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Aspects of priapulid development

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Abstract

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The phylum Priapulida is a small group of marine worms that is allied with the nematodes, kinorhynchs, loriciferans and nematomorphs in a clade called the Cycloneuralia or Introverta. Together with the arthropods they are generally considered to comprise the Ecdysozoa, a clade of moulting animals. A number of recent priapulid species possess features that resemble the predicted Ecdysozoan ancestor. In addition, recent molecular studies have also shown that they are basal within the Ecdysozoa/Cycloneuralia (Garey 2001, Webster et al. 2006). Their putative basal position thus makes priapulids highly interesting research objects for understanding the evolution of Ecdysozoa.

Earlier investigations of the early embryology of the priapulid *Priapulus caudatus* are critically revised with the aid of modern techniques and equipment, confirming earlier studies that the early cleavages are highly symmetrical, total, subequal, radial and stereotypical. New results show that up to the sixth cleavage, the spindles are oriented along the animal/vegetal axis at both poles. This unique cleavage pattern has only limited similarities to other animals. During the sixth cleavage two cells move inwards and gastrulation commences. If the mesoderm is derived from both cells, its origin differs from that of many other protostomes.

Two previously undescribed larval stages of *P. caudatus*; the light bulb shaped hatchling and the first lorica larva are described. The second lorica larva superficially resembles the previously described type 2 lorica larva (Higgins et al 1993). Differences between the second lorica larva and the type 2 lorica larva, with respect to possible ecophenotypical variation and sub-specialization, are described.

Preliminary data are presented on musculature development of *P. caudatus*. Preliminary data have also been obtained on the early development of a second priapulid, *Halicryptus spinulosus*. Comparison of *Halicryptus* and *Priapulus* may help to resolve developmental ground pattern of the priapulids.

Keywords: Priapulus caudatus, Priapulida, Ecdysozoa, larval development, embryology

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Papers included in this thesis

The following papers are included in this thesis:

I **Wennberg, S. A.**, Janssen, R., and Budd, G. E. 2008 Early embryonic development of the priapulid worm, *Priapulus caudatus*. *Evolution & Development*

II **Wennberg, S. A.**, Janssen, R., and Budd, G. E. Hatching and earliest larval stages of the priapulid worm *Priapulus caudatus*. Manuscript **in review**, *Invertebrate biology*

III **Wennberg, S. A.**, Janssen, R., and Budd, G. E. Muscle development in the priapulid worm, *Priapulus caudatus*. Manuscript

In the following text these papers are referred to by their corresponding Roman numeral (I to III).

Statement of authorship

Sofia Wennberg is responsible for the collection of material and processing of data for all of the manuscripts, together with the bulk of the texts. Janssen and Budd read and revised the manuscripts.

Contents

Abbreviations

AO	Acridin Orange
BABB	Benzyl alcohol:Benzyl benzoate, 1:2
dH_2O	Distilled water
EtOH	Ethanol
FA	Formalin
FITC	Fluorescein isothiocyanate
MeOH	Methanol
NaN_3	Sodium Azide
PBS	1M Phosphate buffer
PBS-TX	Phosphate buffer + Triton x-100
PFA	Paraformaldehyde
PI	Propidium Iodide
RT	Room Temperature
SEM	Scanning Electron Microscope
TAAB 812	Epoxy- embedding resin
TEM	Transmission Electron Microscope
TSA	Tyramide Signal Amplification
Vectashield H-1000	Aqueous mounting medium

Introduction

Why study priapulids?

Today 19 extant priapulid species are known (e.g. Shirley and Storch 1999; Todaro and Shirley 2003) and placed within the phylum Priapulida. When one knows where and how to search for these worms, they can be found in high numbers; yet priapulid worms are unknown to most people. The large macrobenthic priapulids (up to 40 cm long) live in cold and deep waters buried in the muddy seafloor, while the smaller meiobenthic species (1mm long or shorter) most commonly reside in gravels close to the shoreline in tropical areas of the world. However, the interest in priapulids and the phyla included in the Introverta (Nielsen 2001) has recently increased among palaeontologists and biologists.

This increased interest in the priapulids is based mainly on two facts: First, the Cambrian fossil record (ca. 520 Mya) reveals putative stem group priapulids bearing morphological characters still found in a number of priapulid species living today (van der Land 1970, Conway Morris 1977, Adrianov and Malakhov 1996, Huang et al. 2004). Morphology and development of many recent priapulids thus bear similarities to the predicted ecdysozoan ancestor, including: (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). Secondly, the Ecdysozoa hypothesis (Aguinaldo et al. 1997) places the Cycloneuralia (including priapulids) in a sister relationship to the Arthropoda. Despite their possible function as “model-organism” for the understanding of Ecdysozoa, not much is known about this group; in particular the development has been poorly investigated.

Priapulus caudatus, my main research organism, is a common priapulid inhabiting the coasts in the northern hemisphere, including Gullmarsfjorden on the west coast of Sweden. I believe that *P. caudatus* is most suitable for initiating developmental studies on priapulids. Not only is it believed to be basal in relation to other priapulids, but also it is historically the most studied priapulid. When I started to collect initial data on the embryonic development in *P. caudatus* I soon became aware of inconsistencies with existing literature on this topic, including the basic data on how it develops (Lang 1949, Zhinkin 1953).

Background

Historical overview

The first priapulid discovered was named *Priapus humanus*. In 1754 Odhelius thought that the worm reminded him of a human penis. Priapos is the Greek god of fertility and protection (Fig. 1) and he is depicted with an oversized phallus. Because of the superficial similarity of the priapulid body with a human penis, Odhelius introduced the genus name "*Priapus*" and the species name "*humanus*". Subsequently, at least eight different names have been assigned to the same animal (see review in Adrianov and Malakhov 1996). However, the name used today is *Priapulus caudatus*, erected by Lamarck in 1816. Lamarck kept the reference to Priapos for the genus but the species name now refers to the caudal appendage. Almost one hundred years after the first finding, Siebold discovered *Halicryptus spinulosus*, a second priapulid species (Siebold 1849). Both *P. caudatus* and *H. spinulosus* are species confined to the northern hemisphere. However, it was not long before a new priapulid species was discovered in the southern hemisphere, *Priapulus tuberculatospinosus* (Baird 1868). Today, nine macrobenthic priapulid species are described (e.g. Todaro and Shirley 2003).

Fig 1. Priapos, the god of garden fertility. Museo Archeologico Nazionale di Napoli, Naples, Italy. Picture recovered from the website: www.theoi.com

As late as 1968 van der Land described the first meiobenthic priapulid, *Tubiluchus corallicola*, from the coral sands of Curaçao. During his work to determine what species he had found van der Land came across a report by Remane and Schulz (1964). They briefly described a priapulid like creature, found in the Red Sea. This report, together with a drawing published by Remane in 1963, lead van der Land to assume that the animals found in the Red Sea probably belonged to the same genus as *T. corallicola* (van der Land 1968) and later this animal was given the name *Tubiluchus remanei* (van der Land 1982). After *T. corallicola* and *T. remanei*, eight more meiobenthic species, have subsequently been described (van der Land 1968, 1982, Shirley and Storch 1999 and Todaro and Shirley 2003).

Priapulids also have a reasonable fossil record, mostly from the Cambrian exceptionally preserved faunas such as the Burgess Shale and Chengjiang (e.g. Conway Morris 1977, Zhang et al. 2006), but a Carboniferous priapulid has also been described (Schram 1973).

Morphology of the adult

How to recognize a priapulid

Despite the difference in size all priapulid worms have a relatively similar morphology. Priapulids have an annulated vermiform body. The body length of different priapulid species ranges from less than 1 mm (with the introvert protruded) up to almost 40 cm (with the introvert inverted) (Shirley and Storch 1999). The introvert refers to the head region and, in some species, it can be completely drawn back into the trunk. On the introvert pharyngeal teeth and scalids surround a terminal mouth. The introvert is generally followed by a more or less prominent neckline, although this character is not present in all species (Adrianov and Malakhov 2001). In some species, one or two caudal appendages extend from the rear end of the trunk. However, there are also species that lack a caudal appendage or have spines instead (van der Land 1968, 1970). A cuticle covers the whole body including the caudal appendage (Scharff 1885). The function of the caudal appendage is not fully known but it has been suggested that, since its cuticle is very thin, it might have a respiratory function (Fänge and Mattisson 1961).

Priapulids show both radial and bilateral symmetry (van der Land 1970, Adrianov and Malakhov 1996). The paired gonads, the ventral nerve cord and the protonephridia display bilateral symmetry, while the introvert, pharynx and the most characteristic part of the nervous system, the circumpharyngeal "brain", show radial symmetry (Storch 1991). The latter, radial characters are secondary.

The pharyngeal teeth are directed posteriorly and followed by scalids and papillae. Teeth and scalids are involved in feeding and moving, and possess sensory functions (van der Land and Nørrevang 1985, Storch et al. 1994). The morphology and size of the teeth and scalids, along with their distribution pattern, are important to distinguish between closely related species (Adrianov and Malakhov 2001).

The cuticle is composed of three layers; the epicuticle, the exocuticle and the endocuticle (Lemburg 1998). Priapulids must moult the cuticle to be able to grow and this process is commonly called ecdysis (Ruppert 1991). Therefore the old, i.e. moulted skin of priapulids, can sometimes be found next to a living priapulid. Freshly moulted priapulids can easily be recognized in culture by their brighter skin colour which is simply because no mud particles have yet attached to the skin (personal observation).

The "Swedish" priapulids

Two priapulid species inhabit the Swedish coasts, *Priapulus caudatus* (Fig. 2A) in salty waters of the Swedish west coast and *Halicryptus spinulosus* (Fig. 2B) in brackish waters of the Baltic Sea. The following text mainly concentrates on these two species, not only because they are my research organisms but also because most information concerning priapulids, and in particular their development, is based on research on *P. caudatus* and *H. spinulosus*.

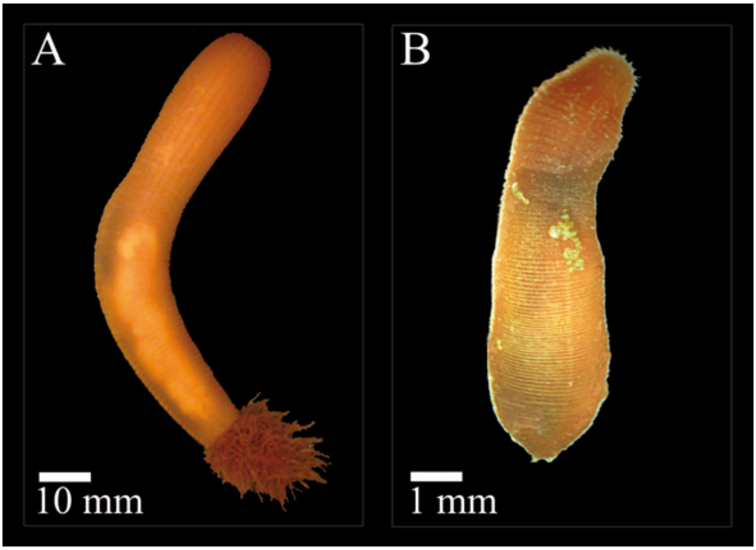


Fig 2. Priapulids found in Swedish waters. A. *Priapulus caudatus* B. *Halicryptus spinulosus*

Both species are relatively large; adult *H. spinulosus* have a maximum body length of 4 cm (often ca. 2 cm) while *P. caudatus* can be longer than 10 cm. The most striking feature in *P. caudatus*, and responsible for the species name, is the large caudal appendage. Such an appendage is not present in *H. spinulosus*; instead two setae are present at the rear end (van der Land 1970). The introvert of *H. spinulosus* is less pronounced than that of *P. caudatus* (Storch et al. 1994). However, both species have a heavy set of pharyngeal teeth and scalids on their introvert.

How to separate the sexes

Priapulids are generally dioecious animals (i.e. separate sexes) but hermaphroditic exceptions include *Halicryptus spinulosus* (pers. observation) and reportedly, *Priapulus caudatus* on occasion (Lang 1948). Only one genus, *Tubiluchus,* shows sexual dimorphism (Hickman 1979). In the males the ventral side of the trunk is covered with specific cuticle structures and setae (van der Land 1970, Storch 1991). In all other species the morphology of the sack shaped gonads is the only morphological feature to separate the sexes; they can be seen and identified through the body wall. The gonads are located pair wise together with the protonephridia within the pseudocoelom (van der Land 1970). The gonads of the large macrobenthic priapulids typically contain up to thousands of oocytes while the gonads of the much smaller meiobenthic species contain only a few.

Gametes, fertilization and development

Eggs and sperm

There are many thousands of oocytes in the gonads of the large macrobenthic priapulids but only a few oocytes in the small meiobenthic priapulids. The developing oocytes of *P. caudatus* are located in the epithelium of the gonads (Storch 1991). Mature oocytes are larger and only surrounded by the basal lamina of the ovary (Nørrevang 1963). This is true for at least *P. caudatus* and other macrobenthic priapulids as well as the meiobenthic *Meiopriapulus fijiensis* (Storch et al 1989). In all except one priapulid species, the mature oocytes have a diameter of ca. 80 µm; the exception being *Meiopriapulus fijienis*. In this species mature oocytes with a diameter of ca. 250 µm have been reported (Higgins and Storch 1991).

The priapulid sperm is thought to be primitive in relation to other “aschelminth” phyla (Introverta plus Rotifera and Gastrotricha) (Franzén 1956, 1983). A short head with a rounded nucleus and a minute acrosome is followed by a short middle piece and the sperm ends in a long filamentous tail. However, *Tubiluchus* is again the exception since sperm morphology of this genus is profoundly different from what is seen in the rest of the priapulids (Alberti and Storch 1983). In fact, it is even unique in the animal kingdom (Alberti and Storch 1988). The sperm is slender rather then globular and the acrosom and nucleus are wrapped together in a pattern that is similar to a corkscrew (Alberti and Storch 1983, 1988). According to Franzén (1956), this is likely to depict a derived state.

Spawning

All macrobenthic priapulids have external fertilization. During spawning the mature gametes are transported through the urogenital ducts to the external environment (van der Land 1975). Lang (1948) dissected the gonads of *P. caudatus* worms from Gullmarsfjorden to see if the eggs had already been released or not. From this data he concluded that the natural spawning season on the Swedish west coast is in December and January (Lang 1948). He also observed spawning worms (kept in aquaria) and found that fertilization occurred in the evening and at night time. Most commonly the sperm was released first followed by the females releasing their eggs (Lang 1953). In 1990, Shirley arranged an ecological study on *P. caudatus* from Auke Bay, Alaska. Following collection of larvae during a four-year period he concluded that the smallest larvae always appeared in December indicating that the breeding period suggested by Lang (1948) was correct.

During my time as a PhD student, I successfully fertilized eggs of *P. caudatus* from Gullmarsfjorden between October and March (Wennberg et al. 2008). In my experiments, the gonads were always mechanically stripped to release eggs and sperm. Therefore, the natural spawning season cannot be confirmed by these studies; but its confinement to December and January can be ruled out, since, if that were the case, mature eggs would not be found after January.

In August 1949, Zhinkin successfully stripped gonads and fertilized eggs of *P. caudatus* and *H. spinulosus* collected in the White Sea. In addition to these observations, Wesenberg-Lund (1929, 1939) commented on *P. caudatus* spawning from the west coast of Greenland, indicating natural spawning during spring at this locality (Wesenberg-Lund 1929, 1939).

The breeding season in *P. caudatus* is thus possibly variable at different locations. Factors that trigger the gametes to mature and the priapulids to spawn are not yet known. The water temperature is probably the main trigger; however a number of additional factors such as light levels might also play a role.

Internal fertilization

Van der Land considered the possibility of internal fertilization within the genus *Tubiluchus*, after he discovered structures that reminded him of sperm heads in the female urogenital duct of specimens of *Tubiluchus corallicola* (van der Land 1970). Moreover, *Tubiluchus corallicola* showed sexual dimorphism, a feature never earlier observed in priapulids. Additionally this priapulid species produce a very low number of gametes each season (in comparison to the macrobenthic priapulids). In 1970, *T. corallicola* was the only described meiobenthic priapulid species and van der Land had no other species to compare with.

In 1983, Nørrevang and van der Land assumed that all six of the meiobenthic priapulid species known at the time possess internal fertilization. This assumption was based on the observations mentioned above, as well as the description of *Tubiluchus corallicola* sperm. The sperm is morphologically derived (Kirsteuer and van der Land 1970), relative to other priapulids and this type of sperm is, according to Franzén (1956), indicative for internal fertilization. However, there are complications with this general assumption, because features used to assign internal fertilization to all meiobenthic priapulids are simply missing in some species; e.g. *Meiopriapulus fijiensis* (described 1981) has a primitive type of sperm indicating external fertilization.

Alberti and Storch (1988) found a more solid proof for internal fertilization, the finding of deeply embedded spermatozoa in the epithelial cells of the urogenital ducts of a female of *Tubiluchus philippinensis* (Alberti and Storch 1988). However, they were not able to give an explanation on how the actual sperm transfer between male and female work.

It is thus still unclear if (and if, how) internal fertilization takes place in meiobenthic priapulids. Therefore, more research on this topic is needed to answer this interesting and fundamental question.

Development

Embryology. There is little information concerning the embryonic development of the meiobenthic priapulid species (van der Land 1975, Storch et al. 1989, Higgins and Storch 1991). This is also partly true for the macrobenthic species; examination of the developing embryos is confined to the early cleavages in *Halicryptus spinulosus* and *Priapulus caudatus* (Lang 1953, Zhinkin 1949, Zhinkin and Korsakova 1953). In addition, the information gained from this pioneering work is very limited; the data are confined to early developmental stages only. More importantly, the existing data on *P. caudatus* is not reliable because there are contradicting results in the two studies Zhinkin (1949) and Lang (1953) carried out. This is partly due to obvious misinterpretation and inadequate cultivation techniques (Lang 1953, Zhinkin 1949, discussed in Wennberg et al 2008). As mentioned earlier, Lang kept the worms in chilled aquaria and waited for the priapulids to spawn. He collected the fertilized eggs and followed their development. Zhinkin collected both *P. caudatus* and *H. spinulosus* embryos from the White Sea but the handling of the adult priapulids is not mentioned in the published papers (Zhinkin 1949; Zhinkin and Korsakova 1953). The first paper presented in this thesis describes the work on the early development of *P. caudatus*. Similar to in the Lang study, the animals were collected in Gullmarsfjorden. In contrast to the Lang study, the gonads were stripped out and the eggs were fertilized in the same manner as Zhinkin describes for *P. caudatus* and *H. spinulosus* (Zhinkin 1949, Zhinkin and Korsakova 1953). For details on embryo preparation see the method section. For my results concerning the early development of *P. caudatus* and a critical discussion of Lang's and Zinkins work, please see paper I.

Larval stages. With the exception of *Meiopriapulus fijiensis*, all priapulids are believed to have larval stages. An initial loricate larva grows stepwise to a certain length and finally undergoes metamorphosis to form a juvenile worm. The lorica larvae of many priapulid species are similar in their appearance and in contrast to what is seen in priapulid embryology relatively many studies have dealt with the morphology of the larvae (e.g. Hammarsten 1915, Purasjoki 1944, Lang 1948, Storch and Alberi 1985, Storch and Higgins 1991, Higgins and Storch 1993, Lemburg 1999, Adrianov and Malakhov 2001). Three main parts can be observed in the lorica larva: the trunk, the neck and the introvert (Fig. 3). A rigid cuticle (i.e. the lorica) covers the trunk and when the introvert is not protruded also the introvert. The introvert, including the pharyngeal teeth and scalids, can only be fully observed when the introvert is protruded (Fig. 3).

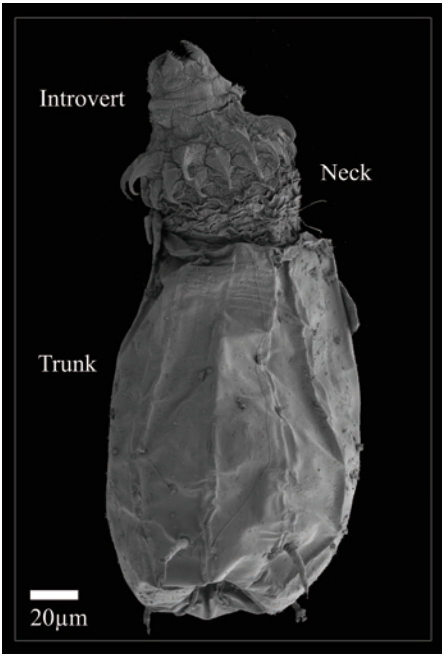


Fig 3. Lateral view on the second lorica larva of *Priapulus caudatus*.

Based on work by Hammarsten (1915), Lang (1948) and Purasjoki (1944) and his own studies in 1970, van der Land (1975) divided the lorica larvae into three categories and this distinction is still used (e.g. van der Land 1975, Higgins and Storch 1989, Storch 1991). Larvae of *Tubiluchus corallicola* possess a rounded lorica with twenty prominent longitudinal ridges and numerous tubuli scattered on the lorica. *Priapulus*, *Priapulopsis* and *Maccabeus* have dorsoventrally flattened lorica larvae with eight longitudinal ridges along their ventral sides. The number of tubuli is less in these genera, only four to eight, and they are all confined to the lateral sides of the lorica (van der Land 1975). The lorica of *Halicryptus* has eight longitudinal ridges along the ventral sides and some small tubuli (Storch 1991). However, the lorica has an alveolus pattern (van der Land 1975).

A closer look at *Priapulus caudatus* earliest larval stages is presented in the paper II in this thesis. For the first time, the hatching larva is described and it turns out that it is profoundly different from the lorica larva described in earlier literature; it has a round cross-section, no rigid lorica and no well-developed lorica spines. In addition, it has no mouth opening and fewer scalids than earlier described for larvae of *P. caudatus*. This first larval stage, described in paper II, is thus clearly a non-feeding stage that has been overlooked in previous studies. This is also true for the second larval stage (the first lorica larva). For a detailed description, see paper II.

Direct development. Higgins and Storch (1991) reported a case of direct development in *Meiopriapulus fijiensis*, the only known case in the priapulid phylum. In addition, they also mention that it is likely that female *M. fijiensis* brood their embryos. The information concerning this developmental mode is mainly based on the finding of a juvenile that was caught in the act of “being born”, i.e. partly protruded from a female (Higgins and Storch 1991). The outer morphology, as well as the organization of the musculature of the “newborn”, resembled that of larger juveniles and adults of the same species. However, only a general outline of the alimentary canal could be located internally and since a functional introvert had still not formed at this developmental stage, they assumed that such a premature specimen could not move or eat properly (Higgins and Storch 1991). It should be mentioned that Higgins and Storch (1991) are not certain if the postembryonic stages they describe are “hatchlings” or if the embryos have been discarded by the female prematurely. Nevertheless, they refer to the kinorhynch *Pycnophyes cryopygus* Higgins and Kristensen (1988), where the earliest juvenile stage also shows a non-functional mouth cone. Given that kinorhynchs and priapulids are closely related phyla (both are cycloneuralians), the appearance of premature larval stages may reflect a common developmental mode for both phyla. In addition, our study of *Priapulus caudatus* larvae (described in paper II) also supports this idea, since the *P. caudatus* hatchling, as described for *M. fijiensis* larva, lacks a mouth.

Brooding of the embryos obviously implies internal fertilization, but as mentioned above the sperm of *M. fijiensis* is of the primitive type, implying external fertilization (Franzen 1956). Again, there are several open questions that have to be answered before we can state that we understand priapulid development in its entirety.

Ecology

Nearly all meiobenthic priapulid species live in tropical shallow waters, inhabiting coves (up to 0.5 m) covered with coral sand or fine grained sand (van der Land 1975, Storch 1991, Adrianov and Malakhov 1996). Only three species do not obey this rule: 1) *Tubiluchus arcticus* inhabits coarse sands in cold arctic waters, at depths of around 100 m. 2) *Maccabeus tentaculatus* is found in muddy sediments at depths going down to 550 m in the Mediterranean Sea. 3) *Maccabeus cirratus* has been found in the Indian Ocean, at depths down to 2500 m (Adrianov and Malakhov 1996). The suggested diet of the meiobenthic priapulids is detritivorous and bacterivorous (Adrianov and Malakhov 1996).

The larger macrobenthic species most commonly inhabit cold waters of the northern and southern hemisphere (van der Land 1970). However, *Priapulus caudatus* has also been found in the Mediterranean Sea (van der Land 1985). The depth range for *P. caudatus* is everything between tens of m down to ca. 4000 m and *Priapulus abyssorum* has been collected from ca. 8000 ms depth (Shapeero 1962, Adrianov and Malakhov 1996). The sediments in which the macrobenthic priapulids are living also consist of sand and gravel, but primarily mud. *P. caudatus* mainly inhabits waters with high salinity but can also be found in some areas of the Baltic Sea with much lower salt content ($7^{o}/_{oo}$). *H. spinulosus* is confined to brackish water environments with a salinity of around 6 $^{o}/_{oo}$. Both *P. caudatus* and *H. spinulosus* can survive in habitats with low oxygen content, 2ml/l respectively 3ml/l (van der Land 1970).

Most ecological observations on priapulids are restricted to *P. caudatus.* Trott (1998) studied the ecology of *P. caudatus* and came to the same conclusion as Lang (1948); *P. caudatus* lives on muddy sea bottoms and is opportunistic, feeding on both living and dead prey. This opportunistic mode of life probably accounts for all the macrobenthic priapulid species.

Adaptation

Priapulids are expected to have a primitively large body size (Adrianov and Malakhov 1995, 1996, Budd 2001). The small size of the meiobenthic priapulids is thought to be a secondary adaptation to the environments they inhabit. A large body size might not be the best for a worm that hides in coral sands of shallow warm waters. In addition to their small size, all meiobenthic species are believed to have internal fertilization. Moreover, *Meiopriapulus fijiensis* is the only priapulid species with an assumed direct development. This, together with internal fertilization, could be a perfect adaptation to the "chaotic" life style in coarse sands and shells in the intertidal zone (Higgins and Storch 1991). In the genus *Tubiluchus*, all species show sexual dimorphism, a different gonad structure and a unique sperm morphology (van der Land 1970, Storch and Alberti 1985, Adrianov and Malakhov 1996), indicating internal fertilization. In addition, all *Tubiluchus* species have a long slender tail; a character thought to be an adaptation to the interstitial mode of life. Features such as internal fertilization and direct development considered to support the derived nature of meiobenthic priapulids. However, it should be kept in mind that some of this information is based on restricted observations only (see discussion on internal fertilization and direct development).

Bipolar distribution

Bipolarity is observed within two of the priapulid genera, *Priapulus* and *Priapulopsis* (van der Land 1970, Schreiber et al. 1995, Adrianov and Malakhov 1996). *Priapulus caudatus* and *Priapulopsis bicaudatus* live in cold waters of the northern hemisphere while *Priapulus tuberculatospinosus* and *Priapulopsis australis* are found in similar habitats but in the southern hemisphere. The external morphology of *Priapulus caudatus* and *Priapulus tuberculatospinosus* is very similar whereas *Priapulopsis bicaudatus* and *Priapulopsis australis* are easier to separate; in particular the tail differs between the species (van der Land 1970). The best explanation for bipolarity is, according to van der Land (1970) and Adrianov and Malakhov (1996), migration through abyssal depths. An ancestral priapulid migrated towards the southern and northern hemisphere, became isolated and finally adapted to its new isolated environment.

Phylogeny

The advent of the Ecdysozoa

The interest in priapulids has increased markedly in recent years. Palaeontologists and biologists find them intriguing owing to the advent of the Ecdysozoa theory (Aguinaldo et al. 1997). After sequencing 18S rDNA from a number of metazoan taxa, Aguinaldo et al. (1997) presented a new view of the phylogenetic relationship within the Protostomia. The Ecdysozoa theory is now considered to be the best-supported hypothesis concerning protostomian relationships. The pros and cons of the Articulata and the newer Ecdysozoa hypothesis are discussed controversially in numerous recent articles (e.g. Schmidt-Rhaesa et al. 1998, Scholtz 2002, Haase et al. 2001, Garey 2001, Giribet 2003, Nielsen 2003, Giribet and Ribera 1998). The phylogenies that support the Articulata concept are mainly based on morphological characters, in particular the segmentation seen in arthropods and annelids. Ecdysis (moulting) is put forward as the main morphological character that unites the cycloneuralians and the arthropods within Ecdysozoa (Schmidt-Rhaesa et al. 1998). However, the Ecdysozoa are most commonly well supported in phylogenies based on sequence data (e.g. Aguinaldo et al. 1997, Garey 2001, Webster et al. 2006).

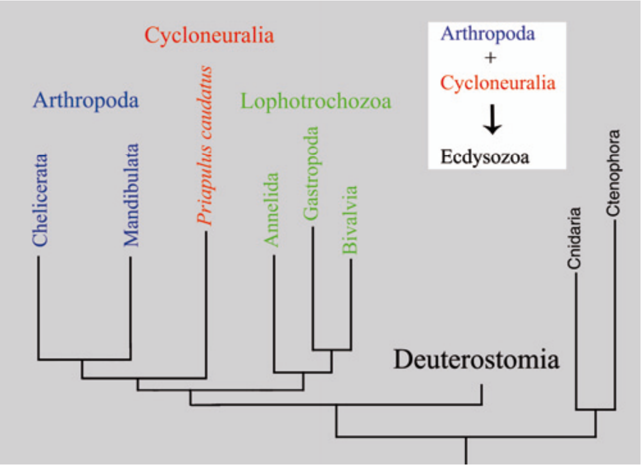


Fig 4. Phylogenetic tree showing *P. caudatus* (Cycloneuralia) in relation to Arthropoda and Lophotrochozoa. Simplified and redrawn from Webster et. al. 2006.

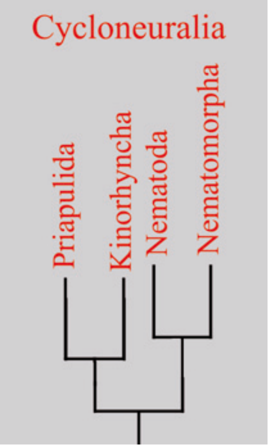


Fig 5. Phylogenetic tree showing the relationship within Cycloneuralia. Simplified and redrawn from Dunn et. al. 2008.

Aims

The overall objective of this thesis is to expand our knowledge concerning priapulid development.

> The aim of **Paper I** is to describe the early embryology of the priapulid worm, *Priapulus caudatus*. In addition, former studies on priapulids are re-evaluated, using for the first time adequate cultivation techniques and modern microscopic equipment (confocal microscopy).
>
> **Paper II** describes the first three larval stages (the hatching larva, the first lorica larva and the second lorica larva) of *Priapulus caudatus*, the first two larval stages entirely unknown and the last previously incompletely described.
>
> The aim of **Paper III** is to map the developing musculature in *Priapulus caudatus*.

Methods

Fieldwork

Gullmarsfjorden

Gullmarsfjorden is located on the west coast of Sweden. It is the only threshold fjord in Sweden and marine field stations have been located in the area since 1877. During my time as a PhD student, I have worked at the Sven Lovén Centre for Marine Sciences (formally known as Kristineberg Marine Research Station) and Klubban Biological Station. For seven field seasons, between the years 2002-2007, I have been trawling for priapulids of the species *Priapulus caudatus*. Most of the fieldwork took place in the month of November, but also earlier (October) and later (March) fieldwork was carried out. The priapulids were caught with a ring trawl in depth ranging between 30 and 60 m. The process of trawling comprises four steps: 1) The ring trawl is filled with dark, slightly anoxic, mud by pulling it over the sea floor. 2) The filled ring trawl is pulled up and left by the railing of the boat, close to the water surface, where an extra net is placed around and under the primary ring trawl. 3) The trawl is dragged behind the moving boat until all mud is washed out. 4) The nets are lifted on deck and priapulids eventually are collected. Work on living material was exclusively conducted at the Sven Lovén Centre for Marine Sciences and Klubban Biological Station.

Askö

Askö is located in the archipelago south of Stockholm, in the Baltic Sea. The Askö Laboratory opened 1961 and it has since then been a part of Stockholm University. At two occasions, February-March 2008, our group collected specimens of *Halicryptus spinulosus* at a number of localities situated around the island of Askö. The water is brackish ($6^{o}/_{oo}$) in this area of the Baltic Sea and the collecting depth ranged between 30 and 60 m. The dredging was carried out with a Lundgren-dredge. Huge quantities of mud were loaded onto the deck and rinsed through a number of sieves of different mesh size. Priapulids of variable size where subsequently collected from the different sieves. The collected priapulids were immediately transported to the laboratory at Uppsala University where the in vitro fertilization experiments took place.

Cultivation techniques

Priapulus caudatus

Prior to fertilization, the worms were kept in aquaria with a constant flow of deepwater. The priapulids were cut open and the gonads were dissected out.

Fig 6. Tools needed to dissect a priapulid

The eggs and sperm sacks were removed from the gonads. The eggs were separated with the help of tweezers and the sperm sacks were macerated to release sperm into 1000 µl of filtered saltwater. After an hour of "incubation" in saltwater, the sperm were activated and started moving. 50 µl of sperm-solution was added to a Petri dish containing 50 ml seawater with ca. 1000 eggs. During all field seasons, the developing embryos were kept in a cold room with constant temperature. The temperature ranged from 8 to 10ºC, depending on the field season (but always constant within each season). At 10ºC the first larvae started hatching after 10 days, but developmental time is extremely variable. A mixture of developmental stages was observed, starting already in the first cleavage. We also exposed embryos to room temperatures (ca. 20ºC) and the result was a huge proportion of deformed embryos; finally almost all embryos died (Wennberg et al. 2008).

A number of fixatives were used to preserve the embryos at all developmental stages for future examination in the lab. During my first two years of fieldwork, I fixed eggs every four hours throughout the developmental time. This assured that embryos and larvae of all developmental stages were fixed and available for future examination. In the two remaining years, the frequency of fixation was once every day. At this point I knew about the non-consistent developmental time in *P. caudatus* embryos, giving me slightly different embryonic stages at a daily fixation anyway.

Halicryptus spinulosus

Prior to fertilization, the worms were kept in glass bottles filled with brackish deep water at a constant temperature of 7ºC. The gonads were stripped and eggs were fertilized in the same way as for *P. caudatus*. The gonads are smaller and the eggs fewer in *H. spinulosus* than in *P. caudatus*. The eggs were separated with the help of tweezers and the sperm sacks were macerated to release the sperm into 1000 µl water. After ca. 24 hours of "incubation" in seawater, the sperm was activated and added to a Petri dish containing 50 ml seawater with ca. 50 eggs. To increase fertilization success, ca. 500 µl of sperm was used for each dish instead of 50 µl as for *P. caudatus*. The developing embryos were kept in the dark at 7ºC in the fridge.

In each fertilization experiment, only a few embryos started to develop. The first few cleavages appeared to be normal (when compared to the development of *P. caudatus*) but after that the developing first became very irregular and finally stopped totally at the 16- or 32-cell stage. This happened although the embryos were basically treated the same way as the embryos of *P. caudatus*. Observation and documentation of the first three cleavages together with a comment on earlier studies can be found in the *Unpublished results* section of this thesis (p. 24).

Remarks on the gonads of *P. caudatus* and *H. spinulosus*

In both *P. caudatus* and *H. spinulosus* the gonads are visible through the cuticle and, in *P. caudatus*, it is even possible to sex the priapulids before dissection (personal observation). However, in *H. spinulosus*, sex determination is more difficult owing to the smaller body size and the fact that all specimens I observed were hermaphrodites.

The dissected female and male gonads of *P. caudatus* are easy to separate by eye only. The female gonad is compact and smooth and the male gonad is branched in an intricate threadlike pattern (Fig 7A-B).

In *H. spinulosus,* a dissecting microscope is necessary to distinguish sperm vesicles from ovaries. The sperm sacks are approximately twice the size of the eggs and under the dissecting microscope they appear transparent/greyish. The sacks are round. In both species, freshly prepared (dissected) eggs are roundish but after some time in seawater, they obtain their typical globate shape (Fig 7C-D) (Zhinkin and Korsakova 1953).

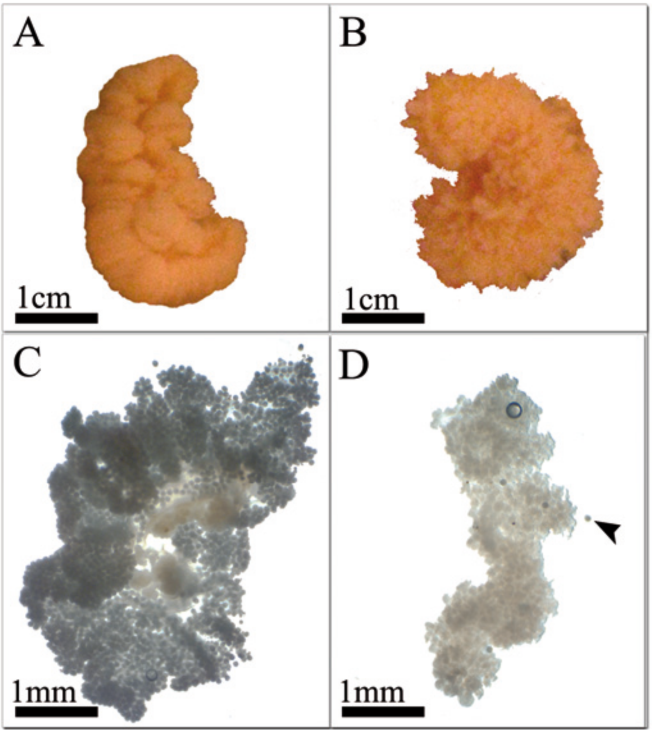


Fig. 7. Gonads A. Female gonad of *Priapulus caudatus* (immature) B. Male gonad of *P. caudatus* C. Female gonad of *Halicryptus spinulosus* D. Male gonad of *H. spinulosus*. Note the color differences of the gonads in *P. caudatus* (pink to yellowish) and, *H. spinulosus* (whitish to grey).

Preparation techniques

Paper I

Acridin Orange (AO) and Propidium Iodide (PI) were used to stain the nuclei and cell borders of embryos. The optimal fixation for this protocol is 4% paraformaldehyde (PFA) buffered in PBS and a fixation time of four hours at room temperature (RT). The fixated embryos are stored in 70% EtOH in the fridge.

Acridin Orange. The embryos were stepwise transferred from 70% EtOH into distilled water (dH_2O), causing an osmotic shock and the embryonic membrane and the eggshell to break open. A toothpick of Acridin Orange was added to 1000 µl distilled water. For each tube containing ca. 30 embryos, 250 µl of the AO-solution was added. The solution was incubated for ca. 15 minutes and after this the superfluous amount of AO was removed by rinsing with dH_2O.

Propidium Iodide. As for AO stainings, the embryos were also stepwise dehydrated in dH_2O. A solution containing 4 µg PI diluted in 1000 ml dH_2O was added to the embryos. After 15 minutes of incubation, the superfluous amount of PI was removed by repeated rinsing with dH_2O.

To visualize the whole embryo under the confocal microscope, BABB (1:2 mixture of benzyl alcohol and benzyl benzoate) was used as a clearing agent.

Paper II

The larvae described in paper II were visualized through Scanning Electron Microscopy (SEM).

SEM preparation. The specimens were fixed in a 1:1 mix of 4% PFA and 2.5% glutaraldehyde (both fixatives were buffered in PBS). In order to visualise the introvert of some specimens, it was pushed out mechanically with a needle. After fixation, the larvae were post-fixed in 1% osmium tetraoxide, stepwise moved into 100% EtOH and critical point dried. After mounting, the larvae were coated with gold palladium coating medium or gold.

In addition to SEM prepared material, larvae were prepared for Transmission Electron Microscopy (TEM) and light microscopy. These specimens were not included in this article but they are now presented in the *Unpublished results* section of this thesis (p. 20-24).

TEM preparation. Larvae were fixed in 2.5% glutaraldehyde for one hour and post fixed in 1% osmiumtetraoxid for one hour. After rinsing in PBS buffer the embryos were embedded in TAAB 812 embedding resin. Embryos were dehydrated stepwise in EtOH and subsequently incubated in acetone. After this, embryos were left to infiltrate in a 1:1 mix of acetone and resin over night. The next day, acetone was removed and the embryos were left in resin overnight. I embedded the embryos in silicon plates and left them to polymerize at 50°C for 48 hours. The thickness of each section was 50 nm.

Light microscopy preparation. Larvae were put into methylcellulose for direct observation under the microscope. In addition, paraffin sections were prepared. Larvae were fixed in 4% paraformaldehyde (PFA buffered in PBS) for four hours at room temperature. The fixed larvae were then stored in 70% EtOH in the fridge before preparation. The protocol starts with dehydration (stepwise and with an extra step of 100% EtOH). After this, the larvae were moved into 100% xylene (change xylene twice). For infiltration, the vial was filled up with paraffin and left overnight at 50°C. The following day the paraffin was heated to allow moving the larvae into the embedding forms. New paraffin was added to the embedding forms, and after cooling, the blocks were cut into sections of 5 µm. The sections were then stained with haematoxylin and eosin.

Paper III

Muscle development in embryos, from gastrulation to hatching, are described in this manuscript. Cell nuclei were stained with Propidium Iodide followed the same protocol as described earlier. In order to visualise the musculature under a confocal microscope, embryos were prepared according to the following protocol. The embryos were left in 4% PFA (in PBS buffer) for 1 hour. After extensive rinsing with PBS, they were stored in the PBS buffer with a trace of NaN_3. Embryos were rinsed twice in PBX (triton x-100 0.01%), transferred to a PBS-phalloidin solution and incubated for 1 hour at RT. After rinsing in PBS, the embryos were mounted in Vectashield mounting medium.

Microscopic methods

Confocal microscopy

I used a confocal microscope for the studies on the cleavage pattern (paper I) and the musculature (paper III). At Biologisk Strukturanalys, Uppsala University I used a Leica TCS SP2 while at Sven Lovén Centre for Marine Sciences and at the University museum of Zoology, Cambridge, I used a Leica TCS SP5. With a confocal microscope it is possible to obtain both 2D and 3D photographs of embryos and larvae.

Light microscopy

Light microscopy was used in paper II to visualize whole mount larvae and in the *Unpublished results* section, I show paraffin sections of larvae. To document whole mounts and sections a Nikon Eclipse E400 light microscope was used and pictures were taken with a Nikon D70 camera attached to the microscope.

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

I used a Scanning Electron Microscop (SEM), i.e. a Zeiss Supra 35VP scanning emission gun to study the larvae of *P. caudatus* (paper I). The same microscope was also used for Transmission Electron Microscope (TEM) investigations presented in the *Unpublished results* section.

Unpublished results

Additional information to papers I and II

At the time of going to press, live larvae of *P. caudatus* are still stored in seawater or mud at a constant temperature of 7°C in our laboratory fridge. These larvae are more than one year old and have not been fed during this time. In comparison to the described first larval stage, the third stage has a proper mouth opening and is hence able to intake food, but showed no change in morphology. We therefore believe that the larvae do not develop any further without food from that stage on but they can easily survive a long period without food. Under natural conditions that might be a requisite to survive until the larvae ends up in a suitable environment. I present some supplementary information concerning these more than one year old second lorica stage larvae. The close-ups show the introvert, with pharyngeal teeth and scalids, of a seven months old larva (Fig. 8A-B).

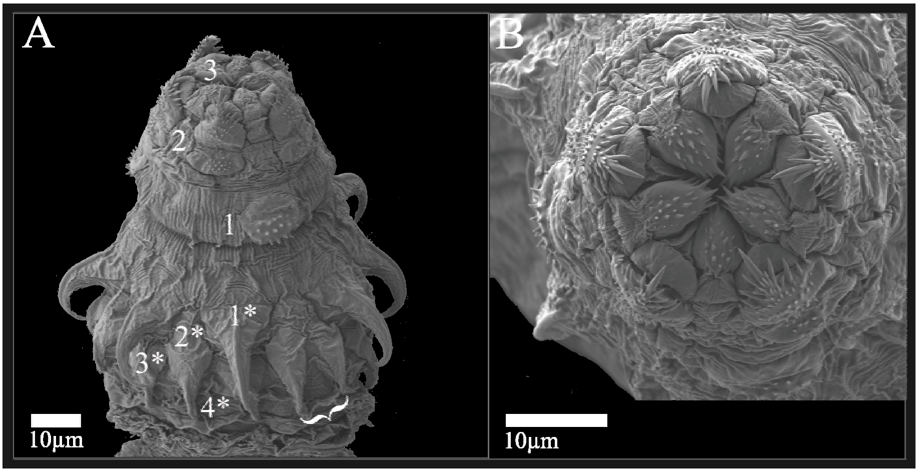


Fig 8. Tooth- and scalid pattern of a seven months old, third larval stage *P. caudatus* larva. A. Ventral view. A first ring-tooth is indicated by 1, a second-ring tooth by 2 and subsequently a third ring-tooth is marked by 3. The scalids are marked by numbers corresponding to their position, first-ring scalid; 1*, second-ring scalid; 2*, third-ring scalid; 3* and fourth-ring scalid; 4*. In addition, a bracket indicates the two neighbouring second-ring scalids. B. Anterior view of the larva seen in A.

In paper II we also mention the presence of the alimentary channel in the first larval stage, the hatchling. I here present a light microscopic picture to support this statement (Fig 9).

Fig 9. Paraffin section of a hatching larva. There is no mouth opening or anus in the first larval stage. However, an alimentary canal is already in place. The purple grains seen in the photograph are nuclei, the pink, smaller, grains are yolk. A star marks the anterior.

In paper II we show that there is no mouth opening present in the first and the second larva. I here support this with four additional photographs of the first three larval stages. As seen in Fig. 9, the alimentary channel is in place in the hatching larva but a mouth opening is not present (Fig. 10A). In a hatching larva with the cuticle removed before coating, it is possible to see a depression where the mouth later is formed (Fig. 10B). However, a functional mouth is not seen in the second lorica stage either (Fig. 10C). It is only in the third larval stage i.e. in the second lorica larva that a true mouth opening appears (Fig. 10D).

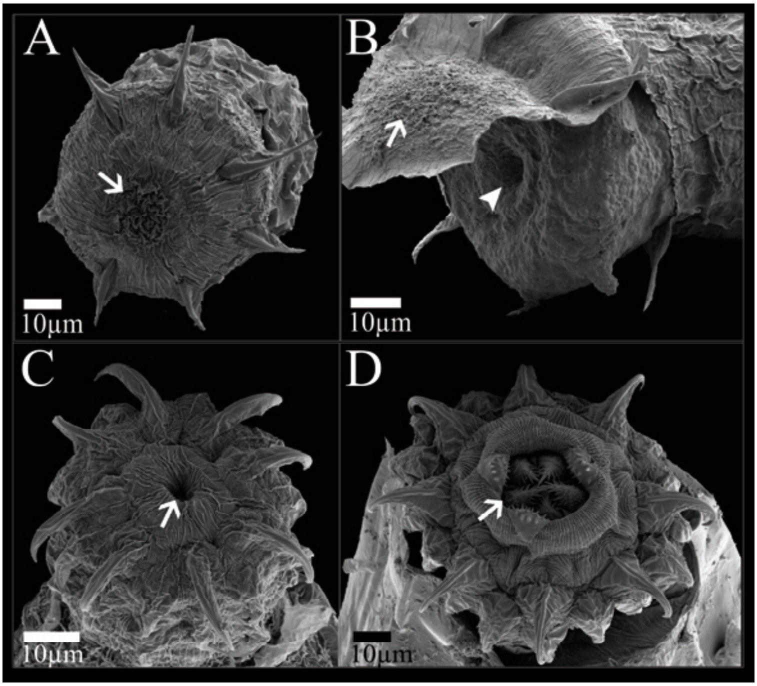


Fig 10. Anterior view of the first three larval stages of *P. caudatus*. The area where the mouth opening later forms / or is formed is marked by a white arrow. A. A hatching larva. B. The cuticle of a hatching larva is removed and the underlying tissue is exposed. Arrowheads point to the area where the mouth later is formed. C. Second stage larva (first lorica larva). A depression in the cuticle is seen where the mouth opening is later observed. D. Third larval stage (second lorica larva). The arrow is pointing at the mouth opening, now fully functional.

In papers **I** and **II** we refer to the high yolk content of the embryos and larvae of *P. caudatus*, but do not show pictures to illustrate this fact. Fig. 11 illustrates this here.

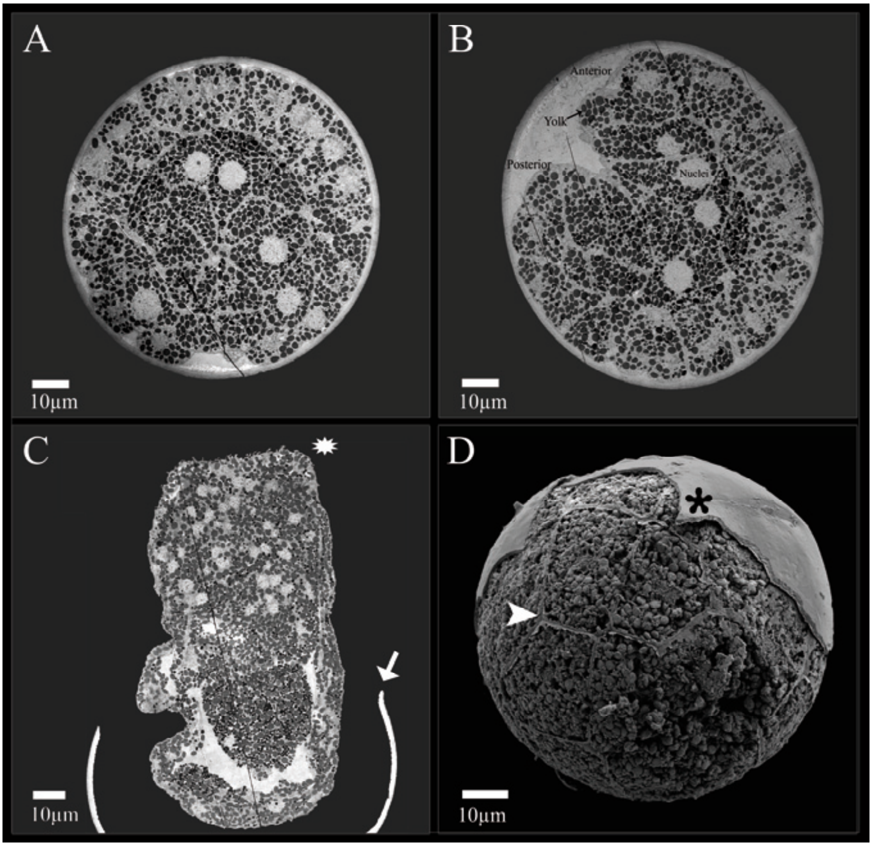


Fig 11. A-C. TEM photographs showing the distribution of yolk (dark grains) in different developmental stages of *P. caudatus*. A. Late gastrulation. B. Prior hatching (9 days). C. A hatching larva. A star marks the anterior and an arrow marks the broken eggshell. D. SEM photograph of a 32- cell stage embryo. The eggshell is marked by (*) and the arrow-head points at a cell membrane.

Cleavage pattern as seen in the priapulid worm Halicryptus spinulosus

The *in vitro* fertilization success rate of *H. spinulosus* is much lower than I describe for *P. caudatus* (paper I). Not only was the number of mature eggs per gonad much smaller, but also not many fully mature gonads were available for my first experiments. This may be due to the wrong, i.e. non-natural fertilization time (fertilizations were only attempted in February and March). A second problem is the apparent relative immobility of the sperm. In contrast to the case in *P. caudatus* I could not obtain many agile sperm. Most sperm were simply not activated (possibly because they were immature) or the spermatozoa stick together, which prevents them from swimming properly and finally fertilizing an egg. Despite this, I managed to obtain a small number of developing eggs. My first attempt was to keep them alive until larvae would hatch. This to assure that the development was natural and no “freaks” would appear. Unfortunately this did not work and all the embryos died shortly after the 8-cell stage. I have observed that the first cleavages are total and possibly slightly subequal (Fig. 12A-C). At the 4-cell stage, a cross furrow forms (Fig. 12B). This has not been reported for *Halicryptus* before (Zhinkin and Korsakova 1953). This cross furrow persists until at least the 8-cell stage (Fig. 12C).

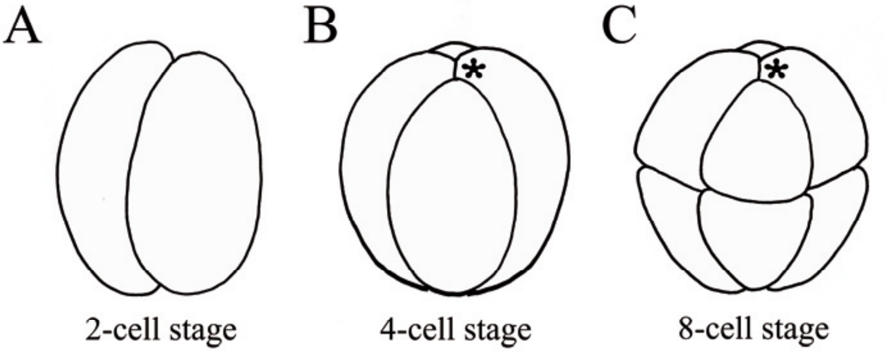


Fig 12. The drawing illustrates the first three cell-stages in *H. spinulosus*. A. The 2-cell stage show two equally sized blastomeres. B. 4-cell stage with the cross-furrow marked (*). C. 8-cell stage, four cells at each pole, the cross furrow is still present (*).

Whether the cross-furrow is in a right angle on the vegetal and animal pole respectively, as it is the case in *P. caudatus*, is currently unclear. After the 8-cell stage, the cleavage pattern becomes very irregular, leading to a number of non-stereotypic cell-patterns resembling the earlier reported embryonic “developmental stages” (Zhinkin and Korsakova 1953; for a discussion see Wennberg et al. 2008). Shortly after the embryos died and started rotting. This either implies, the conditions for the cultivations were not optimal for *H. spinulosus* or that I used non-mature eggs (and possibly also sperm) for the fertilization experiments.

However, since some success was achieved in adequately fertilizing *H. spinulosus*, it seems likely that successful culture of embryos is possible and can be achieved in the future. This should enable me to draw conclusions concerning the developmental features shared between these two priapulids.

Conclusion

* The cleavage pattern in *Priapulus caudatus* is highly symmetrical, total, subequal, radial, and stereotypical up to gastrulation.

* Gastrulation is intermediate between epiboly and invagination. The mesendoderm may be derived from both cells of the first cleavage, thus differing significantly in its origin from that of many other protostomes.

* The cleavage pattern remains highly regular even at later stages, and is unlike any previously studied embryos.

* The insights derived from *Priapulus caudatus* embryology presented in paper I, combined with new data published recently on kinorhynchs (Kozloff 2007) and tardigrades (Eibye-Jacobsen 1997 and Hejnol and Schnabel 2005, 2006), imply substantial developmental diversity among basal ecdysozoans. This weakens the hypothesis that irregular cleavage is plesiomorphic to the entire clade.

* The *hatching larva* of *Priapulus caudatus* differs considerably from previously described larvae, not only in its general body shape, but also in its lack of a proper lorica including the typical lorica tubuli. Moreover, no mouth opening or pharyngeal teeth have yet formed and also the number and arrangement of scalids differ from that of later larvae.

* The *first lorica larva* partially resembles lorica larvae described earlier, especially the *larva-type 1* described by Higgins (Higgins et al. 1993). However, there are a number of important differences between these larvae. The first lorica larva is smaller than previously described lorica larvae of *P. caudatus* and the mouth opening as well as pharyngeal teeth are yet to form.

* The *second lorica larva* is equipped with four rings of pharyngeal teeth. It shows striking similarity to the previously described *larva-type 2* (Higgins et al. 1993) only differing in the described scalid pattern.

*   The cleavage pattern up to and including the 8-cell stage of *Halicryptus spinulosus* is basically the same as described for *Priapulus caudatus* (Wennberg et al. 2008), i.e. cleavage is highly symmetrical, total and possibly subequal. A cleavage furrow is clearly formed at the 4-cell stage and persists until at least the 8-cell stage.

*   Preliminary data on muscle development in *Priapulus caudatus* is presented.

Svensk sammanfattning

Varför studera priapulider?

Under min tid som doktorand har jag studerat embryologin och tidiga larvstadier av priapulidmasken *Priapulus caudatus*. I dag finns det ett 20-tal priapulidarter i våra hav, en spillra av den artrikedom som tidgare funnits. Priapulider som återfinns i kalla vatten och på stora djup är oftast relativt stora (över en centimeter långa) emedan prapulider i varma grunda vatten tillhör arter som inte blir längre än någon eller några millimetrar som vuxna. Priapuliderna återfinns som fossil redan i underkambrium, för ca 520 miljoner år sedan, vilket gör att de är av stort intresse för mig som arbetar med bauplan evolution dvs. utveckling av olika kroppsorganisationer. Flera utav de tidiga flercelliga djuren (c:a 520 miljoner år gamla och yngre) är numera utdöda och kan endast återfinnas som fossil men några få djurgrupper finns fortfarande på vår jord. Studier av dessa ”levande fossil” är mycket viktiga för vår förståelse om de tidiga flercelliga djurens utveckling och släktskap. Noggranna undersökningar av priapulidernas embryonalutveckling kan markant öka vår förståelse för hur bauplan evolutionen har fortgått hos priapuliderna och deras närmaste släktingar.

Släktskap med andra djur

Priapulidernas nära släktingar kallas ofta cycloneuralia och inkluderader av övriga maskformiga djur, rundmaskarna. På senare tid har genetiska analyser presenterats som stödjer ett systerskap mellan cycloneuralia och leddjuren, denna gruppering kallas Ecdysozoa (Aguinaldo et al. 1997). Gruppens namn är taget från Ecdysis – skalömsning, en karaktär som ses hos både cycloneuralia och leddjuren. Ett antal karaktärer som tros förena de nutida priapuliderna med den *hypotetiska* urfadern till alla de djur som är inkluderade i ecdysozoa har presenterats. Karaktärerna är följande: (1) en masklik kroppsform (2) en annulerad, eller till och med segmenterad kropp (3) en relativt stor kroppstorlek hos de vuxna djuren (4) en terminal munöppning (5) en cykloneural hjärna (6) ecdysis - skalömsning (7) radialt klyvningsmönster under embryoutvecklingen (8) direkt utveckling (Budd, 2001). De flesta karaktärerna är lätta att verifiera hos vuxna priapulider emedan andra kräver att vi undersöker priapulidernas utveckling vidare. Detta gör att studier som behandlar olika aspekter av embryonalutvecklingen hos priapuliderna i jämförelse med leddjuren är minst lika intressant som jämförandet med släktingarna inom cycloneuralia. Deras embryologi kan ge oss indikationer om vilka evolutionära processer som har ägt rum, inte bara hos djuren vi studerar men även hos djur som varit utdöda sedan länge.

Fältarbete

Jag har under mina år som doktorand samlat in en stor mängd priapulider av arten *Priapulus caudatus*. Denna art tillhör de stora priapulidarterna och fångas lättast i Gullmarsfjorden, som återfinns på den svenska västkusten. Efter infångandet dissikeras gonaderna ut från han och hondjuren, varefter ägg och sperma blandas i en embryoskål. Embryona förvaras under sin utveckling i ett kylrum med konstant temperatur (8-10°C). På detta sätt har jag fött upp tusentals priapulidembryon under sju fältsäsonger. Olika fixeringsmetoder har använts beroende på om embryon och larver skall studeras med hjälp av ljusmikroskopi, konfokalmikroskopi eller elektronmikroskopi. Mest frekvent användes 4% PFA som fixeringsmedel och embryona förvarades i 70% etanol. Undantaget var de embryon som används till studien av muskulaturen. Muskelfilamenten får ej komma i kontakt med alkohol och därför användes vid denna studie PBS buffer med en tillsatts av NaN_3 som lagringsmedium.

Artiklar

Early embryonic development of the priapulid worm, *Priapulus caudatus*. *Publicerad i Evolution and Development*

Artikeln behandlar de första celldelningarna som sker efter befruktning fram till då embryot består av 64 celler och gastruleringen startar. De fixerade embryona färgades in med akridinorange (en autofloroserande markör som färgar in cellkärnor och membran) och studerades med ett Leica TCS-SP konfokalmikroskop. 3D bearbetning av informationen utfördes i modelleringsprogrammet, Mimics 9.1. Resultatet visar att tidigare studier (Zhinkin 1949 och Lang 1953) var korrekta i sin beskrivning av de första cellklyvningarna. Vidare fylldes de tidigare luckorna i literaturen i med det korrekta mönstret för 32- och 64 cellstadiet. Mönstret vid gastruleringen är en blandning av epiboli och invikning av celler. Resultaten visar, tillsammans med nyutkomna studier kinorhynchernas (introverta) och tardigradernas (leddjur) embryologi, att vad gäller till den tidiga embryonalutvecklingen inom ecdysozoer så är diversiteten stor.

Hatching and earliest larval stages of the priapulid worm *Priapulus caudatus*. *Manuskriptet är inskickat till "Invertebrate Biology"*

Manuskriptet beskriver kläckning av *P. cauadatus* ägg och behandlar vidare de tre första larvstadierna. Efter kläckning förvarades larverna i 4ºC (att jämföra med utvecklingens 10ºC). Vidare var fixeringsmediumet 1:1: 4% PFA : 2,5% glutaraldehyd. En noggrann elektronmikroskopisk studie har dokumenterat kläckning och de tre första larvstadierna. Larverna kläcks 10 dagar efter fertiliseringen. Det första larvstadiet saknar en riktig lorica, det hårda skal som återfinns i de senare larvstadierna. Vidare saknas även en riktig mun när introverten är indragen ser det skenbart ut som en mun. Sju stora taggar-scalids- och ett varierande antal små taggar kan observeras framtill på introverten. Det andra larvstadiet har en lorica och de fyra karaktäristiska laterala lorica taggarna ses parvis vid den bakre delen av larven. Introverten är nu täckt av flera rader av taggar men ingen munöppning är observerad. Det sista larvstadiet har en munöppning och ett imponerande tandgarnityr, detta larvstadie stämmer någorlunda överens med vad som tidigare beskrivits i literaturen. För att förklara likheter och skillnader mellan vår och tidigare studier så tar vi upp problematiken kring material insamling kontra laboratorieuppfödning av djur. Även utbredningen av *P. caudatus* och övriga priapulidarter, samt möjliga förväxlingar av larver diskuteras.

Development of the musculature in the priapulid worm, *Priapulus caudatus*. *Manuskript*

Artikeln beskriver den fortsatta embryonalutvecklingen i *P. caudatus*, efter gastrulering. Cellkärnor färgas in med propidiumjodid (en autofloroserande markör som färgar in cellkärnor) och för att visualisera muskulaturen, aktinfilamenten, användes falloidin kopplat till FITC.

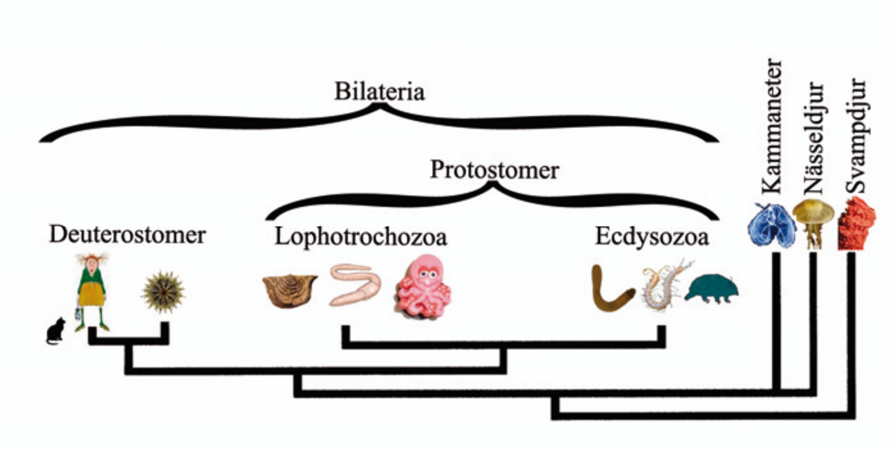
Embryona studerades med ett Leica TCS-SP2 konfokalmikroskop. Resultatet av studien är preliminärt, var vänlig se artikel III för resultat och diskussion.

Populärvetenskaplig sammanfattning

Priapulider - okända djur som fascinerar

Har du någon gång funderat över vilka djur var först på plats på vår jord? Det har ju inte alltid vandrat omkring människor, igelkottar och älgar i naturen. Under livets tidiga historia var många djur maskliknande små kryp. Faktum är att de flesta djur som vi har kring oss idag är ganska masklika de också. Här kommer en introduktion till den mest intressanta masken av dem alla.

De flesta av oss har någon idé om vart vi härstammar ifrån, vilka som är våra föräldrar och morföräldrar och vart de levt och verkat. Vissa människor är mer nyfikna än andra och söker sitt ursprung genom att ta hjälp av släktforskare. Familjeträd kan skapas med hjälp av den information som exempelvis hittas i kyrkböcker eller som gamla fotografier. Paleontologer nöjer sig inte med sin egen släkthistoria. De vill återskapa mänsklighetens, djurens, och i vissa fall, vår jords historia. Ett fylogenetiskt träd beskriver hur olika djur eller växtgrupper är besläktade med varandra. Det visar även när grupperna levde och verkade relativt till varandra, lite som våra egna familjeträd men i större skala.



Figur 1. Exempel på ett fylogenetiskt träd.

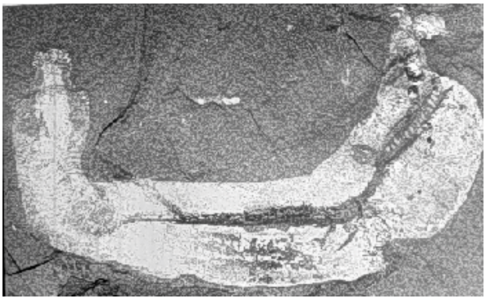
Livets tidiga utveckling

Min forskning fokuserar på den nedre delen av det fylogenetiska trädet som beskriver djurens tidiga utveckling. Under tidsperioden kambrium, för ca 520 miljoner år sedan, började det dyka upp en mängd flercelliga djurgrupper på vår jord. Denna uppblomsting av liv kallas populärt ”den kambriska explosionen”. Vart djuren härstämmar ifrån och hur de är besläktade med varandra är omtvistat. I avsaknad av tidsmaskiner så kan vi endast förlita oss på fossil, djur eller spår av djur som har bevarats i sten genom årmiljonernas gång, eller studera de organismer vi har på vår jord idag.

Figur 2. En priapulid av arten *Priapulus caudatus*. De kan hittas på Sveriges västkust om man letar tillräckligt noga med trål.

Storleken spelar roll

Priapuliderna ser fortfarande i stort sett likadana ut som de priapulidfossil vi hittar från den kambriska tidsperioden. Till detta kan även läggas att priapuliderna har haft en stor kroppstorlek genom hela sin utveckling. I dag är den art jag arbetar med, *Priapulus caudatus*, ungefär en decimeter lång och studerar man kambriska fossil så är de i princip lika stora. Detta är något som skiljer priapuliderna från en mängd andra djur, exempelvis så visar deras närmaste släktingar upp små kroppstorlekar under sin tidigaste evolution. Priapulidernas kroppstorlek i kambrium, och de nulevande arternas likhet med de utdöda arterna, som hittas som fossil, gör att de anses vara en grupp djur som till viss del har ”konserverats” genom historiens gång. De yttre likheterna gör att paleontologer, som jag själv, antar att deras sätt att föröka sig också har bevarats från deras tidigaste utveckling, för ca 540 miljoner år sedan. Men varför är detta antagande så viktigt?



Figur 3. Den utdöda priapuliden *Ottoia* levde mitt under den kambriska explosionen. Likheten med de levande priapuliderna är slående. Tänk dig priapuliden i föregående bild men väldigt lätt att, det är det du ser på denna bild.

Leddjuren och deras segment

Som du läst tidigare så uppstod många av de djurgrupper vi ser i dag under den kambriska explosionen, en av de mer kända grupperna är leddjuren. Leddjuren inkluderar fyra nu levande klasser; insekter, spindlar, kräftor och skorpioner. En klass, trilobiterna, är numera utdöd men många känner till den från de vackra fossil som finns spridda över världen. Karakteristiskt för ett leddjur är uppdelningen i segment. De olika segmenten är anpassade för olika saker, exempelvis är kräftornas framben omvandlade till gripklor medan bakbenen endast används vid förflyttning. En fråga som paleontologer funderat länge över är om leddjuren härstammar från en segmenterad förfader eller om de har utvecklats från ett osegmenterat djur som i ett senare skede blivit segmenterad? För att lösa detta mysterium kan priapuliderna komma att spela en viktig roll.

Figur 4. Ett vackert fossil av en trilobit. De är utdöda sedan länge men troligtvis såg de ut lite som de gråsuggor du idag kan hitta under en vanlig sten i skogen. Storleken varierade kraftigt mellan arterna, vissa kunde bli upp till 70 cm långa!!

Priapulider - nyckeln till mysteriet?

Genom att studera fossil vet vi att priapuliderna är ”konserverade” till kroppstorlek och utseende. Jag, och andra med mig, gör därför ett antagande om att priapuliderna även har ”konserverat” sitt sätt att föröka sig. De priapulider som lever i dag släpper ägg och sperma fritt i vatten precis som deras förfäder gjorde under den kambriska tidsperioden. Tanken att deras embryon har utvecklats på ett likartat sätt under årmiljonernas gång är då inte långt borta. Priapulider och deras närmaste släktingar är placerade nära leddjuren i det fylogenetiska trädet. För det mesta anses de vara en äldre grupp än leddjuren. Detta kan vara forskarna till hjälp då de vill lösa frågan om segmentering fanns hos de tidigaste leddjuren eller ej. I dag är priapuliderna inte ledade, de uppvisar inga segment, och om de har haft segment under sin tidigaste utveckling vet vi inte. Hur priapulidernas embryon utvecklas har i stort sett varit okänt fram tills nu, då jag har börjat med att kartlägga cellernas utveckling från befruktningen av ägget fram till och med då larverna kläcks. Om jag kan finna något i embryonas utveckling som tyder på att priapulidernas tidigaste förfäder var segmenterade så kan detta vara en indikation på att priapuliderna och leddjuren härstammar från ett segmenterat djur. Om detta stämmer har priapuliderna, förlorat segmenteringen under årmiljonernas gång. Däremot, om inga tecken på segmentering ses i priapulidembryona så var troligtvis den gemensamma förfadern till de båda grupperna osegmenterad. Segmentering har då uppstått vid ett senare skede hos leddjuren.

En pusselbit i livets träd

Det kan ty sig märkligt att en liten grupp av forskare bryr sig om och ägnar sina liv åt segmentationens vara eller icke vara i leddjurens tidiga utveckling. Trots att det är en liten pusselbit i det gigantiska fylogenetiska trädet så behövs den likväl för att vi någon gång i framtiden skall kunna rita upp ett komplett släktträd över de djurarter som vi har på vår jord i dag.

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