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Early embryonic development of the priapulid worm,

***Priapulus caudatus* Lam.**

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SUMMARY

The early cleavage up to gastrulation is described here for the priapulid worm *Priapulius caudatus*, contradicting and clarifying earlier partial reports on this topic. The cleavage pattern up to gastrulation is highly symmetrical, total, sub-equal, radial and stereotypical. Gastrulation is intermediate between epiboly and invagination, and the mesendoderm may be derived from both cells of the first cleavage, thus differing significantly in its origin from that of many other protostomes. Priapulids occupy an increasingly important position in studies of animal evolution as they appear to be relatively basal within the new clade Ecdysozoa (panarthropods plus cycloneuralians); and have been described as both morphological and genetic living fossils. The insights derived from priapulids combined with new data published recently on kinorhynchs and tardigrades imply a substantial developmental diversity among basal ecdysozoans, and weakens the hypothesis that irregular cleavage is plesiomorphic to the entire clade. Further study is required to reconstruct basal cleavage patterns in both this clade, and indeed, the Bilateria as a whole.

INTRODUCTION

The phylum Priapulida is a small group of marine worms that includes no more than 19 described species (Shirley and Storch 1999; Todaro and Shirley 2003). Despite their low species number (one of the smallest of any phylum), they are ecologically diverse. They occur in both warm tropical and cold arctic waters and in depths ranging from the inter-tidal zone down to several thousand meters, with the largest extant species reaching almost 40 cm, whereas some species do not even reach 1 mm (Shirley and Storch 1999). Although the persistence of such small numbers in a clade has proved problematic to account for, one possibility is that extant priapulids represent the relicts of a former much more species-rich group. This is documented by a substantial stem-group priapulid and scalidophoran fossil record from the Cambrian (e.g. Conway Morris 1977; Wills 1998; Budd and Jensen 2000; Budd 2004; Dong et al. 2004; c.f. Strathmann and Slatkin 1983). However, with the notable exception of the Carboniferous Mazon Creek biota (Schram 1973), the post-Cambrian record is extremely poor. The excellent Cambrian fossil record shows that the morphology of some extant priapulids was broadly represented in the Cambrian era from at least 515 million years ago, giving rise to the view of the extant ones as “living fossils”. Moreover, priapulids broadly resemble the predicted ecdysozoan ancestor, which is thought to be of (1) vermiform shape with (2) an annulated or even segmented body of (3) relatively large adult size (4), radial cleavage (5), a terminal mouth, (6) cycloneuralian brain, (7) direct development and (8) ecdysis (e.g. Budd, 2001). Altogether, these are characteristics of at least some extant priapulids like *P. caudatus*.

Although they have languished as a “minor phylum” for many years, interest in priapulids has increased in recent years with the advent of the Ecdysozoa theory (Aguinaldo et al. 1997). Priapulids and their relatives included in the Cycloneuralia (Ehlers et al. 1996), i.e. nematodes, nematomorphs, and scalidophorans (kinorhynchs, loriciferans and priapulids), are

now considered to be the sister group of the arthropods *s.l.* (Aguinaldo et al. 1997). A number of molecular phylogenetic studies further suggest that the scalidophorans are the sister group to the Nematoidea (i.e. nematodes plus nematomorphs) or to the Nematoidea plus Panarthropoda. In the latter case the priapulids would occupy a relatively basal position within the Ecdysozoa (Mallatt and Giribet 2006). For these reasons the scalidophorans, in particular the priapulids, are currently in the spotlight of evolutionary and developmental interest (Park et al. 2006; Webster et al. 2006, 2007; this paper). Indeed, the argument has been made that priapulids represent the closest approximation we have to the last common ancestor of the Ecdysozoa, based on genetic as well as morphological studies mentioned above (Webster et al. 2006). Whilst extrapolation from a single taxon to the node that subtends it in a tree is hazardous, priapulids may thus be able to offer important comparative data on all aspects of ecdysozoan evolution, not least because they are relatively basal compared to both *Caenorhabditis elegans* and *Drosophila melanogaster*, the two classical ecdysozoan model organisms. Lastly the recent approval of the sequencing of the *Priapulus caudatus* genome (<http://www.genome.gov/10002154>) places an urgent demand on increased knowledge of all aspects of priapulid biology, not least their development. For example, little can be gained from comparative studies of gene expressions without a basic knowledge of the developing anatomy.

Despite their wide distribution, large size and potential importance for evo-devo questions, comparatively little is known about priapulid structure (e.g. Storch and Alberti 1985; Lemburg 1999; Adrianov and Malakhov 2001a,b; Rothe et al. 2006), ecology (Land 1970; Huang et al. 2004) and development (Zhinkin 1949; Lang 1953; Zhinkin and Korsakova 1953), which is based mostly on two species, *Priapulus caudatus* and *Halicryptus spinulosus*. As part of a larger study to document the morphological and molecular development of a species of the Priapulida then, the early embryology of the priapulid *P. caudatus* is described with modern morphological techniques and proper cultivation conditions for the first time.

The main aim of the study is not only to re-evaluate the pioneering work on priapulid development that was carried out more than fifty years ago (Zhinkin 1949; Lang 1953), but also to begin the contribution to the reconstruction of the last common ancestor of the Ecdysozoa (Budd 2001) that priapulids may uniquely be able to offer.

MATERIALS AND METHODS

Animal collection, fertilization and handling of embryos

Specimens of *Priapulius caudatus* were collected from Gullmarsfjorden (Fiskebäckskil) on the west coast of Sweden in November. Mud from depths of between 35 to 60 meters was collected with a ring dredge and then sieved behind the boat with an extra net. Gonads were removed from mature females, placed in filtered seawater, and eggs shaken off the ovaries under a dissecting microscope; a large female typically produces many tens of thousands of eggs. The male gonads were removed and pressed through a fine mesh into filtered seawater. The vitality of the sperm was checked after approximately one hour under a microscope. A small quantity of sperm was mixed into 1000 µl of filtered seawater and used to fertilize the eggs (approximately 50µl of sperm solution to 50 ml of eggs in seawater). Several batches of eggs from different females were fertilized with sperm of several different males to avoid fertilization specific artefacts. Fertilized eggs were kept in petri dishes filled with filtered deep seawater, and kept at a constant temperature of 9°C. To keep the dishes free from contamination with bacteria, fungi and protozoans and to keep the level of metabolites low, the water was exchanged every second day.

Large numbers of embryos were available for study, although some stages (e.g. the four cell stage) seem to be rather fleeting. A few embryos of each stage were selected for detailed study as described below, after surveys showed them to be representative of normal development.

Fixation methods

4% paraformaldehyde in PBS was used as fixative and the time interval for fixation was ten hours, starting at the moment of fertilization and continued until the larvae hatched. Embryos were left in the fixative for four hours at room temperature or overnight in the cold room at 8°C. After PBS washing embryos were kept in 70% ethanol at 4°C. For SEM microscopy the embryos were additionally post-fixed in 1% osmium tetroxide (OSO_4) for one hour. After this post-fixation step the embryos were washed in PBS and stored in 70% ethanol at 4 °C.

Staining methods

Acridin Orange (AO) Fluorescence (Merck) is a versatile stain of nucleic acids and other cell constituents such as the spindle apparatus and cell boundaries (Darzynkiewicz and Kapuscinski 1990). Embryos were taken stepwise down to deionised water (dH_2O). A toothpick of AO was added to 1000 μl dH_2O and 250 μl of the solution was added to each tube containing ca. 30 embryos. Embryos were incubated for 15 minutes in the staining solution. The superfluous amount of AO was removed from the embryos through repeated rinsing with dH_2O . After rinsing the eggs were stepwise dehydrated in ethanol.

Propidium iodide (PI) (Calbiochem) is a specific stain for nucleic acids. Embryos were stepwise rehydrated in dH_2O and 4 μg PI was added to 1000 μl dH_2O . Subsequently the embryos were incubated for 15 minutes in the PI solution. The superfluous amount of PI was removed from the embryos through repeated rinsing with dH_2O .

In order to visualize embryos under the confocal microscope they were cleared with a 1:2 mixture of benzyl alcohol and benzyl benzoate (BABB).

Documentation techniques

We used a Leica TCS-SP confocal microscope to visualize and document the stained embryos. Preparation of picture series and conversion into 3D images was carried out in the Leica confocal software and ImageJ (Abramoff et al. 2004). To produce the reconstructions of the different embryonic stages shown in Fig. 3, Mimics 9.1, a 3D image-processing program (Materialise) was used. Confocal stacks were imported, and nuclei, spindles and cell boundaries of interest were marked manually. SEM pictures were taken with a Zeiss Supra 35VP scanning electron microscope with field emission gun. Final editing, for both confocal and SEM pictures, was carried out in Adobe Photoshop CS.

NOMENCLATURE

A nomenclature based on that adopted by Herztler and Clark (1992) and Alwes and Scholtz (2004) was used (Fig. 1). The two cells arising from the first cleavage are labelled **AB** and **CD**, respectively, which give rise to the four cells **A**, **B**, **C** and **D** after the second cleavage. Subsequent daughter cells are labelled with the subscript I and II according to whether they meet at the cross furrow or not, respectively. Further longitudinal cleavages are labelled a or p for anterior or posterior daughters, based on the vegetal pole being anterior. Cells that divide along latitude are labelled with e for equatorial as they all lie away from the poles. Subsequent daughters of these cells are labelled h or b according to whether they lie towards the hinge or the break of the hatching cap. Important cells at the vegetal pole involved in gastrulation are labelled with capital letters **U-Z** as discussed in the text.

Although there is no apparent distinction between **AB** and **CD**, and between the two pairs of cells these cells give rise to, we distinguish between them by their relationship to the hatching cap (see below). During the early stage of the second cleavage, the hatching cap lies over the cell labelled **AB**, with the hinge coincident with the cleavage plane (Fig. 1); the margin of the

cap along which it will eventually break lies over **AB**. After the rotation of the egg described below, the cap lies over the vegetal pole, the hinge over the furrow between **C** and **D**, and the break between **A** and **B** (Fig. 1). We have no evidence that there is any other kind of movement of the cap (for example, rotation around the axis of the embryo) or the presence of mirror image embryos, although neither of these can be ruled out without definitive cell markers. We have chosen the nomenclature so that the two cells meeting at the vegetal pole cross furrow are **B** and **D** as in both of the arthropod studies mentioned above. This reflects the view (e.g. Hejnol and Schnabel 2006) that early cleavage patterns of the type represented here are homologous within the Ecdysozoa.

RESULTS

Pre-fertilization procedures

The natural spawning time for priapulids on the west coast of Sweden seems to be in December (Lang 1953). However, we have succeeded in obtaining viable sperm and eggs that can be successfully fertilized from October to March; and fertilization outside these times may be possible too. Since no reliable method to induce spawning exists for priapulids, gonad stripping is the best method to obtain viable gametes. It is possible to sex mature specimens of *Priapulius caudatus* by examining the form of the paired gonads through the semi-transparent body wall. Both the male and female gonads are sack-shaped. However, the male gonads are multiply branched and thread-like whereas the female gonads are composed of convoluted tubes. For the female gonads the grade of convolution marks the stage of maturation with less compactly convoluted gonads being more mature than tight ones. It is crucial for the correct development of the later embryos to strip the gonads at the right time. The use of slightly immature eggs can indeed lead to initial development of the embryos, but soon after the first cleavages development becomes irregular and finally results in a die-off of the embryos.

Similar effects have been observed in embryos taken from mature females that were kept in captivity for several days prior to gonad stripping. The stress of captivity interferes with the proper development/storage of oocytes in the female gonads. Comparable effects have never been observed for prepared male gonads. Even in females close to natural spawning a number of eggs are not mature. These eggs are smaller than mature ones and can be of irregular shape. Eggs that were obviously immature were removed prior to fertilization to avoid the later documentation of artefacts.

Fertilization and polar bodies

Our method to fertilize exclusively mature eggs of females that are close to natural spawning is very efficient, with more than 90% of eggs undergoing complete embryonic development and only a very small number of irregularly developing embryos (ca. 2%; ca. 8% of the eggs remain unfertilized or fail to undergo the first cleavage). The mature fertilized egg is round with a diameter of ca. 50 μm . The size of the egg increases to 70-80 μm during the first cleavages and remains relatively constant until the larva hatches. The priapulid embryos themselves contain large amounts of yolk granules that are evenly spread within the cells (not shown). We have observed only one obvious polar body. It is located at right angle to the first cleavage plane and approximately 4 μm in diameter, with a central nucleus (Figs. 2A, B and 3A). The fact that we were not able to observe polar bodies in the vast majority of our samples implies that they detach easily from the embryo during preparation (but see also comments on the included granules, below). The correctly developing embryo is surrounded by two membranes: a robust eggshell (ca. 2 μm thick), which protects the embryo from mechanical influences, and a fertilization membrane. The latter becomes visible with fertilization and encloses the embryo tightly throughout embryonic development (Lang 1953, personal observation).

First and second cleavages

At 9°C the first cleavage takes place approximately 15 hours after fertilization. Within the eggshell the structure of a “*hatching cap*” becomes visible before the first cleavage takes place (not shown). The inner side of this cap is covered by a number of small pustules, whilst no pustules are present on the inner surface of the rest of the eggshell (not shown). The hatching cap represents a line of weakness in the shell and the shell is observed to break at one side of it while the other side acts as a hinge (see also nomenclature, above). By the end of the first cleavage the hatching cap lies to one side of the cleavage furrow with the hinge coinciding with it (Fig. 3BI); the cap itself lies over **AB**.

The 2-cell stage consists of two equally sized blastomeres (Figs 2C and 4A) labelled **AB** and **CD**, of which the nuclei are also of approximately the same dimensions. Cleavage is holoblastic throughout. At second cleavage the nuclei divide simultaneously in both cells (Figs 2C, 3BI-II). During metaphase the spindles are slightly skewed. The angle between the separate spindles and the boundary between the two cells is approximately 10° to 15°. The angle between the two spindles is approximately 40° (Fig. 3BI). However, by late anaphase, the spindles have reoriented to lie parallel to each other, at right angles to the animal vegetal pole, and slightly toward the animal pole (Fig. 3BII). By the end of this cleavage the vegetal pole is now marked by the centre of the hatching cap: the hinge lies over the furrow between **C** and **D**; and the break point over the furrow between **A** and **B**.

The second cleavage results in a situation where only two of the four cells, **B** and **D**, meet at the poles, so that cross furrows are formed between them (Figs 2D and 4B); the cleavage is thus slightly unequal. The furrows at both animal and vegetal poles are parallel; however, the four cells all meet at the centre of the embryo. From the second cleavage stage onwards a brightly stained granule is observed in the central part of the embryo that does not depict a

proper nucleus (Fig. 2C, D, F, H, I); it lies in a small central cavity between the dividing cells (data not shown).

Third cleavage and 8-cell stage

Up to this point the initiation of the cleavage is synchronous in all blastomeres of a given embryo (Figs 2D, and 3C). During the third division the spindles are broadly oriented along the animal-vegetal axis (Figs 2D, 3C and 4B). All four spindles tilt slightly towards the animal pole; those within **A** and **C** sit slightly higher in their cells relative to those of **B** and **D**. The result of the third cleavage is an 8-cell stage with four blastomeres at the vegetal pole, **A_{II}**, **B_I**, **C_{II}** and **D_I**, and four blastomeres at the animal pole, **A_I**, **B_{II}**, **C_I** and **D_{II}**, in a typical radial cleavage pattern (Figs. 2E and 4C). The blastomeres located closer to the vegetal pole are slightly larger than the others (data not shown; Fig. 2E). The cells meet in the innermost part of the embryo. The cross furrow at the animal pole is now at 90 degrees to that at the vegetal pole. However, sections through these embryos show that the furrow is in fact parallel from just below the animal pole downwards, suggesting that the furrow at the animal pole has changed direction by **A** and **C** slightly overgrowing **B** and **D** very close to this pole. The view that the furrow direction at the animal pole changes is reflected in our nomenclature (Fig. 1). The 8-cell stage thus technically consists of two interlocking chains of cells: **A_{II}**, **A_I**, **C_I**, **C_{II}**; and **B_{II}**, **B_I**, **D_I**, **D_{II}**, although this is not obvious because both cross furrows are short. Spindle directions are similar along each chain.

Fourth cleavage and 16-cell stage

The fourth division is again almost synchronous. As in the third cleavage the spindles are once more broadly oriented along the longitudinal axis and tilting towards the closest pole (Figs 3D and 4D). The 16-cell stage is characterised by four rows of four cells (Figs 2F and

4D). The eight cells located closer to the vegetal pole are now notably larger with a diameter of ca. 25µm; at the animal pole the cells have a diameter of only ca. 15 µm. As before, the embryo can also be considered to consist of two interlocking bands of cells, i.e. **A_{IIa}**, **A_{IIp}**, **A_{Ia}**, **A_{Ip}**, **C_{Ia}**, **C_{IIp}**, **C_{IIa}**; and **B_{IIp}**, **B_{IIa}**, **B_{Ip}**, **B_{Ia}**, **D_{Ia}**, **D_{Ip}**, **D_{IIa}**, **D_{IIp}**, each generated with similar spindle directions.

Fifth cleavage and 32- cell stage

At this point of development the cleavage pattern changes. As in the previous stage the spindles of the four cells surrounding each pole point towards the respective pole. Because the cells at the animal pole are now notably smaller than those at the vegetal pole, their spindles are at a higher angle to the animal vegetal axis (Figs 2G, H and 3E). In the two cell rows around the equator of the embryo, the spindles are oriented tangentially along the equator (Fig. 3E). This results in a 32-cell stage with two rows of four cells close to each pole (Fig. 4E); **A_{Ipp}**, **B_{Ipp}**, **C_{Ipp}**, **D_{Ipp}** and **A_{Ipa}**, **B_{Ipa}**, **C_{Ipa}**, **D_{Ipa}** at the animal pole and **A_{IIaa}**, **B_{Iaa}**, **C_{IIaa}**, **D_{Iaa}** and **A_{IIap}**, **B_{Iap}**, **C_{IIap}**, **D_{Iap}** at the vegetal pole. Between these two double quartets, there are two rows of eight cells around the equator (Fig. 1). As at earlier stages the cells at the vegetal pole are larger than the cells at the animal pole. The change in style of cleavage between the poles and equator means that the spindle directions are no longer similar throughout the two chains described above.

Sixth cleavage and 64-cell stage

The sixth cleavage is also approximately synchronous, although less so than in previous stages. Once more, the spindles in the four cells surrounding each pole are oriented towards the respective pole, with spindles again at a higher angle at the slightly cramped animal pole (Figs 2I and 3F). In contrast to this the spindles within the two remaining rows of four cells

and the two rows of eight cells are oriented equatorially. At the 64-cell stage rows of four cells thus still surround the animal and vegetal poles, i.e. **A_{Ippp}**, **B_{IIppp}**, **C_{Ippp}**, **D_{IIppp}** and **A_{IIaaa}**, **B_{Iaaa}**, **C_{IIaaa}**, **D_{Iaaa}** (i.e. **Y**, **X**, **W**, **Z**) respectively (Fig. 4F). A row of eight cells underlies the cells at each pole (Fig. 4F). Finally, two rows of 16 cells lie between the two rows of eight cells (Figs 1, 4F). The cells at the vegetal pole remain larger than the cells at the animal pole.

Coeloblastula and Gastrulation

During the fourth cleavage, after approximately 30 hours, a small blastocoel emerges, and by the fifth cleavage it is clearly visible (Fig. 5A and B). At this stage two small cells lie inside the blastocoel (Figs 5C and Fig. 6). The blastocoel does not persist for long since gastrulation commences shortly after its formation, at the 64-cell stage (Figs 5C-E). The process of gastrulation shows some similarities to invagination, but also to epiboly as the blastocoel is small and rapidly filled, starting with two enlarged cells migrating inwards (Fig. 5D). As these cells meet at the pole in polar sections, they are likely to be the cells labelled **X** and **Z** in Fig. 1. Short after the start of gastrulation the entire blastocoel is filled up by these two cells (Fig. 5D, E). As the gastrulation process continues, two additional cells migrate into the blastocoel (Fig. 5E)'. These cells lie lateral to **X** and **Z**, and are thus likely to correspond to **U** and **V** in Fig. 1. Cells **W** and **Y** do not appear to be immediately involved in gastrulation. Finally the cells inside the blastocoel undergo several cleavages resulting in two rows of cells (Fig. 5F). The blastomeres that are not involved in gastrulation divide continuously during this time.

DISCUSSION

Inconsistencies in previous studies of the early development of *Priapulus caudatus*

The only previous studies on the early embryonic development of *P. caudatus* date back to the middle of the last century (Zhinkin 1949; Lang 1953). As might be expected from such pioneering work, the quality of the results obtained is variable, not least because Zhinkin's work is only illustrated with a few drawings that are not fully consistent with the text. The description of the 2- to 8-cell stages of *P. caudatus* by Lang (1953) and Zhinkin (1949) agree both with each other and with our findings, basically coming to the conclusion that the cleavage is total, radial and subequal. However, their studies of later stages do not agree at all.

Critical disagreements between Zhinkin (1949) and Lang (1953) concerning the early stages of *P. caudatus* embryology up to gastrulation are as follows: 1) According to Zhinkin it takes around 9 hours to reach the 2-cell stage, but according to Lang it takes around 20 hours. 2) The proportions of the polar body shown by Zhinkin (drawing at the 1-cell stage) and Lang (photography at the 8-cell stage) differ considerably. 3) The description of the 32-cell and 64-cell stages differs remarkably in both publications. Lang presents a photograph showing a very regular cell pattern, although one of the two "32-cell stage" embryos he presents is in fact a 64-cell stage embryo. In Zhinkin's hand drawings the cells appear of irregular shape and number at the 32-cell stage. Assuming that the cleavage pattern is still symmetrical at this point, Zhinkin's drawing does not match the number of cells one would expect if the cleavages were still synchronous at the 16- and 32-cell stage. In the text, however, Zhinkin describes the fourth cleavage with cells dividing latitudinally, which would result in the same synchronous pattern we see in *P. caudatus*. Zhinkin mentions a subsequent replacement of blastomeres that could explain the strange 16-cell pattern he presents in the drawings. 4) In Zhinkin's hand drawings all cells seem to be of different shape and size at the blastula and early gastrula stage. Additionally, the two invaginating cells at the edge of the invagination side appear to be elongated. In contrast to this, the cells documented by Lang at this stage appear to be of regular shape and similar size.

Artificial results and misinterpretation in earlier studies

Zhinkin (1949) studied and cultivated developing embryos of *P. caudatus* under a low magnification microscope, presumably at room temperature. Our own control experiments show that such a treatment results in an unnaturally rapid development of the embryos, a large number of misshaped embryos and finally the death of almost all embryos - as Zhinkin himself admitted. Zhinkin's observation that the first cleavage takes place after 9 hours can hence easily be explained by the cultivation of embryos at too high temperatures ($> 10^{\circ}\text{C}$), since the tempo in which fertilized eggs of *P. caudatus* develop is highly dependent on the temperature. We have observed that the first cleavage does not take place earlier than 15 hours after fertilization. However longer times between fertilization and first cleavage have also been observed, in agreement with Lang's observation (Lang 1953). Lang kept adult priapulids as well as embryos in aquaria with a constant flow of seawater collected from 30 meters depth. As a consequence of Zhinkin's cultivation method only a few embryos remained (ca. 20) for further observation after the first few cell divisions (Zhinkin 1949), and the irregular shape and distribution of blastomeres at his 16- and 32-cell stages seem to be attributable to this too. Degradation of embryo viability also occurs whenever initially keeping adult females in captivity prior to gonad stripping (own observations) as conducted by Zhinkin. To avoid this problem we started fertilization within few hours of catching the priapulids.

With regard to the embryonic stages around gastrulation our data tend to support Zhinkin in his finding that the invaginating cells are elongated and that the blastocoel is quickly filled up with invaginated cells. Lang illustrates a classical invagination gastrula, which is unlike any gastrula we have ever observed for *P. caudatus*. We suspect that Lang's

documentation of the neatly invaginating cells, forming a classical coelogastrula, may be owing to fixation artefacts.

Zhinkin (1949) illustrated a fertilized egg of *P. caudatus* with one polar body at the animal pole (in the text he mentions a second). The same result is presented three years later in his studies on a second priapulid, *Halicryptus spinulosus* (Zhinkin and Korsakova 1953). Lang (1953) describes the attachment of a single polar body in association with fertilization that should be attached to the embryo up to the 8-cell stage, but this appears to be artificial, for two reasons. First, the size of Lang's so-called "polar body" is far too big compared with our own findings (Figs. 2A,B and 3A) and Zhinkin's observation. Second, we found a number of embryos in our preparations showing exactly the same structure as the one Lang claims to depict a polar body. As it turned out this is simply the result of a broken eggshell with a small amount of leaked yolk mass.

Inclusions in *P. caudatus* embryos

An unexpected result of our studies is the presence of granules found within the majority of the embryos at stages up to 32 cells (Fig. 2C, D, F, H). These granules do not depict proper chromosomes, and do not lie within membranes, but stain intensively for acridine orange (AO), which *inter alia* stains DNA. We also successfully stained them with propidium iodide (PI) (not shown), which is known to be a more specific marker for DNA than AO (e.g. Tas and Westerneng 1981; Darzynkiewicz and Kapuscinski 1990). By the 32-64 cell stage, we detected two compact IP- and AO-positive granules within the blastocoel (Figs. 2I, 5C and 6). These granules are clearly surrounded by membranes, which is not the case for the early granules described before. Furthermore the granules each have a small bulge or attachment that also stains IP/AO positive (Fig. 6).

The identity of these structures, and whether the early single granules give rise to the later, membrane-bound ones, is not currently clear. One possibility is that they represent the remnants of internalised polar bodies, which would explain the general lack of external ones: the size of the later structures is comparable to that of the polar body seen at the zygotic stage (Fig. 2A, B). Another possibility is that they can be related to various structures seen in nematode embryos. For example, Goldstein (1977, 1981) described “accessory nuclei” formed by budding off other nuclei in oocytes and early embryonic stages, which resemble the bulging structure in the later membrane-bound granules. Unfortunately, it has not yet proved possible to find a TEM section through one of these structures.

Cell lineage and gastrulation in *P. caudatus*.

The pattern of cleavage, although highly regular and stereotyped, with little variation from one embryo to the next, shows some peculiarities. During the second cleavage both the orientation of the spindles and the egg seem to shift. The spindles start skewed (Fig. 3BI) and later become parallel (Fig. 3BII). The sequence of these events makes it clear that **AB** gives rise to **A** and **B**, and **CD** gives rise to **C** and **D**. At the same time the embryo can be inferred to rotate so that the vegetal pole moves from the edge to the centre of the hatching cap where it remains up to and beyond gastrulation. Such a rotation has been directly observed during the first cleavage of the tardigrade *Thulinia stephaniae* (Hejnol and Schnabel 2005). Another unusual feature is that the cells at the poles divide for several successive rounds longitudinally, and the more equatorial cells latitudinally, in contrast to most other embryos where cells tend to divide longitudinally and latitudinally in alternate cleavage cycles (as discussed in Alwes and Scholtz 2004).. The vegetal pole lies along the first cleavage plane, and after the second cleavage lies in the centre of the cross furrow. The third cleavage, giving rise to the slightly larger vegetal cells, morphologically indicates the anterior-posterior body

axis. The two cells that flank the furrow, i.e. **B_I** and **D_I** and their successive most anterior daughters, eventually give rise to **X** (i.e. **B_{Iaaa}**) and **Z** (i.e. **D_{Iaaa}**), and it is these two cells in our interpretation that first move into the blastocoel, probably followed by their neighbours **U** (i.e. **B_{Iaap}**) and **V** (i.e. **D_{Iaap}**): it is likely that from these cells the presumptive mesendoderm is derived. Thus, priapulid gastrulation seems to be significantly more symmetrical than that of many other protostomes: both of the two first cells give rise to the first cells involved in gastrulation.

Comparison with embryonic development in other cycloneurians

Embryological evidence has thought to be of importance in understanding morphological and developmental evolution in metazoans (e.g. Haeckel 1874; Siewing 1979; Nielsen 1987; Valentine 1997; Scholtz 2002). It has long been considered that to draw reasonable phylogenetic conclusions from embryology the ground pattern for the clade in question needs to be established. However for many groups of animals this has still not been achieved. Nevertheless, the recent tendency to perform analyses on exemplars rather than hypothetical ground patterns (e.g. Prendini 2001) means that any new data from extant species are likely to be of help in reconstructing phylogenetic distribution of characters.

Scalidophora

Priapulida. Only the embryology of *Halicryptus spinulosus* has been studied beside that of *Priapulius caudatus* (Zhinkin and Korsakova 1953). In Zhinkin and Korsakova (1953), no information is given concerning handling and preparation of the *H. spinulosus* embryos. It is therefore likely that embryos of *H. spinulosus* have been handled in the same way as described in the article concerning *P. caudatus* embryology (Zhinkin 1949). For reasons explained before, the data are likely to be influenced by inadequate experimental conditions. However, in *H. spinulosus* the first polar body is observed by the animal pole and the first,

holoblastic, cleavage is along the animal-vegetal axis. Our observations of *P. caudatus* cannot confirm the location of the polar body but the first cleavage plane is the same for both. The cross furrow separating two cells at each pole in *P. caudatus* at the 4-cell stage has not been described for *H. spinulosus* (Zhinkin and Korsakova 1953). In addition it is reported for *H. spinulosus* that the division of one cell is delayed at this stage, which is not the case for *P. caudatus*. At the 2-cell and 4-cell stage some cells are larger than the others in *H. spinulosus*, which is also not the case for *P. caudatus*. The outcome of the third cleavage is almost the same in both species. In contrast to *P. caudatus* only two of the cells at the vegetal pole are enlarged in *H. spinulosus*. During the fourth cleavage the cleavage furrows are oriented latitudinally in *H. spinulosus*, which results in the same “4-4-4-4” pattern described for *P. caudatus* (Fig. 4D) (Zhinkin and Korsakova 1953). At the same time the blastocoel is formed - as in *P. caudatus*. The later stages are described with a very unsymmetrical distribution of cells, in a very similar way as the same authors also described the later stages in *P. caudatus* (Zhinkin 1949; Zhinkin and Korsakova 1953). As the present study shows, this may be the outcome of an inappropriate handling of embryos. The information concerning *H. spinulosus* is hence also scarce and only the description of the first three cleavages appears to be fully reliable. The description up to the 16-cell stage in *H. spinulosus* is very similar to the pattern seen in *P. caudatus* and could therefore be a part of the priapulid ground pattern. However, a more reliable study on the early development of *H. spinulosus* is clearly needed to confirm this and to draw conclusions about the ground plan of priapulid development at later stages.

Other Scalidophora. Data concerning the embryonic development in scalidophorans other than priapulids (i.e. kinorhynchs and loriciferans), have until recently been extremely limited. The embryology of kinorhynchs has largely been unknown apart from the information available from Kozloff (1972). He described the ellipsoid eggs of *Echinoderes*. Besides the shape; only the egg size (75 µm by 65 µm or 80 µm by 70 µm), and the fact that the egg envelope is transparent, is included in his description. However, new work by the same author

(Kozloff 2007) has substantially expanded these data. After the 8-cell stage, cleavage in *Echinoderes* seems to radically diverge from that of *P. caudatus*, with two layers of eight cells being formed; and the later stages are not easily compared to what is observed in priapulids.

Data concerning the loriciferans are limited to the findings in one species that the first cleavages are holoblastic and unequal. The outcome is macromeres located at the vegetal pole and micromeres at the animal pole (Gad 2005). This pattern bears no similarities to our observations on *P. caudatus* development.

Nematoidea

Most information on cycloneuralian embryology is known from nematodes and nematomorphs (i.e. Nematoidea) (Ehlers et al. 1996).

Nematoda. Most nematodes generate five somatic founder cells via a series of unequal cleavages, plus a germline cell; this holds true for all nematodes except for the subclass Enoplia (Schierenberg 2000). Included in the enoplians is an order of free-swimming marine nematodes, the enoplidans, which are assumed to be most basal among the nematodes in many aspects, based on both embryological (Malakhov 1994) and phylogenetic analysis (Blaxter et al. 1998). Among them are species (e.g. *Enoplus brevis*) with a slow developmental rate and total and equal cleavage, as it is the case for the priapulid *P. caudatus* (Voronov and Panchin 1998). Although the second cleavage in enoplians is already characterised by a number of pattern variations, there is also a pattern that is similar to that in *P. caudatus* (Malakhov 1994; Voronov and Panchin 1998). Later stages do not show any similarities to the development of *P. caudatus*.

Nematomorpha. The difficulties of keeping adult nematomorphs alive has meant that their embryology has been relatively poorly studied, with the most important studies being those of Inoue (1958) on *Chordodes* and Malakhov and Spridinov (1984) on *Gordius* (see Bresciani (1991) and Nielsen (2001) for summaries). Cleavage here is total and usually equal,

and gives rise to somewhat variable blastomeres. The embryology of nematomorphs is interesting since larval morphology resembles morphological traits also found in priapulid and kinorhynch larvae (e.g. Hou and Bergström 1994). Some authors have therefore considered them to be closely related phyla (e.g. Malakhov 1994). However, despite morphological similarities in larval and adult stages, the early embryology of the Gordioidea and *P. caudatus* does not show many similarities. In this context it would be interesting to know the embryonic development of the marine *Nectonema*. This is the only genus of the second order of nematomorphs, the Nectonematoidea; and their embryology has never been described (Nielsen 2001).

Panarthropoda

Tardigrada. The embryology of tardigrades has until recently been little studied (but see e.g. Marcus 1929), despite their interesting potential phylogenetic position as a sister group to the euarthropods. However, recent work by Eibye-Jacobsen (1997) and Hejnol and Schnabel (2005, 2006) has contributed significant new data. In heterotardigrades (*Echiniscoides sigismundi* (Eibye-Jacobsen 1997)) and eutardigrades (*Thulinia stephaniae* (Hejnol and Schnabel 2005, 2006)), cleavage is total and equal. The first two cleavages produce a 4-cell stage identical to that in the priapulids. During the third cleavage the blastomeres of *E. sigismundi* do not divide synchronously (Dewel and Eibye-Jacobsen 2006) and from the 8-cell stage on the cleavage pattern differs significantly from the pattern seen in *P. caudatus*. This is also true for *T. stephaniae*: in this species the location of the eight cells varies even between individual embryos (Hejnol and Schnabel 2005). Thus, tardigrade development appears to be significantly less stereotypical than that of priapulids.

Onychophora. Onychophorans are often positioned as sister-taxa to the euarthropods or to the tactopods (i.e. euarthropods plus tardigrades; e.g. Aguinaldo et al 1997; Schmidt-Rhaesa et al. 1998; Budd 2001). Within the onychophorans many different cleavage patterns

are found (Anderson 1973). So far no published data suggest any resemblances between onychophoran and *P. caudatus* embryology. However unpublished data (Eriksson pers. com.) concerning the cleavage pattern of *Euperipatoides kanangrensis* show some similarities to what is seen in *P. caudatus*. Although superficial (Manton 1949; Eriksson pers. com.) the cleavage pattern up to the 16-cell stage is essentially the same as we describe here for *P. caudatus* (“4-4-4-4” pattern). The presence of this pattern at the base of the Panarthropoda (i.e. in an onychophoran) and at the base of the Ecdysozoa (i.e. in a priapulid) may imply that it is plesiomorphic among the Ecdysozoa.

Euarthropoda

A survey of cleavage patterns in the euarthropods is necessarily complicated by the presence of highly yolky eggs and interlecithal and superficial cleavage (e.g. Scholtz 1997); and which of these states is basal is unclear. Further, in embryos that are inferred to have secondarily lost their high yolk content and returned to total cleavage, the cleavage pattern may not easily be comparable to that of other embryos (e.g. as argued by Anderson 1973). Total cleavage is scattered in the arthropod tree (e.g. Anderson 1973; Scholtz 1997). Generally, arthropods with relatively small eggs show total cleavage (Anderson 1973). However there are exceptions, as eggs of amphipods (Crustacea) and diplopods (Myriapoda) for example are very large but nevertheless show total cleavage (e.g. Dohle 1964; Wolff and Scholtz 2002).

P. caudatus shows some intriguing similarities and differences to totally-cleaving arthropod eggs such as from the crustaceans *Sicyonia* and *Meganyctiphanes* (e.g. Hertzler and Clark 1992; Alwes and Scholtz 2004) and myriapods (Dohle 1964). In both cases, early cleavage is essentially radial and sub equal. However at the second cleavage the spindles are at least initially skewed in both priapulids and in the crustaceans mentioned above. At the 8- and 16-cell stages, the embryos consist of two interlocking bands of cells. Nevertheless, *P. caudatus* shows some significant differences from the features suggested to be plesiomorphic for the

arthropods (Scholtz 1997). First, a small blastocoel forms in *P. caudatus* which is not yolk filled; second, the nuclei in *P. caudatus* do not migrate to the outer margins as they typically do even in holoblastic euarthropods (e.g. Scholtz 1997); third, the cell divisions remain essentially synchronous in *P. caudatus* up until at least the 64-cell stage; there is no retardation of division of a large yolky cell that will subsequently be involved in gastrulation; fourth, the pattern of interlocking bands is lost after the 16-cells stage as the cleavages patterns diverge within different cells.

Finally, the cleavage in *P. caudatus* seems to be significantly more stereotyped than the irregular patterns suggested to be basal in both the panarthropods (Scholtz 1997; Hejnol and Schnabel 2005, 2006) and indeed Ecdysozoa (Hejnol and Schnabel 2005, 2006). Thus, it seems premature to generalise irregular cleavage to the base of the Ecdysozoa with current information.

Given the variety of developmental modes now known, the plesiomorphic mode of development within the Ecdysozoa (or even the Cycloneuralia or Scalidophora) is still unclear. Further studies of the priapulids and their closest relatives are thus critical. The apparent dissimilarities between the two examined priapulid species *P. caudatus* and *H. spinulosus* (Zhinkin and Korsakova 1953) is likely to be based on misinterpretations in earlier studies (Zhinkin and Korsakova 1953). Thus, further studies on the early cleavage pattern in priapulids other than *P. caudatus* and including *H. spinulosus* are needed to determine the priapulid ground pattern which may give important insights into that of the Scalidophora, Cycloneuralia or even Ecdysozoa.

Priapulid development from a palaeobiological perspective

The recent description of stem-group scalidophoran embryos from the Cambrian (Dong et al. 2004; Budd 2004; Donoghue et al. 2006a, b) offers a rare opportunity for direct comparison of

developmental modes more than 500 million years apart. At the moment only later embryonic stages from these fossils have been described, but the description here of the distinctive development of priapulids suggests that similar embryos could be detected and recognised in the fossil record. The evolution of ecdysozoan embryology may in the near future thus be tackled with a comparative phylogenetic direction encompassing both stem and crown group representatives from the various groups within the clade.

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FIGURES

Fig. 1. Nomenclature for cells and implied cell lineages in *P. caudatus* embryos used herein, showing views from animal and vegetal poles (cf. Fig. 4). The position of the hatching cap is indicated by the shaded circle in the two- and four-cell stages of the vegetal view. The position of the hinge of the cap is marked by the black dot, and of the break point by the black square. Cells at the vegetal pole discussed in relation to gastrulation are **U** (**B_{Iaap}**); **V** (**D_{Iaap}**); **W** (**C_{IIaaa}**); **X** (**B_{Iaaa}**); **Y** (**A_{IIaaa}**) and **Z** (**D_{Iaaa}**). See text for basis of nomenclature. Notice rotation of animal pole cross furrow from four to eight cell stages (cf. Fig. 4B, C).

Fig. 2. Early cell stages and cleavages in *P. caudatus*. Embryos stained with acridine orange and processed with Leica confocal software to form 2-D projections of z-stacks. The exception is seen in (E) which was processed with Adobe Photoshop CS. White arrows indicate an extra-cellular DNA-granule seen in all early embryonic stages. Note that the structure in I may be the membrane-bound structure seen in later stages (Fig. 6). (A) 1st cleavage, metaphase. Polar body indicated by asterisk. (B) Close up on the polar body (asterisk) located 90° from the 1st cleavage furrow; mem = polar body cell membrane; nuc = polar body nucleus. (C) 2nd cleavage, metaphase. Future vegetal pole is facing down. Note skewed spindles; they will be parallel by late anaphase (Fig. 3B). (D) View over the vegetal pole, 3rd cleavage, metaphase. (E) 8-cell stage. (F) 16-cell stage. Larger blastomeres at the vegetal pole. (G) 5th cleavage. View on the animal pole, most cells in anaphase. (H) 5th cleavage side view with vegetal pole facing down, metaphase. (I) 6th cleavage. Side view with vegetal pole facing down, most cells in metaphase. The spindle orientation of this egg is more clearly illustrated in Fig. 3F.

Fig. 3. Reconstructions of spindle orientation in the first to sixth cleavage . Vegetal pole facing down in B-F. (A) First cleavage, polar body indicated by white arrow. The model is based on the embryo in Fig. 2A, B. (B) Second cleavage. Cell boundary of first cleavage marked in red; hatching cap in light blue, with breaking point arrowed. (I) Metaphase at second cleavage. Note skewed spindle orientations, and position of hatching cap to one side, with the hinge coinciding with the cell boundary. (II) Late anaphase. Cleavage direction is now oriented into the plane of the page and parallel in both cells; spindles have almost disappeared (not shown) but the second cleavage furrow has yet to form. Note that the hatching cap now lies symmetrically beneath the cell boundary; its centre will mark the vegetal pole in later stages. (C) Third cleavage, with emerging daughter cells labelled. All spindles are along the animal vegetal axis but angled slightly towards the animal pole (at top). The spindles connecting **A_I-A_{II}** and **C_I-C_{II}** sit slightly higher in the embryo than those of **B_I-B_{II}** and **D_I-D_{II}**. (D) Fourth cleavage. All spindles are broadly along the animal vegetal axis but angled towards the nearest pole. Cleavage of the nucleus DII is slightly delayed. (E) Fifth cleavage. Spindles at the poles are along the animal vegetal axis and angled towards closest pole (blue arrows); at the animal pole the spindles are slightly more angled than the spindles at the vegetal pole. In the two middle rows the spindles are along the equator of the embryo (red arrows). Model based on the embryo shown in 2H. (F) Sixth cleavage. The spindles at each pole continue to divide longitudinally (blue arrows). The remaining four rows are arranged as follows: two rows with four cells encircling each of the poles (red arrows), and two rows with eight cells, found either side of the equator of the embryo (yellow arrows). In all of these rows the spindles divide along the equator of the embryo. Model based on embryo shown in Fig. 2I. In the model, cells are lacking in the fourth row down; this is not due to absence of cells but the result of weak staining.

Fig. 4. Drawings illustrating the early cleavage stages of *P. caudatus*. Vegetal pole facing down and to the left. Black bars indicate spindle directions; hollow bars indicate sister cell relationships. (A) 2-cell stage. The cleavage is total and equal. Spindles rotate to become parallel and at right angles to cell boundary by end of anaphase (Fig 3B). (B) 4-cell stage. Two cells (**B** and **D**) meet to form a cross furrow (asterisk); the cross furrows at both poles are parallel (vegetal pole not seen here). The spindle plane direction is perpendicular to that at the 2-cell stage. (C) 8-cell stage. Four cells are symmetrically located at each pole (“4-4 pattern”). For nomenclature, see Fig. 1 (note that the cells meeting at the cross furrow at the animal pole are now **A_I** and **C_I**). Cells at the vegetal pole are slightly larger than those at the animal pole. (D) 16-cell stage. Four rows of four cells (“4-4-4-4” pattern). Cells in the vegetal hemisphere are larger. (E) 32-cell stage. Two rows of four cells at each pole with two rows of eight cells between them (“4-4-8-8-4-4” pattern). The same cleavage pattern as described for the 16-cell stage. Cells at the poles divide longitudinally; cells in between divide equatorially. (F) 64-cell stage. The cells are located in a “4-4-8-16-16-8-4-4” pattern. Arrowheads point to the equator. Note the symmetry of the cleavage pattern/ cell distribution at all described stages.

Fig. 5. Cross-sections through embryos of *P. caudatus*. Blastocoel formation and gastrulation. Next to each embryo is a schematic drawing of the position of the cross-section. Position of hatching cap marked by brown line; and break point by asterisk. (A) 16-cell stage. A small blastocoel is visible in the middle of the embryo (arrowed). (B) Fifth cleavage. The blastocoel is enlarged and clearly visible now (arrowed). (C) Sixth cleavage. Same embryo as in Fig. 2I and Fig 3F. Gastrulation has not yet commenced. Blastocoel marked by arrow. The two cells (**X** and **Z** of Fig. 1) that will later migrate into the blastocoel are marked by white dots. The membrane-bound granules are marked by the arrowhead. (D-E) Gastrulation. Cross-sections through different levels of two different embryos, at a slightly later stage than seen in C (E and E' different views of same embryo). Two cells **X** and **Z** are migrating into the blastocoel

(white dots). In this embryo one additional cell is seen lateral to one of the larger cells (arrowed). (F-F'') Late gastrulation stage. Three cross-sections at different levels through an embryo, showing the bilaterally symmetrical nature of the cell bands within the blastocoel. Note that the blastocoel is completely filled.

Fig. 6. (A) Propidium iodide stained sixth cleavage stage embryo. Note the two membrane-bound granules within the blastocoel. (a) close-up showing the apparent budding structures on the granules.