrostomum hystricinum* (Turbellaria, Macrostomida). Am Zool 31:25a

Rieger RM, Gehlen M, Haszprunar G, Holmlund M, Legniti A, Salvenmoser W, Tyler S (1988) Laboratory cultures of marine Macrostomida (Turbellaria). Fortschr Zool 36:523

Rieger RM, Salvenmoser W, Legnitti A, Reindl S, Adam H, Simonsberger P, Tyler S (1991a) Organization and differentiation of the bodywall musculature in Macrostomum (Turbellaria, Macrostomida). Hydrobiologia 227:119–129

Rieger RM, Tyler S, Smith JPS, Rieger G (1991b) Plathyhelminthes: Turbellaria (Chapter 2). In: Harrisson FW, Bogitsch BJ (eds) Plathyhelminthes and Nemertinea. (Microscopic anatomy of invertebrates, vol 3) Wiley-Liss, New York, pp 7–140

Rieger RM, Salvenmoser W, Legniti A, Tyler S (1994) Rhodamine-phalloidin preparations of *Macrostomum hystricinum marinum* (Plathelminthes) morphology and postembryonic development. Zoomorphology 114:133–147

Rieger RM, Salvenmoser W, Reiter D, Boyer BC (1995) Differentiation of the body wall musculature in *Macrostomum* and *Haploplana* ("Turbellaria", Plathyhelminthes) (abstract). Hydrobiologia 305:225

Seilern-Aspang F (1957) Die Entwicklung von *Macrostomum appendiculatum* (Fabricius). Zool Jahrb Anat 76:311–330

Stefanini M (1967) Fixation of ejaculated spermatozoa for electron microscopy. Nature 216:173

Torrance SA, Stuart DK (1986) Gangliogenesis in leech embryos: migration of neural precursor cells. J Neurosci 6:2736–2746

Tyler S (1981) Development of cilia in embryos of the turbellarian *Macrostomum*. Hydrobiologia 84:231–239

Tyler S, Hyra GS, Rieger RM (1994) Three-dimensional visualization of musculature in the acoelomate worm *Convoluta pulchra* (Turbellaria). Am Zool 33:115