Postembryonic development of dorsoventral and longitudinal musculature in Pycnophyes kielensis (Kinorhyncha, Homalorhagida)

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Synopsis
We investigated the development of dorsoventral and longitudinal musculature in all postembryonic stages of the kinorhynch Pycnophyes kielensis. Although the earliest stages have only 8 externally separated trunk segments, they already possess dorsoventral muscles for 10 (prospective) trunk segments. The last, 11th, pair is added in the third juvenile stage. Longitudinal musculature, in contrast, is slower to develop and reaches its full length only in the adult. In several juvenile individuals, single fibers project from the longitudinal musculature into the following segments. In all juvenile stages, longitudinal muscles are continuous between segments, whereas in adults they are segmentally separated from each other. Such late occurrence of a segmental pattern in the longitudinal musculature is in contrast to patterns of muscle development in arthropods and annelids.

Introduction
Kinorhyncha, with approximately 180 described species, are remarkable among related groups within the Cycloneuralia (nematodes, nematomorphs, priapulids, and loriciferans) in expressing segmental patterns in several organ systems. Adult kinorhynchs always have an introvert that can be completely withdrawn into the trunk, a neck region that acts as a closure apparatus when the introvert is withdrawn, and 11 trunk segments (Fig. 1A).

The external segmentation is caused by the cuticle, which is dorsally and ventrally subdivided into thicker regions covering the segments. In the intersegmental regions, the cuticle is very thin. In addition, each dorsal and ventral part of the cuticle is curved inward or thickened. This structure is called the pachycyclus. Pachycycli are ideal attachment points for musculature, and in adult kinorhynchs longitudinal musculature attaches to the posterior margin of a pachycyclus and runs to the anterior margin of the following pachycyclus (Kristensen and Higgins 1991; Adrianov and Malakhov 1994; Neuhaus and Higgins 2002; Müller and Schmidt-Rhaesa 2003; Rothe and Schmidt-Rhaesa 2004). In addition to its presence in the cuticle and the musculature, this segmental organization is also present in the nervous system (Kristensen and Higgins 1991; Adrianov and Malakhov 1994) and in the distribution of glands (GaOrdónez and others 2000).

There is no convincing definition of the terms “segment” and “segmentation,” in contrast to “metamery.” Segmentation is often restricted to the pattern of body organization found in annelids and arthropods. Therefore, kinorhynch researchers have always struggled between calling the iterated trunk regions “segments” (Kristensen and Higgins 1991) and “zonites” (Zelinka 1928). As an iteration is present in several organs, lacking only coelomic cavities and excretory organs, we regard the term “segment” as appropriate for kinorhynch body regions. However, the use of this term is not meant to imply an a priori homology of iterated body regions in kinorhynchs, arthropods, and annelids.

As it has been hypothesized (Aguinaldo and others 1997) and further corroborated (for example, Garey 2001) that arthropods may be closely related to cycloneuralians in a taxon Ecdysozoa, the evolution of segmental patterns is of central interest. Apart from raising the question of whether segmentation evolved independently in annelids and arthropods or was inherited from a common ancestor, the Ecdysozoa hypothesis asks whether segmentation in kinorhynchs and in arthropods is comparable, that is, whether it was inherited from a common ancestor or evolved separately.

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To address this question, we studied the complete architecture of the musculature, first in an adult cyclorhagid kinorhynch, *Antygomonas* sp. (Müller and Schmidt-Rhaesa 2003), and then in the homalorhagid species *Pycnophyes kielensis* and *P. dentatus* (Rothe and Schmidt-Rhaesa 2004). In the latter publication, a comparison of the last juvenile stage with the adult indicated that the segmental pattern in adult animals likely arises from a continuous band of longitudinal musculature. We have now investigated all postembryonic stages of *P. kielensis* and describe here the development of the longitudinal musculature.

Development in kinorhynchs falls into 2 phases. Early development takes place within the egg. It has been possible to observe these stages only very rarely (Kozloff 1972). From the eggs, kinorhynchs hatch with 8 trunk segments (in the sense of completely separated units) already externally visible and develop through 6 postembryonic stages to the adult. For *P. kielensis*, the species investigated here,
postembryonic development has been documented by Neuhaus (1993). Stages are called juvenile 1–6 (J1–J6). Trunk segments are generally numbered 3–13, because the introvert and neck are regarded as representing 2 modified segments. Regardless of the validity of this assumption, we use the standard numbering 3–13 for the 11 trunk segments.

Materials and methods
Specimens of *P. kielensis* were collected from muddy sediments in the intertidal close to Braderup, Island of Sylt, in the North Sea (54°56′5″N/08°21′37″W) on several occasions and at different times of the year. Sediments were also kept in a seawater aquarium for several months, and all different juvenile stages could be extracted from these sediments. Extraction from sediments followed the “bubbling method” of Higgins and Thiel (1988).

For fixation, specimens were relaxed for 10 min with 8% MgCl₂ solution and then incubated in freshly made 5% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) at pH 7.4. Fixations were carried out at 0°C overnight. After fixation, specimens were washed several times with 0.01 M PBS and stored at 4°C in 0.01 M PBS containing 0.05% NaN₃ for up to several weeks.

For staining, specimens were first preincubated for 24 h at 4°C in a preincubation buffer containing 0.1% Triton-X 100, 0.25% bovine serum albumin, and 0.05% NaN₃ in PBS. Specimens were then incubated in FITC-phalloidin (Sigma) (2 μM 3.8 μM solution in 50 μl preincubation buffer) for 16–24 h, depending on the developmental stage of the animals. Then specimens were rinsed in PBS several times and embedded in Citifluor on microscopical slides. A Leica TCS 2 confocal laser scanning microscope (CLSM) was used for the investigations. Series of optical sections were projected onto 1 maximum-projection image.

We investigated the following numbers of specimens using the CLSM: J1: 12 specimens, J2: 7, J3: 2, J4: 4, J5: 11, J6: 14.

For transmission electron microscopy, specimens were fixed for 8–16 h at 4°C in 2.5% glutaraldehyde in 0.1 M sodium-cacodylate buffer, including a few crystals of ruthenium red. Postfixation was performed with 1% OsO₄ in 0.1 M sodium-cacodylate buffer for 2 h at 4°C. Fixed specimens were dehydrated in an acetone series and embedded in Araldite. Ultrathin sections (~70 nm) were made with an Ultracut E (Reichert); sections were automatically contrasted with uranyl acetate and lead citrate. A Philips CM 100 transmission electron microscope was used to investigate the sections at 60 and 80 kV.

Results
The different juvenile stages correspond to the ones described by Neuhaus (1993). In the following, we use the shorthand J1–J6, for juvenile stage 1 (the first postembryonic stage) to juvenile stage 6 (the last juvenile stage before molt to adult).

Described here are muscles present in all or almost all trunk segments in the adult: dorsoventral muscles and longitudinal muscles. Dorsoventral muscles attach close to the ventral midline and run to a more lateral attachment on the dorsal side (Fig. 1E). Longitudinal muscles fall into 2 portions: paired ventral and paired dorsal portions. The dorsal longitudinal muscles run close to the dorsal midline, whereas the ventral ones are more lateral in position (Fig. 1E). All dorsoventral and longitudinal musculature is distinctly cross-striated (Fig. 1D).

J1/J2
Externally, the 2 earliest postembryonic stages show 8 trunk segments (Fig. 2A). The last segment (segment 10) is distinctly larger than the previous ones and already carries spines typical of the posterior segments of later stages. A superficial transverse furrow is also present but does not completely separate adjacent segments. Stages J1 and J2 differ only in their length and number of spines. Internally, the musculature is identical.

Within the large segment 10, there are 3 pairs of dorsoventral muscles, indicating the prospective muscles of segments 10–12 (Fig. 2B and C). In almost all specimens, each segmental dorsoventral muscle consists of 2 distinctly separated muscle bundles. The bundles of the prospective segments 10 and 11 are close together, whereas they run clearly separate in the prospective segment 12. Both bundles are interpreted as belonging to segment 12 because they are anterior to the intestinal sphincter that marks the boundary between prospective segments 12 and 13 (Fig. 2C). The ventral and dorsal longitudinal musculature runs from segment 3 to the border of segments 9 and 10; it may terminate in the anteriormost region of segment 10, but never further posterior. The longitudinal muscles are not segmentally arranged; they run continuously over the segmental borders (see Fig. 2E for a similar pattern in J6). Within each segment, 2 sarcomeres are present.

J3
Comparable to the first 2 stages, 8 trunk segments (3–10) are externally visible. In addition to the
dorsoventral muscles described for J1 and J2, the dorsoventral muscle of segment 13 is formed. The 2 specimens investigated at this stage differ slightly in this respect. Whereas in 1 specimen there is only 1 bundle (on each side of the segment), there are 2 bundles in the other specimen. As in the previous stages, ventral and dorsal longitudinal musculature terminates at the border of segments 9 and 10.

J4
Externally, 9 trunk segments are visible (segments 3–11); a transverse furrow on the dorsal side of the last segment indicates a later separation between segments 11 and 12. Internally, 11 pairs of dorsoventral muscles are present, that is, 1 for each prospective trunk segment (3–13). The last pair of dorsoventral muscles is present in all specimens investigated, but to varying extents. In 1 specimen, this dorsoventral muscle is clearly separated into 2 bundles of muscle fibrils on each side, whereas another specimen shows only a single bundle on each side. In a third specimen, this last pair of muscles is asymmetrical, with a weak bundle on one side and a stronger bundle on the other side.

Longitudinal musculature is present in trunk segments 3–9. In a few specimens, longitudinal...
musculature also runs into the 10th segment, but specimens vary in this respect. One specimen shows only 1 fiber of the left dorsal, whereas all other longitudinal muscles end at the posterior margin of segment 9. Another specimen has fibers running from both ventral and the left dorsal longitudinal muscle into segment 10. The remaining specimens show intermediate states.

**J5**

Externally, J5 shows, comparable to J4, 11 trunk segments, with an incomplete separation of the 12th segment. Dorsoventral musculature is present in all 11 trunk segments (3–13), and on each side it consists of 2 bundles per segment. The longitudinal musculature (dorsal and ventral) runs from segment 3 to segment 10 and ends in the posterior region of segment 10. Of the 11 specimens investigated, 2 had thin fibers of longitudinal musculature also in segment 11. One specimen had a fiber from both ventral and dorsal muscles, whereas in the other specimen both the dorsal and the left ventral muscles were present, and the right ventral muscle was absent. The longitudinal muscles are composed of 3–4 sarcomeres per segment, each between 8 and 9 μm in length.

**J6**

Externally, all 11 trunk segments are visible, although the separation between segments 12 and 13 is not complete. Internally, all pairs of dorsoventral muscles are present, most with a pair of bundles on either side of the segment (Fig. 2D). Some specimens show only 1 larger bundle per side in several segments. The longitudinal musculature runs to the posterior margin of segment 11 (Fig. 2D), but a few specimens show weak musculature also in segment 12. In these cases, only single fibrils of musculature are detected, either on the dorsal or on the ventral side. As has been described for J1/J2, and as is valid for all other post-embryonic stages, in J6 the longitudinal muscles also run continuously over segmental borders (Fig. 2E).

**Adultus**

In the adult stage, all 11 trunk segments (3–13) are clearly separated (Fig. 1A). Internally, dorsoventral muscles are present in all trunk segments (Fig. 1B). In a few specimens, likely young adults, dorsoventral muscles consist of a pair of bundles on each side of the segments, whereas in most specimens (presumably older adults) only 1 bundle is present on each side. The longitudinal musculature (ventral and dorsal) runs from segment 3 to segment 12 and ends at the posterior margin of segment 12 (Fig. 1B). In contrast to the juvenile stages, the longitudinal musculature is now separated by clear gaps at the transitions between adjacent segments (Fig. 1C). These gaps correspond to the pachycycli, internal curvatures of the cuticle. Longitudinal musculature attaches to the posterior margin of one segmental pachycycle and runs to the anterior margin of the following pachycycle (Fig. 3A).

In a few cases, a phalloidin signal can be detected between segments. This corresponds to cases in which a portion of a longitudinal muscle runs over the pachycycle. We found cases in which 2 muscle cells are in contact directly over a pachycycle (Fig. 3B) but also cases in which muscle cells run completely over the pachycycle (Fig. 3C).

**Discussion**

Although externally visible segments develop consecutively during postembryonic development of *P. kielensis* (compare Neuhaus 1993), the development of the trunk musculature is either faster (dorsoventral muscles) or slower (longitudinal muscles). The final number of dorsoventral muscles is present from stage J3 onward, when there are still only 8 externally visible trunk segments. Dorsoventral muscles are first formed as a pair of bundles on each side of a segment; later these 2 bundles fuse to form 1 larger bundle. This fusion may appear in J6 or in the adult stage; in general, older adult specimens can be distinguished from younger ones by this characteristic.

The longitudinal musculature remains quite short during several stages (J1–J4). It ends in segment 9, although in some specimens fibers already extend into the following segment. From J5, longitudinal musculature runs into 1 following segment per stage. The investigation of several specimens per developmental stage showed that there may be deviations from a general pattern. In several specimens, small fibers were found to extend from one segment into the subsequent segment. In several such cases, there were asymmetries, with a fiber being present either on the dorsal or on the ventral side. It does not appear that either ventral or dorsal longitudinal musculature develops faster.

The longitudinal musculature appears to run continuously over the segmental borders. This has previously been observed in J6 of *P. kielensis* (Rothe and Schmidt-Rhaesa 2004) and was briefly mentioned by Zelinka (1928) and Remane (1936) in their monographs on kinorhynchs. The segmental character of the longitudinal musculature seems to be functionally linked to the extent to which pachycycli extend into the interior of the animals. In adults, this inward curvature of the cuticle is much stronger than in the juvenile
stages. In addition, the cuticle itself is distinctly thicker in adults. However, it remains probable that muscle cells in earlier juvenile stages do not run over segmental borders but attach directly over these borders to each other. This would support the hypothesis for an early segmental arrangement of the longitudinal musculature. In those cases where muscles still ran over the pachycycli in adults, some individuals showed a border between muscle cells over the pachycyclus, but the complete running of a longitudinal muscle cell over a pachycyclus in other specimens shows that the arrangement of muscle cells can also be non-segmental. This means that the segmental pattern in the musculature appears quite late during development.

In comparison with the development of segmental patterns in arthropods and also annelids, the timing of mesodermal segmentation appears to be different. In arthropods and annelids, mesodermal material originates from a posterior growth zone and is very soon subdivided into segmental regions (see, for example, Scholtz 1997 for crustaceans and Weisblad and Huang 2001 for leeches). Within these regions, myogenesis occurs comparatively late (Manton 1949; Anderson 1973). For example, in onychophorans the mesodermal is already segmented and coelomic cavities are present, but the coelomic wall cells are still undifferentiated (Bartolomaeus and Ruhberg 1999). Therefore, these developmental data from kinorhynchs may be taken

**Fig. 3** *P. kielensis*, adults, longitudinal section, transmission electron microscopy. (A) Two longitudinal muscles (mc1 and mc2) attach at anterior and posterior margin of pachycyclus (pa), respectively. (B) Two longitudinal muscle cells contact each other over a pachycyclus (arrow); cell border is directly over the pachycyclus. (C) One muscle cell runs completely over a pachycyclus.
as a first indication of a different mode of segmentation compared with arthropods and would support the idea of the independent evolution of segmental patterns in kinorhynchs and arthropods. This should, however, be confirmed by investigating the development of other segmental organs, for example, the nervous system, and the expression of genes involved in segment formation in arthropods and annelids.

References