MORPHOLOGY OF THE TESTES AND SPERMATOGENESIS IN ACOELA & NEMERTODERMATIDA

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“But morphology is a much more complex subject than it at first appears”

Darwin, 1872:584

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CHAPTER 1: INTRODUCTION

1. GENERAL INTRODUCTION

Phylogenetic relationships between animal taxa have always intrigued scientists. As the raw data of phylogenetic studies shifted from morphology to molecules, a major reorganization in our insights on the animal tree of life took place. Some positions within that tree are quite well resolved, as many molecular studies are congruent on them, while the phylogenetic position of other taxa still remains obscure. It is remarkable that taxa that have puzzled morphologists for years, appear to be just as puzzling to molecular phylogeneticists.

Acoela and Nemertodermatida are two examples of taxa that have been placed at various positions in the tree of life. They were initially placed within the taxon Platyhelminthes (Steinböck 1930-1931), but their exact phylogenetic position was already at that time puzzling. Recent molecular studies attribute a position at the base of the Bilateria for both Acoela and Nemertodermatida, making them pivotal for the reconstruction of ancestral bilaterian features.

In this introduction, the characteristics of Acoela and Nemertodermatida are described, after which their various phylogenetic positions are summarized. The third part of the introduction deals with character evolution and its consequences. In the last part of the introduction, the definition of the germ line & gonads is outlined.

2. CHARACTERISTICS OF ACOELA AND NEMERTODERMATIDA

2.1. ACOELA

Acoela is a group of soft-bodied, marine, unsegmented bilaterian organisms. The group has around 380 species. Representatives of Acoela have a great morphological diversity (Mamkaev 1967). Although they are triploblastic, they do not have a coelomic cavity, hence their name. They lack some other structures as well: a circulatory system, an excretory system, a true brain and an anus. With the absence of an anus, they lack a through gut, and the morphology of this gut is also quite different from other animal groups: acoels digest their food through a syncytial digestive parenchyma. The mouth is in some species modified as a pharynx; the mouth opening can be positioned ventrally (anterior, central or posterior) or caudally. Acoela have a frontal organ and a statocyst containing a single statholith, which is used for balancing their body. Some species have eyes, but these comprise only 2 cells per eye: a pigment cell and a receptor cell. Pairs of nerve cords run along the body, and an anterior nerve ring serves as brain. Ultrastructural research by Bery et al. (2010) has shown that the juvenile brain of *Symsagittifera roscoffensis* is structured like a typical invertebrate ganglion: a dense mass of neuronal cell bodies surrounding a central neuropile. A glandular frontal organ is found at the anterior part of the body. Acoels have a multiciliated epidermis, with an extensive and complex ciliary rootlet system, which is believed to replace the function of an extra-cellular matrix (ECM), which is lacking in this taxon (Schmidt-Rhaesa 2007). Tyler & Rieger (1999) interpret small amounts of material found between epidermal and muscle cells as remnants of extra-cellular matrix (Schmidt-Rhaesa 2007). The body-wall musculature is very variable: some Acoela have a simple orthogonal grid of longitudinal and circular fibres, while other have distinct pattern of ventral and dorsal body-wall musculature containing longitudinal, circular, U-shaped and diagonal muscles (Hooge 2001, Chiodin et al. 2011). Acoela have a pool of somatic stem cells (neoblasts), responsible for growth, cell renewal and regeneration.

Most Acoela are hermaphroditic, meaning that they have both male and female organs. They reproduce sexually, after internal fertilization. Acoels have a variety of copulatory organs (Hooge & Tyler 2005; Raikova et al. 2006). After copulation, sperm can be stored in the seminal bursa, but in other species, the sperm lies freely in the

parenchyma after hypodermic impregnation. All acoels have elongated sperm cells with two incorporated and inverted axonemes (Hendelberg 1969, 1977, Tyler et al. 1986, Justine et al. 1998, Raikova et al. 1998, 2001; Raikova and Justine 1999, Petrov et al. 2004, Tekle et al. 2007, Boone et al. 2011). Fertilized eggs leave the body through the female pore, the mouth or ruptures in the body wall.

Acoel embryos have a unique duet cleavage. When the eggs hatch, miniature worms emerge, hence larval stages are absent. Some species reproduce asexually by fragmentation or fission (e.g. *Convolutriloba longifissura*, Bartolomaeus & Balzer 1997) or budding (e.g. *Convoluta retrogemma*, Hendelberg & Åkesson 1991). A pool of neoblasts is responsible for this fission.

The majority of known Acoela are part of the benthic marine meiofauna (e.g. *Childia macroposthium*, *Isodiametra pulchra*). Some members of Convolutida (e.g. *Symsagittifera roscoffensis* and *Convoluta convoluta*) possess algal symbionts (Douglas 1992, Achatz et al. 2010). *Hofstenia miamia* is an acoel that crawls on mangrove leaves, and there are two species which inhabit freshwater (Schockaert et al. 2008).

2.2. NEMERTODERMATIDA

At first glance, species of Nemertodermatida appear similar to acoels. Like Acoela, they are soft-bodied, marine and bilaterian. A taxonomic revision of the group was made by Sterrer (1998); the taxon contains only 9 species. Representatives of Nemertodermatida lack a circulatory and excretory system. They do have an epithelial gut but no anus. No known species of Nemertodermatida has a pharynx, and some species even do not have a mouth. Typical of this group is their statocyst containing at least 2 statoliths, which serves as a static sense organ. No nervous projections were found in the statocyst, so the exact mechanism of the transduction of the signal is unknown. Nemertodermatida have a peripherous intra- or basiepidermal nervous system, and a ringlike concentration of nerves in the brain. The epidermis is thick, rich in glands and has an elaborate system of ciliary rootlets. Nemertodermatida are hermaphroditic, but all species lack accessory organs. Nemertodermatida are not known to reproduce asexually. Their sperm is filiform, has an elongated nucleus in the head, mitochondrial derivatives, an axial filament in the middle piece, and a flagellum which extends as a tail. The early cleavage of eggs from *N. westbladi* is characterized by duets of macromeres and micromeres at the 4-cell stage. However, during later divisions, cleavage pattern differs from the acoel duet pattern. All known nemertodermatids are part of the benthic meiofauna, except *Meara stichopi*, which lives inside the sea cucumber *Stichopus tremulus*.

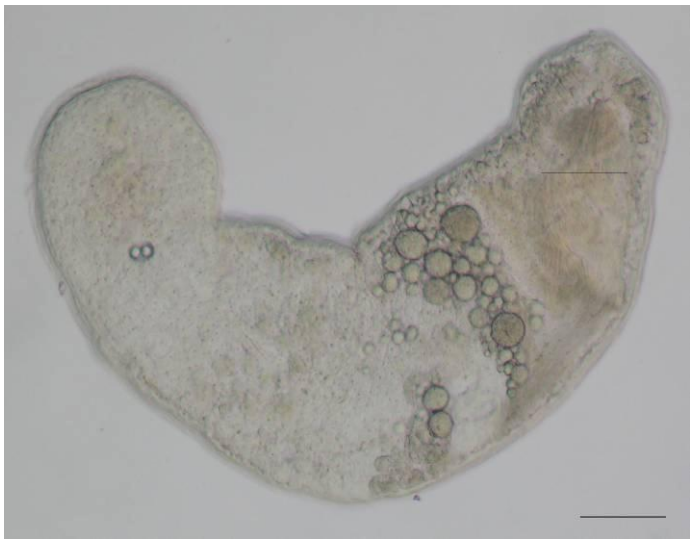

Fig. 1: light microscopic image of *Flagellophora apelti*, a member of the taxon Nemertodermatida. Scale bar: 100 µm.

3. PHYLOGENY

3.1. PHYLOGENETIC POSITION OF ACOELA AND NEMERTODERMATIDA

The phylogenetic position of Acoela and Nemertodermatida is heavily debated. Both morphological and molecular data have provided arguments for the placement of both groups at different branches of the bilaterian tree of life. Hypotheses on the phylogeny of the Metazoa has changed drastically during the past decades, and becomes increasingsly vexing as most molecular studies provide results very different from the trees based on morphological data (Zrzavý et al. 1998).

Initially, Acoela and Nemertodermatida were placed together within the Platyhelminthes (Steinböck 1930-1931). However, different authors have pointed out the special features of Acoela and Nemertodermatida, such as the ciliary rootlet system and the lack of an excretory system, indicating possible other phylogenetic positions.

In a paper on the reproduction of *Amphiscolops langerhansi*, Libbie Hyman states that the simplicity of their morphology is in all probability primitive and not the consequence of degradation (Hyman 1937). In part I of her series on the invertebrates (Hyman 1940), she attributes the position of ancestor to all Bilateria to “acoel flatworms” (Hyman 1940). In part II of her series on invertebrates (Hyman 1951), Hyman notes that *Nemertoderma bathycola* combines primitive (epidermal nerve plexus, no female duct, no penis papilla) and advanced characters (clear intestinal lumen and well-defined testes).

Karling (1940; 1974) considered Acoela and Nemertodermatida to be substantially different from each other in morphology of the gut and the statocyst, and placed the taxon Nemertodermatida outside Acoela (Karling 1940).

In the first phylogenetic analysis of Platyhelminthes by Ehlers (1985), Nemertodermatida formed a sister group relationship with Acoela, together forming the taxon Acoelomorpha, at that time considered to be the sister-group of Rhabditophora. Both Acoela and Nemertodermatida have a complex system of ciliary rootlets: a strong anterior and a weaker posterior rootlet from which two fibrous bundles originate. As the rootlet structures contact each other, they form a complex

network that has a structural function. Acoela differ in their anterior rootlets, which have two additional branches (Hendelberg & Hedlund 1974). Both taxa also have the same modified cilia with a shelfed tip and both lack protonephridia (Tyler & Rieger 1977).

This view of Acoela and Nemertodermatida being grouped as Acoelomorpha was followed in most subsequent morphological studies (for a review: see Lundin 1997).

However, Smith and Tyler (1985) concluded from ultrastructural characters of the body wall, parenchyma and digestive tract that the Acoela are derived, and cannot be seen as models of primitiveness or as ancestral coelomates, while Nemertodermatida can. Smith et al. (1986) suggested the possible polyphyly of the taxon Platyhelminthes, based on the absence of clear synapomorphies, and viewed the Acoelomorpha as one of the three well-defined monophyletic groups within Platyhelminthes, the two others being Catenulida and Rhabditophora. Different molecular phylogenetic studies (Ruiz-Trillo et al. 1999, 2002, Jondelius et al. 2002, Telford et al. 2003) corroborate the polyphyly of the Platyhelminthes and they appoint a position as early diverging taxa within the Bilateria for the Nemertodermatida and the Acoela. Non-acoelomorph Bilateria were considered a monophyletic group, Nephrozoa (Jondelius et al. 2002, Zrzavý et al. 2003). The presence of the small Hox cluster in both Acoela (Cook et al. 2004) and Nemertodermatida (Jimenez-Guri et al. 2006) is concordant with this phylogenetic position.

The sister-group relation of Acoela and Nemertodermatida was challenged by studies based on 18S small subunit rRNA and mitochondrial genes (Jondelius et al. 2002), on nuclear protein coding myosin heavy chain type II (Ruiz-Trillo et al. 2002), 18S rRNA and 28S rRNA genes (Wallberg et al. 2007), and on a combination of several molecular markers (Baguña et al. 2008, Zzravy et al. 1998). According to these studies, Acoelomorpha appeared to be paraphyletic, hence the taxon was dismissed: Nemertodermatida and Acoela are separate branches at the base of the Bilateria.

Wallberg et al. (2007) also noted the considerable morphological differences between Acoela and Nemertodermatida. They differ in the structure of the intestine, the statocyst and the mature sperm cells. The gut is epithelialized in Nemertodermatida, while Acoela have a digestive parenchyma. Acoels have only one statolith inside their statocyst, while Nemertodermatida have at least two.

Nemertodermatid sperm is filiform, with an elongated nucleus, a middle piece and a flagellum that extends as a tail; a very different morphology compared to acoel sperm, which is also filiform but has no clear middle piece, and has two flagella which are incorporated and inverted.

However, a recent phylogeny based on a large number of taxa and multiple nuclear genes, however, provides support for a monophyletic Acoelomorpha, albeit also positioned at the base of the Bilateria (Paps et al. 2009). Hejnol et al. (2009) not only reunite acoels and nemertodermatids, but also show a sister-group relation of Acoelomorpha with Xenoturbellida and attribute a basal[1] position within the Bilateria for these taxa. The view that the Acoelomorpha are the earliest divergent extant lineage of Bilateria was also supported by the complete mitochondrial genome data of the acoel *Symsagittifera roscoffensis* (Mwinyi et al. 2010). However, Philippe et al. (2011) suggest that the taxon Xenacoelomorpha should be situated within Deuterostomia, as a sister-group to Ambulacraria, based on several molecular data sets. No single of their data set clinches the case for placing acoels within the deuterostomes, but they all point in the same direction (Maxmen 2011). However, critics of this paper say that the key branches of the tree are not strongly statistically supported, therefore making the tree suggestive but not definitive. Moreover, the study has left out data that would have weakened the conclusions (Maxmen 2011). Furthermore, the paper of Philippe et al. (2011) is largely based on microRNA analysis, the power of which is doubted by some, as it has only recently been adopted for evolutionary studies (Maxmen 2011). Edgecombe et al. (2011) agree that Acoelomorpha and Xenoturbellida are sister taxa, but acknowledge that the position of these taxa is not yet settled, and can be either within Deuterostomia or as a sister group to Nephrozoa.

It is clear that the debate on the exact positions of Acoela and Nemertodermatida is still ongoing, and that no consensus has been reached yet. This advocates the study of their morphological and developmental features.

[1] We note that it is more correct to use the term “early diverging” instead of “basal” (as extant taxa are never basal); however, as “basal” is an established expression, we use this term throughout the thesis.

3.2. INTERNAL PHYLOGENETIC RELATIONSHIPS

3.2.1. ACOELA

Until recently, Acoela was subdivided in 21 families without a reference to the internal relationships between these families (see Wallberg 2009 for references). Hooge et al. (2002) used partial 18S rDNA sequences from 32 species of 10 families of Acoela to infer internal phylogenetic relationships. They found that only four of these families were monophyletic: Paratomellidae, Mecynostomidae, Sagittiferidae and Anaperdia. The families Actinoposthiidae, Convolutidae and Haploposthiidae were found to be polyphyletic, while the status of the three remaining families in their study remained undetermined. Their study showed that body musculature and certain features of the spermatozoa reflect the branching pattern in the tree well. Jondelius et al. (2011) made the first large-scale phylogenetic analysis of the internal relationships within the Acoela, using 18S rDNA, 28S rDNA and COI genes. This study revealed 10 monophyletic families: Diopisthoporidae, Paratomellidae, Hallangiidae, Hofsteniidae, Solenofilomorphidae, Proporidae, Isodiametridae, Dakuidae, Mecynostomidae and Convolutidae (fig. 3).

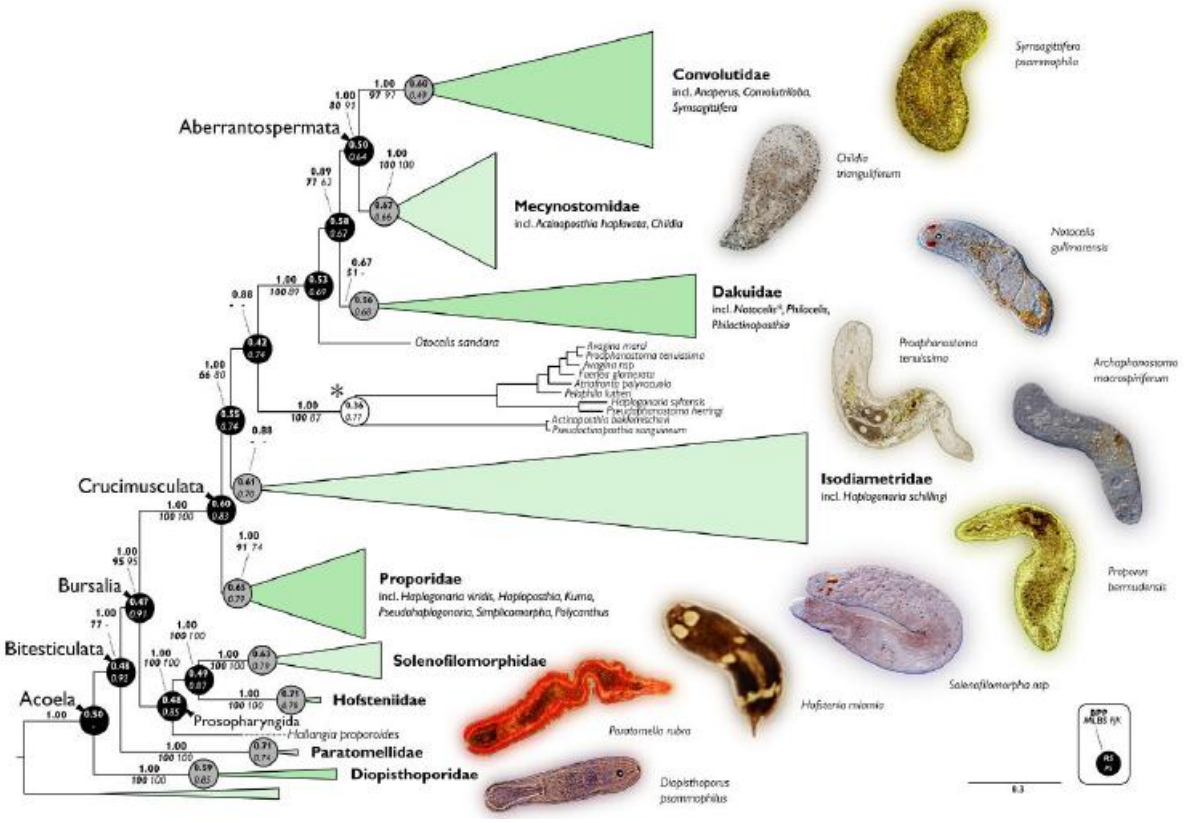

Fig. 3: Overview of acoel phylogeny by Jondelius et al. 2011: total-evidence Bayesian majority-rule consensus tree inferred from 18S, 28S and COI sequences.

3.2.2. NEMERTODERMATIDA

Sterrer (1998) made the most recent taxonomic revision of the Nemertodermatida. He delineated two families within the taxon: Ascopariidae and Nemertodermatidae. *Flagellophora apelti*, *Ascoparia neglecta* and *A. secunda* are representatives of the family Ascopariidae, while *Nemertoderma westbladi*, *N. bathycola*, *Sterreria psammicola*, *Nemertinoides elongatus* and *Meara stichopi* are members of the Nemertodermatidae.

Lundin (2000) and Lundin & Sterrer (2001) made a cladistical analysis of the Nemertodermatida based on morphological data. Their results support the monophyly of Nemertodermatida, Nemertodermatidae and Ascopariidae.

3.3. IMPORTANCE AND IMPLICATIONS OF PHYLOGENETIC POSITION

As certain nodes in the phylogenetic tree of Metazoa become increasingly supported by molecular sequences, speculations are made as to the morphology of the ancestral organisms represented there (Valentine 2006). Hypothetical clade ancestors have disparate living representatives. This way, homologies in these groups can be used to infer some features in the clade ancestors and the last common ancestor, while differences in their molecular sequences can be used to estimate a molecular clock to date the last common ancestor (Valentine 2006). As Acoela and Nemertodermatida are usually positioned at the base of the bilaterian tree and their characteristics could provide important clues to the understanding of the evolution of these characteristics in the Bilateria.

Philippe et al. (2011) remark that Acoela might not be placed at the base of the Bilateria, but at the base of the Deuterostomes. In this particular scenario, they must have evolved from an ancestor with a body cavity, a through gut and a central nervous system (Maxmen 2011). However, it is remarkable that loss of several different structures occurs in three taxa, which group together (Acoela, Nemertodermatida and Xenoturbellida). The placement of Xenacoelomorpha within Deuterostomia has several implications: early deuterostomes must be secondarily simplified (as mentioned before) and a remaining puzzle at the base of the Bilateria (Lowe and Pani 2011) as the evolutionary step between non-bilaterians and bilaterians is missing (Maxmen 2011).

Mark that the fact that acoels and nemertodermatids lack certain structures, makes it difficult to infer phylogenetic relationships on the basis of morphology, as the lack of a structure can represent an initial absence or a secondary loss.

3.4. CHARACTER EVOLUTION

Comparative data sampled from different species, can be used to supply the data basis for the reconstruction of a phylogenetic hypothesis, or to be mapped on an existing phylogenetic tree in order to extract an evolutionary interpretation (Schmidt-Rhaesa 2003). As phylogenetic trees describe the pattern of descent amongst a group of taxa, the combination of a phylogenetic tree and species information can be used to infer past states (Pagel 1999, see also 3.3.: the importance of a phylogenetic position). Character evolution is tracing and understanding the evolutionary history of characters along the phylogenetic tree (Wallberg 2009). When a morphological character state is mapped onto a phylogenetic tree, the evolution of this character can be inferred.

Detailed and comparative morphological studies are a prerequisite for mapping morphological characters on a phylogenetic tree: they can shed light on the evolutionary origins of important novelties (Jenner 2006). For many organisms and many organ systems, very isolated and sketchy information is available and it is important to gain more information on the diversity of structures within particular taxa; choosing the right data of comparative morphology is an important but hard part of this process (Schmidt-Rhaesa 2007). Smith and Turner (2005) put it this way: “Even a cursory review of many current morphological and phylogenetic based research programs and journals would reveal that a great deal of the useful morphological diversity of the earth’s biota, present and past, has not been scrutinized.” The choice and number of species should be done carefully, especially when members of a taxon are very diverse.

The quality of the input data is also pivotal. Due to a shift in emphasis from observation as the source of a datamatrix to what can be inferred from the data matrix (Jenner 2001), one should always be careful and critical about the quality of morphological characters gathered by different authors when using them as raw data for new phylogenetic studies. They can contain different flaws, such as scoring without empirical support, inconsistencies or imprecise character definitions (Jenner

2001). Examples of other problems in morphological analyses are the questions whether and how to weight characters and the choice of an appropriate coding method (Schmidt-Rhaesa 2007). Character coding must be done with great caution. As clades age, homoplasies increase in frequency. Moreover, estimates of homoplasy based on morphological phylogenetic studies are probably underestimates (Jenner & Littlewood 2008).

When assessing correspondence of structures, it is crucial to analyze the individual parts of a complex structure. Superficial morphological incongruence owing to character conceptualization can be resolved by studying the morphological characters at the ultrastructural level (Ragsdale & Baldwin 2010).

In certain circumstances, as form and function affect each other, it is also valuable to apply a functional approach to comparative morphology (Jenner 2004). Once the characters have been studied and plotted onto the tree, it is important to recognize that the properties of the organism are both the cause and the effect of natural selection (Lewontin 2001).

It is important to keep in mind that complexity of organisms does not necessarily increase during evolution; there should be no distinction of “simple” versus “complex” taxa or “lower / minor” versus “higher” taxa. When a comparison is made between a recent sponge and a human, it should not be forgotten that both lineages passed the same evolutionary time since their separation from a common ancestor, and both have been adapting to their environment (Smith-Rhaesa 2007). All extant animals feature a mix of ancestral characters and derived characters. Taxa can change indefinitely, and the only thing that they share is a common ancestor (Ghiselin 2005). As not only more derived clades change evolutionary, basal branches are far from infallible guides to ancestral characters states (Jenner 2006). There is no such thing as a “living fossil”, so what is ancestral or derived has to be inferred from comparative studies between different extant animals (Bourlat & Hejnol 2009).

Reconstruction of ancestral character states makes it possible to discover the most probable characteristics of ancestral species, to describe what the past was like, how traits evolve and helps to understand function. In addition, comparative studies can suggest probable causal pathways (Pagel 1999).

4. GERM LINE & GONADS

In this thesis, the male germ line and the male gonads in representatives of Acoela and Nemertodermatida are studied. The germ line of a mature or developing individual is the line of germ cells containing genetic material that may be passed to the offspring. All gametes (eggs and spermatozoa) are part of the germline, as well as their precursors. Cells which are not part of the germline, are called somatic.

The gonad is a reproductive gland that produces the gametes. According to the definition of Schmidt-Rhaesa (2007), gonads are organs bordered by epithelia and surrounding the gametes. This definition excludes cases in which gametes are present between epithelia or between parenchymal cells.

Sexual reproduction can rage from gonochorism to hermaphroditism. Gonads in different animal taxa have different morphologies, and even within a taxa, the structure of the gonad can vary.

CTENOPHORA

According to Hyman (1940), coelenterates (Ctenophora and Cnidaria) are at the "tissue grade of construction": functionally and morphologically specialized cells are grouped into recognizable tissues, which perform various life-supporting functions but are not themselves organized further into organs.

Ctenophora possess certain locations with concentrations of gametes but no further specializations. As such, the term gonad is inappropriate and many authors prefer to talk about gametogenic areas, although these areas can show some epithelial elaborations (Schmidt-Rhaesa 2007).

CNIDARIA

Concerning the gonadal structures, Cnidaria resemble Ctenophora: gametes are concentrated in gametogenic areas but there are not sufficient specializations to appoint these areas as true gonads (Schmidt-Rhaesa 2007).

In Hydrozoa, the testis are merely accumulations of different gametogenic cells and their interstitial stem cell precursors, covered by unspecialized epidermis of the body column (Thomas and Edwards 1991).

The structures referred to as gonads in Anthozoa are simply gametogenic areas of mesenteries (Fautin and Mariscal 1991); the details of the morphology differ among the orders. Maturation of sperm proceeds peripherically in the sperm follicle (Fautin and Mariscal 1991). All or some mesenteries may be gametogenic, an attribute that has systematic significance. Some are gonochoristic, while others are sequential or simultaneous hermaphrodites. In the latter case, it is possible that both male and female gametes occur in the same mesenterie. Gonadal packets in *Urticina lofotensis* contain the spermatogonia at the periphery of the gonadal packet, while the mature sperm cells are found on the inside (Wedi & Dunn 1983).

Scyphozoan gonads develop from cell aggregations that separate from the gastric gastrodermis and are translocated into the mesoglea immediately adjacent to the gastric filaments (Lesh-Laurie and Suchy 1991). Widersten (1965) distinguishes three layers in the scyphozoan gonad: 1) a layer contiguous with the gastric gastrodermis, 2) an acellular genital mesoglea and 3) a genital ciliated epithelium. Sperm of *Cyanea* develops in follicles, which are formed as cells migrate from the genital epithelium into the genital mesoglea. The follicles retain their continuity with the genital epithelium.

PLATYHELMINTHES

The structure of the testes in members of the Platyhelminthes differs greatly among different taxa.

The testes in species of Catenulida lack wrapping parenchymal cells around differentiating spermatids, although wrapping cells around older germinative cells do exist. Members of the Lecitoepitheliata have sacular[2] gonads. However, the cellular tunica might be incomplete. Testes can have different morphologies: compact and paired testes as in Gnosonesimida, follicular and unpaired testes as in Prorhynchida. Representatives of Prolecitophora show a large variety of male gonadal structures: the testes can be asacular or sacular, mixed with the ovary, or spreaded throughout the parenchyma in groups of germ cells. Hence, the testes can be diffuse or follicular, but without a tunica. Within several parallel evolutionary lines, the formation of a

[2] For definitions: see chapter 5, material & methods

tunica-bounded, compact testis is evident. The principal unit wihin the male gonad appears to be primary follicles, which usually include a cytophore. Testes of Macrostomorpha are compact and sacular in all forms, and are layered by cuboidal or flattened cells. Polyclads have sacular and follicular testes. In the Seriata, gonads are secular: gonads are bound by an epithelium and a thin layer of extracellular matrix. In the Tricladida and some members of the Proseriata, the testes are follicular. In most Rhabdocoela and Temnocephalida, the testes are sacular, paired and compact. They may become secondarily subsivided in a number of follicles (Mesostominae) or enlarged and lobed (Umagillidae) or diffuse. Most Platyhelminthes have an epithelialized canal system (Rieger et al. 1991).

XENOTURBELLA

In *Xenoturbella bocki* Westblad 1949. spermatid clusters occur in the parenchymal or gastrodermal tissues (Obst et al. 2011). So far, no delimited gonads or organs associated with germ cell development have been found in this species.

ECHINODERMATA

Echinoderm testes are found in the perivisceral or genital coelom. They are sac-like, and the gonad wall is composed of several layers. The shape and size of each layer varies according to the season (Riesgo et al. 2011). Spermatogenesis occurs in spermatogenic colums, which consist of a central interstitial cell surrounded by spermatogenic cells that develop along the axis towards the lumen of the testis (Riesgo et al. 2011). This implies that spermatogonia and spermatocytes are found at the base of the columns, while spermatids can be found near the tips of the columns. Mature spermatozoa are located freely in the lumen of the testis. In echinoderms, exogenous nutrition enters the gonads from the perivisceral coelom by the hemal system and accumulates in accessory or auxiliary cells (nutritive phagocytes). These accessory cells provide nutrition to spermatogenic cells (Reunov et al. 2010).

UROCHORDATA

Most species of Urochordata are hermaphroditic. In hermaphrodites, testes and ovaries can be either separate and single or form composite sexual organs. The number of gonads can vary between one to nine (Jorgensen & Lutzig 1997). Each

testis consists of a few to hundreds of pea- or club-shaped testicular follicles, each of which tapers into a narrow vas efferens. The testicular follicles are lined by a non-germinal epithelium; separating germ cells from surrounding connective tissue or haemal sinuses. This epithelium can be ciliated. The cavity within the follicle is filled with a regularly polystratified layer of all stages of spermatogenesis. When spermatozoa are present, they are found in the innermost part of the follicle. A thin basal membrane separated the follicles from the haemal spaces. After the reproductive season, epithelial cells migrate from the follicle wall to the interior of the testis follicle, where they phagocytize male germ cells (Jorgensen & Lutzig 1997).

ACOELA AND NEMERTODERMATIDA

Although it is known that the gonads in Acoela and Nemertodermatida are variable and complex (Rieger et al. 1991), no ultrastructural studies have been performed on the structure of the testis, the complete process of spermatogenesis and how these stages are organized within the testes. Moreover, to asses homology of structures, it is crucial to analyze the individual parts of a complex structure, as homology proposals can be discovered at the ultrastructural level (Ragsdale & Baldwin 2010).

5. AIMS

The main goals of this PhD are:

1) increasing the general and detailed morphological knowledge on Acoela and Nemertodermatida, 2) inferring the evolution of the morphology of the testes in Acoela, and 3) comparing the morphology of the testes of Acoela and Nemertodermatida.

First, we intend to increase the morphological knowledge on Acoela and Nemertodermatida, by performing detailed ultrastructural studies on the testes of different species. It is known that the testes of Acoela and Nemertodermatida can vary greatly in morphology, but details of the structure and organization are lacking, despite their important phylogenetic position. This indespensible fundamental information can also serve as a valuable background for e.g. in situ hybridizations.

The second main goal of this PhD is to trace the evolution of the morphology of the testes in Acoela, by coding the morphological characteristics of the testes and mapping them onto the phylogenetic tree, consequently inferring the character polarity of the analyzed morphological traits.

The third main goal is a comparative study of the male germ line in Acoela and Nemertodermatida and related taxa, in order to shed new light on the phylogenetic position of these two intriguing groups.

CHAPTER 2: FIRST RECORD OF NEMERTODERMATIDA FROM BELGIAN MARINE WATERS

Modified from:

Boone M., Houthoofd W., Bert W., Artois T. (2011). First record of Nemertodermatida from Belgian marine waters. Belgian Journal of Zoology 141 (1): 62-64.

1

BACKGROUND

Collecting specimens is evidently the first step to perform morphological studies. This was done by collection of new material as well as analyzing existing material in order to cover as many species as possible. One of the sampling campaigns took place in the Belgian part of the North Sea, and the results of this campaign are bundled in this publication.

INTRODUCTION

Nemertodermatida is a small taxon of marine worms, comprising only eight described species. They are characterised by a nemertine-like epidermis (hence the name) and a statocyst containing (mostly) two statoliths (Karling 1974). The phylogenetic relationships of the taxon have been (and still are) the subject of debate. Initially, they were placed within the acoel Platyhelminthes (Steinböck 1930), but Karling (1940, 1974) considered them to be substantially different from the acoels in morphology of the gut and the statocyst, and placed the taxon Nemertodermatida outside the Acoela (Karling 1940). In the first phylogenetic analysis of the Platyhelminthes by Ehlers (1985), Nemertodermatida formed a sister group relationship with Acoela, together forming the taxon Acoelomorpha, at that time considered to be the sister group of Rhabditophora (Ehlers 1985). This view was followed in most subsequent morphological studies (for a review: see Lundin 1997). Smith *et al.* (1986) suggested the possible polyphyly of the taxon Platyhelminthes, based on the absence of clear synapomorphies, and viewed the Acoelomorpha as one of the three well-defined monophyletic groups within the Platyhelminthes, the two others being Catenulida and Rhabditophora. Recent molecular phylogenetic studies (Jondelius et al. 2002, Ruiz-Trillo et al. 2002) corroborate the polyphyly of the Platyhelminthes and show the Nemertodermatida and the Acoela in a basal position within Bilateria. The presence of the small Hox cluster in both Acoela (Cook et al. 2004) and Nemertodermatida (Jimenez-Guri et al. 2006) is concordant with this phylogenetic position. Based on analyses of 18S rRNA and mitochondrial genes (Jondelius et al. 2002) and 18S and 28S rRNA sequences (Wallberg et al. 2007), the taxon Acoelomorpha was dismissed because it appeared to be paraphyletic; Nemertodermatida and Acoela are separate branches at the base of the Bilateria. A recent phylogeny based on a large number of taxa and multiple nuclear genes, however, provides support for a monophyletic

Acoelomorpha, albeit also positioned at the base of the Bilateria (Paps et al. 2009). Because of this basal phylogenetic position, the study of Nemertodermatida is important for the understanding of the characteristics of the bilaterian ancestor (Wallberg et al. 2007) and it makes them important study-objects for morphologists and molecular biologists alike. Note that the latest study on the phylogenetic position of Acoelomorpha (Philippe et al. 2011) challenges their basal position and places them within the Deuterostomia, together with *Xenoturbella*. In the most recent comprehensive taxonomic overview of Nemertodermatida (Sterrer 1998), Sterrer revealed considerable intraspecific variation for characters such as size, colour, presence/absence of glands, etc. Hence, species delimitation is often problematic and also cryptic diversity cannot be excluded, which makes nemertodermatids also interesting from a (molecular) taxonomical point of view. Regrettably, Nemertodermatida are known from a few distinct sampling spots only: the Swedish west coast, the east coast of North America, the Adriatic and the Mediterranean, which suggests that representatives of the taxon are difficult to collect.

The Belgian Continental Shelf, situated in the southern bight of the North Sea and characterised by a mainly sandy substrate, has not previously been sampled for turbellarians specifically, in contrast to the sandy beaches of the Belgian coast, the turbellarian fauna of which is very well known (Schockaert et al. 1989, Vandepitte et al. 2010).

MATERIAL & METHODS

During a two-year period (2007 - 2009), researchers from the UHasselt and UGent sampled the Belgian continental shelf, in order to investigate the turbellarian fauna of sublittoral sandy sediments. The samples were taken with the research vessel “Zeeleeuw” of the Flanders Marine Institute (VLIZ) in different seasons: autumn (October 2007), winter (December 2007 and January 2008), summer (July 2008 and September 2008) and spring (March 2009). Sublittoral sand samples were collected with a Van Veen grab, after which the samples were left to rest for a few days. Afterwards, subsamples were scooped and animals were extracted from these subsamples following the $MgCl_2$ method (Sterrer 1971). Animals were collected under a stereomicroscope and identified using an Olympus BX 51 microscope.

RESULTS

In these samples, a total of 60 specimens of Nemertodermatida were discovered, occurring in 10 sampling stations (Fig. 1).

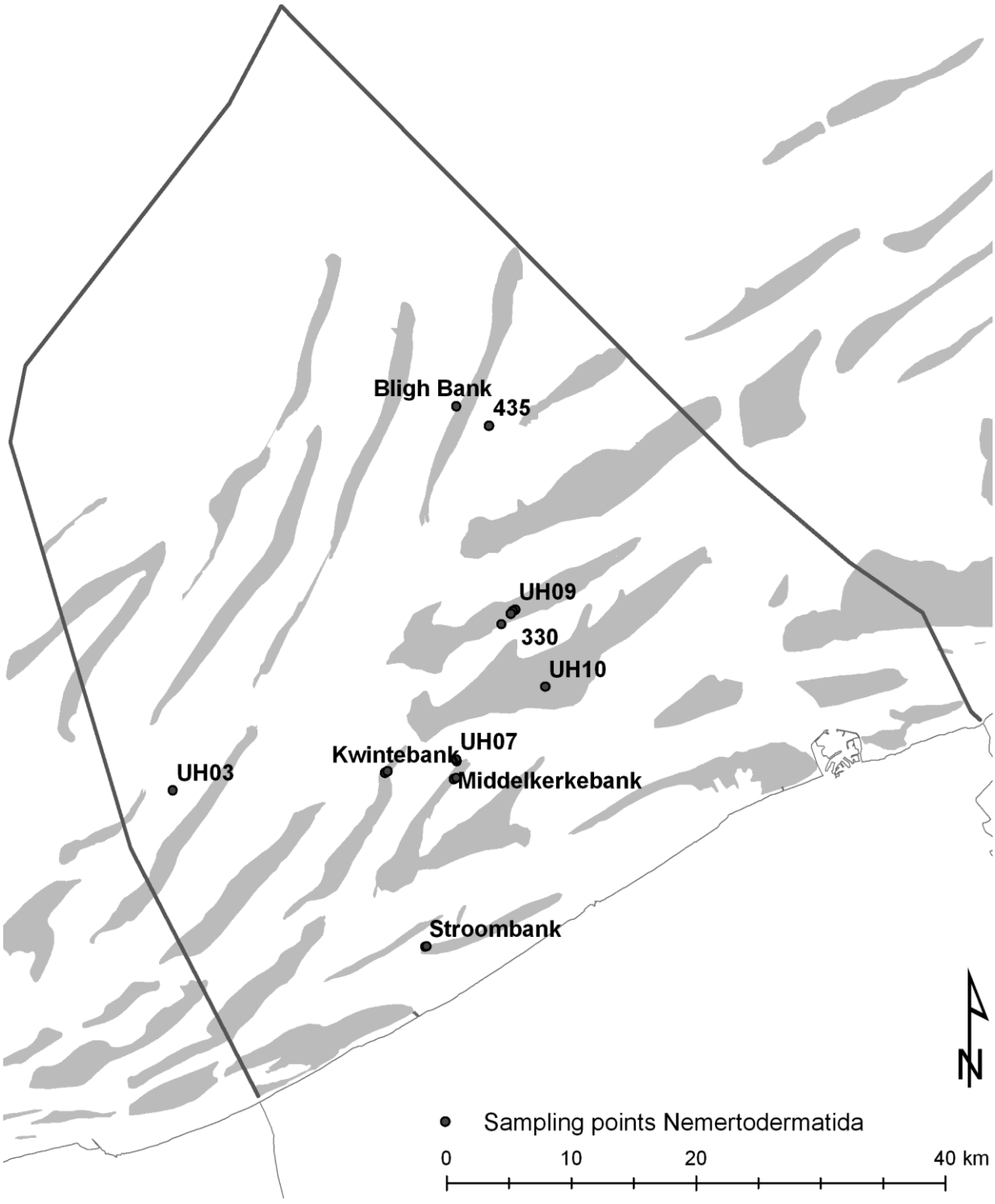

Figure 1: Map of the Belgian Continental Shelf and its sandbanks (dark grey) with the locations of the Nemertodermatida records (black dots).

Three different species representing both families of Nemertodermatida could be discerned (already mentioned in Vandepitte et al. 2010): *Sterreria psammicola* (Sterrer, 1970) Lundin, 2000 (fam. Nemertodermatidae), *Nemertinoides elongatus* Riser 1987 (fam. Nemertodermatidae) and *Flagellophora apelti* Faubel & Dörjes 1978 (fam. Ascopariidae). Table 1 gives an overview of the sampling sites showing the number of specimens of each species at each of the sampling sites, depth and date of the sampling. In total, 11 specimens of *N. elongatus*, 2 specimens of *S. psammicola* and 20 specimens of *F. apelti* were found. In addition, 27 specimens were found that could not be identified, either because they were fragmented or not yet (fully) mature. Sterrer (1998) also mentioned these same problems concerning species identification.

Date	Sampling site	Latitude	Longitude	Depth	Species			
					A	B	C	D
17/10/2007	330	51°26.111	2°48.565	23m	2			
17/10/2007	Bleigh Bank	51°35.475	2°45.120	sublittoral				2
17/10/2007	435	51°34.662	2°47.424	31-33m				1
30/10/2007	UH09	51°26.772	2°49.500	17-19m	4		5	4
30/10/2007	UH07	51°20.272	2°45.641	16m				1
30/10/2007	UH10	51°23.471	2°51.677	15m			3	4
17/12/2008	330	51°26.111	2°48.565	22-24m	2	1		4
21/01/2008	Kwintebank	51°19.635	2°40.737	sublittoral				2
21/01/2008	Middelkerkebank	51°19.411	2°45.509	12m			2	
21/01/2008	Stroombank	51°12.174	2°43.799	10m		1		2
03/07/2008	UH07	51°20.272	2°45.641	16m			3	2
01/09/2008	Middelkerkebank	51°19.411	2°45.509	sublittoral				1
01/09/2008	UH09	51°26.772	2°49.500	20m			2	1
17/03/2009	UH09	51°26.772	2°49.500	21m	2			
17/03/2009	UH07	51°20.272	2°45.641	20-21m	1		3	2
17/03/2009	UH03	51°18.638	2°36.126	18-23m			2	1
		Subtotal			11	2	20	27
		Total number: 60						

Table 1: overview of the sampling dates, the sampling sites with their depth and the number of each species found at every site. Species A: *Nemertinoides elongatus*, species B: *Sterreria psammicola*, species C: *Flagellophora apelti*, species D: unknown / unidentifiable.

DISCUSSION

Despite the fact that the Belgian Continental Shelf is the subject of many scientific studies, this is the first record of Nemertodermatida in this area, thus expanding their known geographical distribution. An overview of the worldwide distribution of each of the three species can be found following these links:

Sterreria psammicola:
http://maps.google.nl/maps/ms?ie=UTF8&hl=nl&msa=0&msid=100800064963713004739.00046b093b8f2cfa9dfa0&ll=10.983886,-87.553265&spn=173.668929,360&z=1
Nemertinoides elongatus:
http://maps.google.nl/maps/ms?ie=UTF8&hl=nl&msa=0&msid=100800064963713004739.00046af8c2fae451945c7&z=3
Flagellophora apelti:
http://maps.google.nl/maps/ms?ie=UTF8&hl=nl&msa=0&msid=100800064963713004739.0004694f3788119941f72&ll=10.983886,-87.553265&spn=173.668929,360&z=1

The obvious explanation for the fact that Nemertodermatida have not been discovered earlier in the Belgian part of the North Sea is the lack of study on these animals in this area (and many other areas worldwide). Their occurrence in this area is, however, not unexpected, since the investigated sediments are of the same type as of the records elsewhere in the world. All three species have been found earlier in sandy bottoms, ranging from fine sand to coarse sand containing shell gravel (Tyler et al. 2006), and this sediment is the main sediment type on the Belgian Continental Shelf. As to depth (see table 1), the records for *F. apelti* in Belgium fall in the depth range of 2m to 400m mentioned in earlier literature (Sterrer 1998), while we found specimens of *S. psammicola* and *N. elongatus* deeper than was known from earlier records (Sterrer 1998). The presence of the genus *Ascoparia* Sterrer, 1998, a genus also occurring in sandy substrates, could not be proven in this study, but given the large fraction of unidentifiable specimens (27 out of 60), we cannot rule out their occurrence in this part of the North Sea. Further research is needed to determine whether the species typical of soft, muddy bottoms (*Nemertoderma bathycola* Steinböck, 1930 and *N. westbladi* Steinböck, 1938) are present on the Belgian Continental Shelf. This present study indicates that nemertodermatids could be present in many more localities than their known distribution suggests.

ACKNOWLEDGEMENTS

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CHAPTER 3: SPERMATOGENESIS AND THE STRUCTURE OF THE TESTES IN NEMERTODERMATIDA

Modified from:

Boone M., Bert W., Claeys M., Houthoofd W., Artois T. (2011). Spermatogenesis and the structure of the testes in Nemertodermatida. Zoomorphology 130: 273-282.

1

BACKGROUND

After sampling (chapter 2), we performed detailed ultrastructural studies on the testes of two species of Nemertodermatida: *Flagellophora apelti* and *Nemertoderma westbladi*. The results of these studies were concentrated in a paper, which was submitted for publication on 22 April 2011 to Zoomorphology, and accepted for publication in September 2011.

ABSTRACT

The ultrastructure of the testes in two representatives of the enigmatic taxon Nemertodermatida was studied using transmission electron microscopy. *Nemertoderma westbladi* has paired testes, which are delineated by lining cells. Within each testis, different follicles, each surrounded by a membrane-like structure, are found. *Flagellophora apelti* has genuinely follicular testes, consisting of several follicles, each containing a certain stage of spermatogenesis. As the gametes are not enclosed by a structure that can be called a true gonad, the structure of the testes differs from most bilaterian animals, but resembles the organization of gametogenic areas of ctenophores. Each stage of spermatogenesis in *F. apelti* is described, enabling the inference of the origin of the structures seen in mature spermatozoa. The overall structure of the mature spermatozoa is similar in all nemertodermatids and unique within the Metazoa: an elongated head containing the nucleus; a middle piece containing an axoneme, mitochondrial derivatives and in *F. apelti* granular derivatives; and a flagellar tail.

INTRODUCTION

Nemertodermatida is a small taxon of marine organisms whose most striking feature is a statocyst containing (mostly) two statoliths. Members lack protonephridia, and their well-defined, sac-like gut has only a single opening—the mouth.

The most recent study on the phylogenetic position of Nemertodermatida, based on three independent datasets, places Nemertodermatida together with Acoela in a taxon Acoelomorpha, which shows a sister group relationship with *Xenoturbella*; the clade of Acoelomorpha. *Xenoturbella* is found within Deuterostomia as the sister group of the Ambulacraria (Philippe et al. 2011). However, the phylogenetic position of Nemertodermatida is far from settled. Originally considered to be part of the Acoela (Steinbo¨ck 1930), it was later seen as a separate taxon, Nemertodermatida, sister group of Acoela, with which it formed the taxon Acoelomorpha within the Plathelminthes (Ehlers 1985). Studies based on 18S small subunit rRNA and mitochondrial genes (Jondelius et al. 2002), on the nuclear protein coding myosin heavy chain type II (Ruiz- Trillo et al. 2002), on 18S rRNA and 28S rRNA genes (Wallberg et al. 2007) and on a combination of several molecular markers (Bagun˜a` et al. 2008) all show a paraphyletic Acoelomorpha, with Nemertodermatida and Acoela appearing as separate branches at the base of the Bilateria. In a recent study, however, based on phylogenomic methods, Acoelomorpha is monophyletic and is the sister taxon of the Nephrozoa (all remaining Bilateria) (Hejnol et al. 2009; Edgecombe et al. 2011). Hejnol et al. (2009) also suggested a sister group relationship between Acoelomorpha and the enigmatic taxon *Xenoturbella*. Complete mitochondrial genome data of an acoel also support the view that the Acoelomorpha are the early diverging extant lineage of Bilateria (Mwinyi et al. 2010). Although the exact phylogenetic position of Nemertodermatida is still debated, it is clear that they are one of the key taxa to understanding early bilaterian evolution and, more specifically, the evolution of early bilaterian morphology.

Studies on different aspects of the morphology of Nemertodermatida have already been conducted: on the parenchyma (Rieger et al. 1991), the nervous system (Raikova et al. 2000, 2004; Reuter et al. 2001), the mature sperm (Tyler and Rieger 1975, 1977; Hendelberg 1977; Lundin and Hendelberg 1998), the epidermis (Tyler and Rieger 1977; Lundin and Hendelberg 1995, 1996; Lundin 1997, 1998, 2001), the

proboscis (Rieger et al. 1991) and the gut (Tyler and Rieger 1977). A taxonomic revision of the Nemertodermatida was made by Sterrer (1998). In order to resolve internal relationships within the Nemertodermatida, Lundin (2000) conducted a cladistical analysis using 72 morphological characters. A slightly altered version of this cladistical analysis was published by Lundin and Sterrer (2001). Although the adult body plan of Nemertodermatida is already relatively well documented, many aspects remain unknown, such as the ultrastructure of the testes and spermatogenesis. Lundin and Hendelberg (1998) described the spermiogenesis in *Meara stichopi* Westblad 1949 (Nemertodermatidae), but not the early stages, while Rieger et al. (1991) illustrated the position of the male and female germinative zone in *Flagellophora apelti* Faubel and Dörjes 1978.

Here, we give ultrastructural details on the morphology of the testis in *F. apelti* and in *Nemertoderma westbladi* Westblad 1937, two representatives of different nemertodermatidan subgroups. We describe the mature spermatozoon of both species in detail. For *F. apelti*, moreover, we describe the complete spermatogenesis. Our material of *N. westbladi* did not allow such a study for this species. The results of this study will allow a comparison with spermiogenesis and testis morphology in other taxa.

MATERIAL AND METHODS

Specimens of *F. apelti* were extracted from sand samples collected with a Van Veen grab on the Belgian Continental Shelf at depths of 12–23 m in 2007 and 2008. Specimens of *N. westbladi* were extracted from mud samples collected around Essvik, Kristineberg, Sweden in 2008. The collected specimens of both species were fixed in a mixture of glutaraldehyde fixative and osmium fixative (Eisenman and Alfert 1982) for approximately 10 min at 4°C. The glutaraldehyde fixative was 4% glutaraldehyde in buffer a (100 mL: 0.2 M sodium cacodylate with 0.58 g NaCl and 11.97 g sucrose; pH 7.2), and the osmium fixative was 1% osmium tetroxide in buffer b (100 mL: 0.2 M sodium cacodylate with 3.48 g NaCl; pH 7.2). After fixation with the cocktail, the specimens were fixed in glutaraldehyde fixative for 1 h at 4°C and then post-fixed with osmium fixative for 1 h at 4°C. After rinsing in buffer b for 10 min at 4°C and rinsing for 5 min in double distilled water, they were dehydrated, using ethanol of increasing concentrations. After dehydration in an ethanol series,

specimens of both species were subsequently infiltrated with a low-viscosity embedding medium (Spurr 1969) then polymerized at 70°C for 8 h. Semi-thin sections of 1 and 2 lm and ultra-thin sections of 60 nm were made on a Reichert-Jung Ultracut E or a Leica-Reichert Ultracut S (Leica, Vienna, Austria) equipped with a diamond knife. Specimens were sectioned semi-thin until the region of interest was reached, after which ultra-thin sections were made. Ultra-thin sections were post-stained with uranyl acetate (40 min at 20°C) and lead citrate (10 min at 20°C) and studied with a Jeol JEM-1010 transmission electron microscope (Jeol Ltd., Tokyo, Japan) operating at 60 kV. Photomicrographs were digitized using a Ditabis system (Pforzheim, Germany). Schematic figures were drawn from photomicrographs using Adobe Illustrator CS software.

RESULTS

STRUCTURE OF THE TESTES

The single testis in *F. apelti* (Fig. 1a) is completely separated from the ovary. The testis is follicular: it comprises primary follicles, in which cells are densely packed. Although the cells in a follicle are lying closely together, no intercellular bridges could be observed. The different follicles are not grouped within a saccular structure but lie scattered in the posterior part of the body, spatially separated from each other. They are not surrounded by specialized cells, but lie adjacent to gut cells, stromal cells, and parenchymal cells. Within each follicle, only a single stage of spermatogenesis can be found. Different testicular follicles contain successive stages of spermatogenesis, those containing early stages (spermatogonia and spermatocytes) being found more anteriorly than those containing later stages (spermatids and mature spermatozoa).

The testes in *N. westbladi* (Fig. 1b) are paired, and situated dorso-laterally from the ovaries, partly enfolding them. The testes are clearly delimited by flattened lining cells which have a large nucleus with a prominent nucleolus; the cytoplasm of these lining cells is packed with networks of rough endoplasmatic reticulum. Within each testis, separate follicles can be found, in which cells of the same stages or spermatogenesis are lying closely together. Each follicle is surrounded by a

membrane-like structure; which is presumably derived from the testes lining cells. Different stages of spermatogenesis can be found in neighboring follicles.

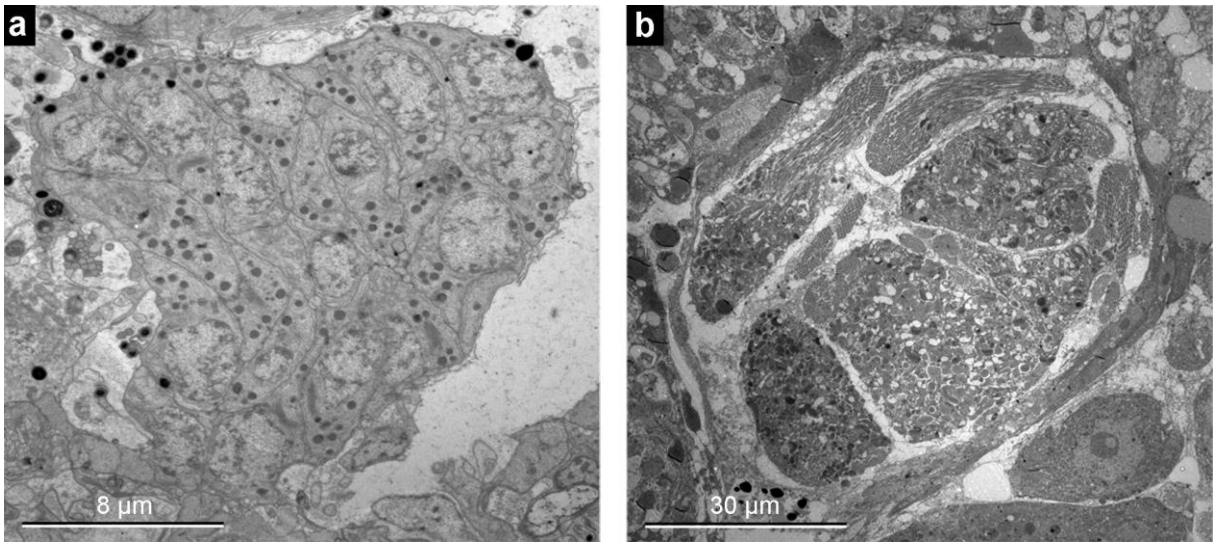

Fig. 1 Structure of the testes in Nemertodermatida. **a**: TEM image of part of the testis in *Flagellophora apelti*: a testis follicle containing secondary spermatocytes; **b**: TEM image of one of the two testes in *Nemertoderma westbladi*, the testis contains several delineated follicles.

SPERMATOGENESIS IN FLAGELLOPHORA APELTI

THE SPERMATOGONIUM (FIG. 2A)

Typical of these cells is the high nucleo-cytoplasmic ratio. The nucleus has a nucleolus and small clumps of heterochromatin. The cytoplasm contains ribosomes, a Golgi complex, mitochondria and a pair of centrioles. The centrioles are arranged perpendicularly, forming a diplosome, which is located in proximity to the nucleus.

THE PRIMARY SPERMATOCYTE (FIG. 2B)

Primary spermatocytes have a relatively undifferentiated nucleus and cytoplasm. The nucleus has a round to ovoid shape and takes up a large part of the cell. It contains few clumps of heterochromatin and synaptonemal complexes, the latter indicating that the cells are in prophase I (Fig. 2b, arrowhead). The cytoplasm contains mitochondria, ribosomes, a Golgi complex, some swollen endoplasmatic reticulum and a pair of centrioles close to the nucleus. There are approximately 16–20 primary spermatocytes in a follicle.

THE SECONDARY SPERMATOCYTE (FIG. 2C & 2D)

Secondary spermatocytes have nuclei that still have a round-ovoid shape but contain more heterochromatin than nuclei of primary spermatocytes. The heterochromatin is more condensed than in earlier stages. As in the primary spermatocytes, several small mitochondria, ribosomes, swollen endoplasmatic reticulum, a Golgi complex, and a perpendicularly organized pair of centrioles (Fig. 2c) can be found. The large Golgi complex and the pair of centrioles are located in proximity to the nucleus. In the cytoplasm of the secondary spermatocyte, there are also vesicles containing large, electron-dense granules. In this stage, each vesicle contains one electron-dense granule; in some of the granules, more electron-dense structures can be found. A follicle of secondary spermatocytes contains usually between 32 and 40 cells.

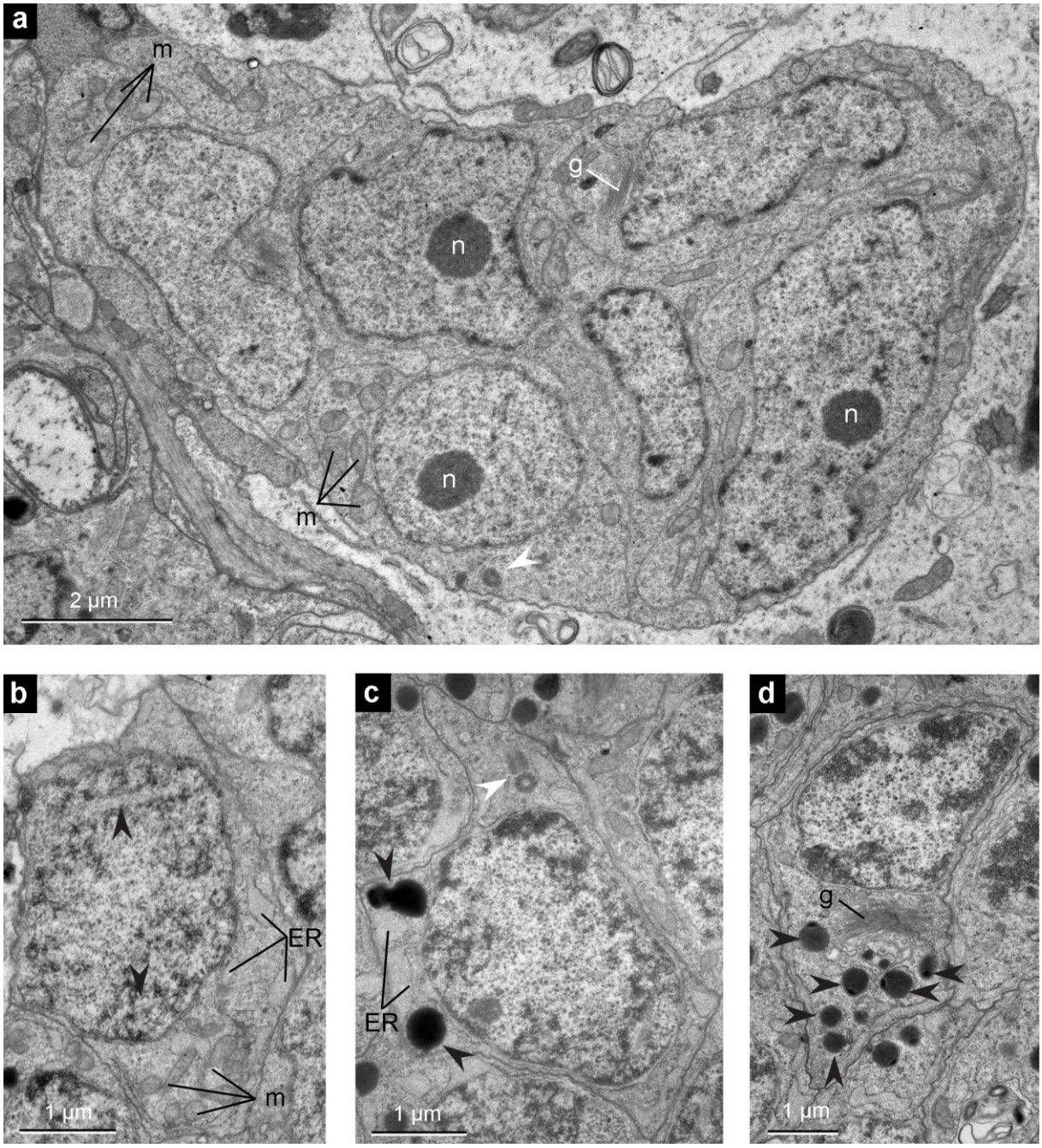

Fig. 2 Early stages of spermatogenesis in *F. apelti*. **a**: spermatogonia. A nucleolus (n) can be seen in the nucleus. The cytoplasm contains ribosomes, Golgi complexes (*g*), a pair of centrioles (*white arrowhead*) and mitochondria (*m*). **b**: primary spermatocyte. The large nucleus contains scattered clumps of heterochromatin and synaptonemal complexes (*arrowhead*). The cytoplasm contains Golgi complexes, mitochondria (*m*), ribosomes and swollen endoplasmatic reticulum (*ER*). **c** & **d**: secondary spermatocytes. The large nucleus contains scattered clumps of heterochromatin. Centrioles (*white arrowhead*), electron-dense granules (*black arrowheads*), a Golgi complex (*g*), mitochondria, ribosomes, and swollen endoplasmatic reticulum (*ER*) are all found in the cytoplasm.

THE SPERMATID (FIG. 3)

Elongation of the cell starts after the second meiotic division and its round-ovoid shape is becoming more elongated during the spermatid stage. The round nucleus, containing scattered clumps of heterochromatin in the early stages (Fig. 3a), changes shape to an electron-dense structure (Fig. 3b, c), which will elongate. Various organelles can be found in the cytoplasm: mitochondria, a large Golgi complex, ribosomes and swollen endoplasmatic reticulum. The Golgi complex is found in the vicinity of the nucleus. The electron-dense granules are more grouped in this early spermatid stage; the multiple vesicles each containing a single granule fuse so that in the late spermatid, only one clearly delimited vesicle can be found, containing multiple granules (Fig. 3a, c, black arrowhead). The mitochondrion starts to elongate: the shape changes from ovoid to more elongated (Fig. 3a, d, white arrowhead). In most cases, a single axoneme grows outwards within the elongating spermatid from a centriolar region that remains near the nucleus (Fig. 3b). Aberrant spermatids with two axonemes were observed, but we never found more than one aberrant spermatid (Fig. 3c) per follicle. Follicles of spermatids contain approximately 64–80 cells.

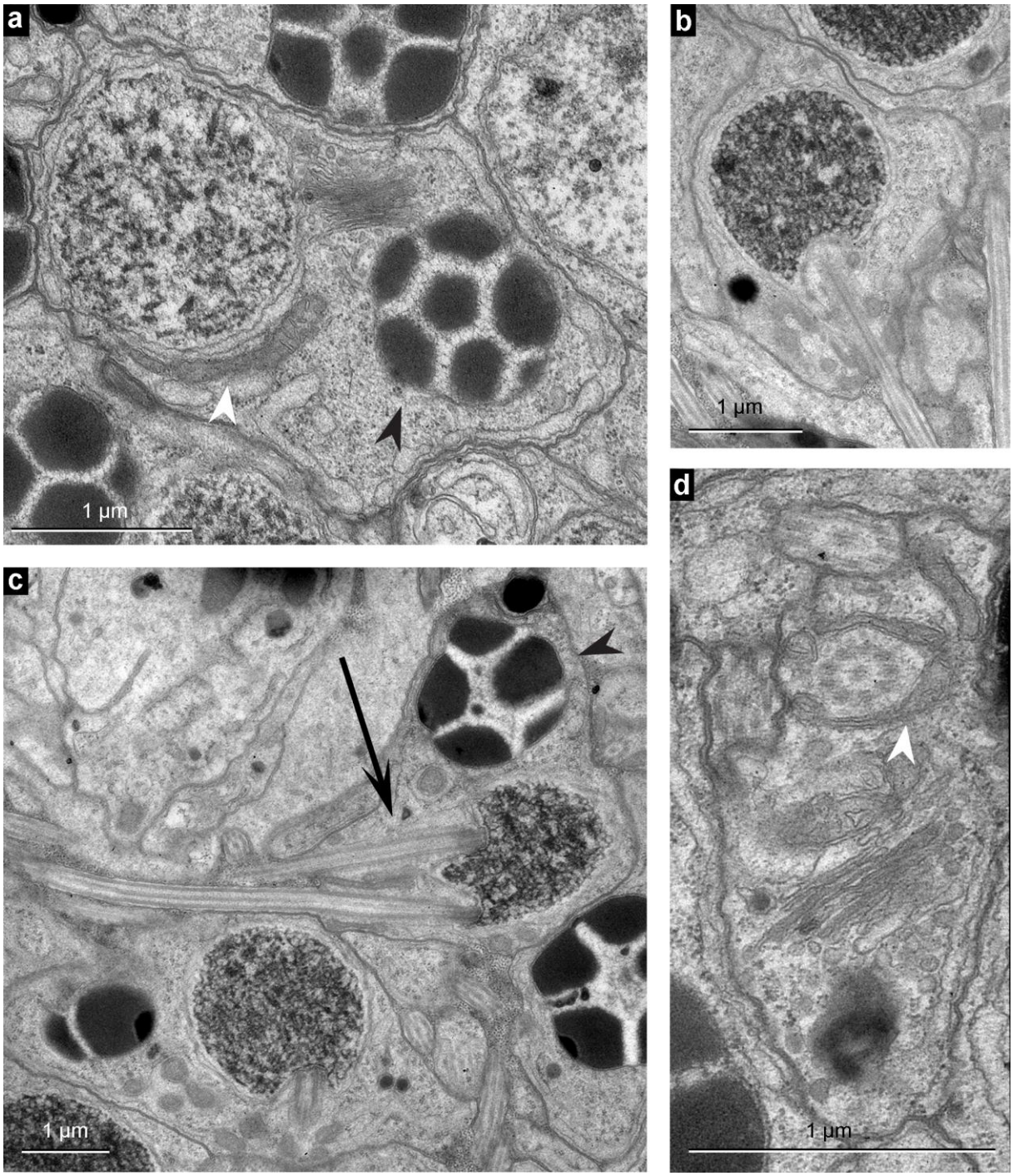

Fig. 3 Spermatids in *F. apelti*. **a**: early spermatid. The nucleus contains scattered clumps of heterochromatin, the mitochondria (*white arrowhead*) are more elongated, the electron-dense granules are grouped (*black arrowhead*) and the endoplasmatic reticulum is swollen. Note the Golgi complex near the nucleus. **b**: the nucleus in a later spermatid stage is more electron-dense and a single axoneme grows outwards from a centriolar region that remains near the nucleus. **c:** aberrant spermatid with two axonemes (*arrow*). Note the grouped electron-dense granules (*black arrowhead*). **d**: Section of a spermatid where the elongated shape of the mitochondrion is visible (*white arrowhead*).

THE MATURE SPERMATOZOON *F. APELTI* (FIG. 4A, B, C AND 5A)

The mature spermatozoon in *F. apelti* is divided into three regions: a head, a middle piece and a tail.

The head is tipped with an acrosome (Fig. 5a) and contains the electron-dense nucleus, which shows a single helical groove along its entire length. The nucleus is approximately 6 µm long and has a diameter of 0.3 µm. The helical groove has a depth of 0.12 µm; there are 4 revolutions per µm.

The middle piece measures approximately 5.5 µm and contains the basal body where the axoneme is attached, the axoneme itself, mitochondrial derivatives (Fig. 4b, c, black arrowheads), and 8–10 electron-dense granular derivates (Fig. 4a, b, white arrowheads). Peripheral fibers are found on the outside of the microtubule doublets in the basal region of the axoneme. The granular derivatives are 0.4–0.6 µm long and 0.35–0.4 µm wide and partly surround the axoneme. They contain an ovoid substructure, which is more electron-dense. In cross sections, the granular derivatives are bean-shaped and cover about half of the section; the other half is covered by the axoneme and the mitochondrial derivative. The mitochondrial derivatives are elongated and flattened. They occur at the same level as the granular derivatives, but extend more at the distal end.

The single axoneme has a 9 x 2 + 2 pattern, and its free part extends as a flagellar tail. Toward the distal tip, the 9 x 2 + 2 structure disintegrates.

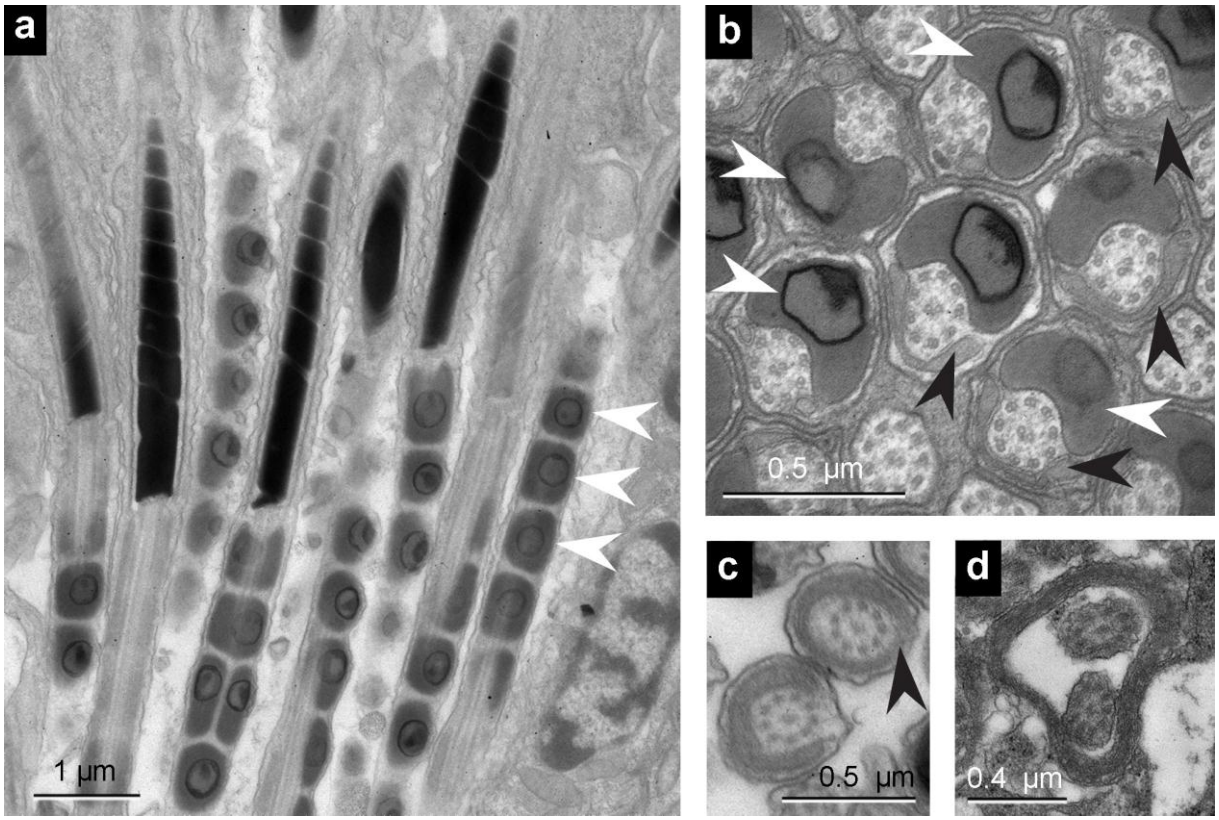

Fig. 4 Mature spermatozoa in the testis of *F. apelti* (**a-c**) *and N. westbladi* (**d**). **a**: longitudinal sections of mature sperm in *F. apelti*. The nucleus with a single helical groove is localized in the head. The middle piece contains an axial filament, mitochondrial derivatives and granular derivatives (*white arrowheads*). **b**: transverse sections of the mature sperm cell of *F. apelti,* including mitochondrial derivatives (*black arrowheads*) and granular derivatives (*white arrowheads*). **c**: the mitochondrial derivatives at the posterior end of the middle piece of the sperm cell in *F. apelti* have a tubular shape (*black arrowhead*). **d**: middle piece of an aberrant spermatozoon in *N. westbladi*. Two axial filaments are surrounded by one mitochondrial derivative.

MATURE SPERMATOZOON IN *N. WESTBLADI* (FIG. 4D AND 5B)

Figure 5b gives a schematic overview of the mature sperm in *N. westbladi*. Normal spermatozoa in *N. westbladi* have an elongated, electron-dense nucleus, a middle piece containing an axial filament surrounded by a tubular mitochondrial derivative and a tail (free axial filament). We occasionally found aberrant spermatozoa with two axonemes instead of one in their middle piece and tail, surrounded by a single mitochondrial derivative (Fig. 4d).

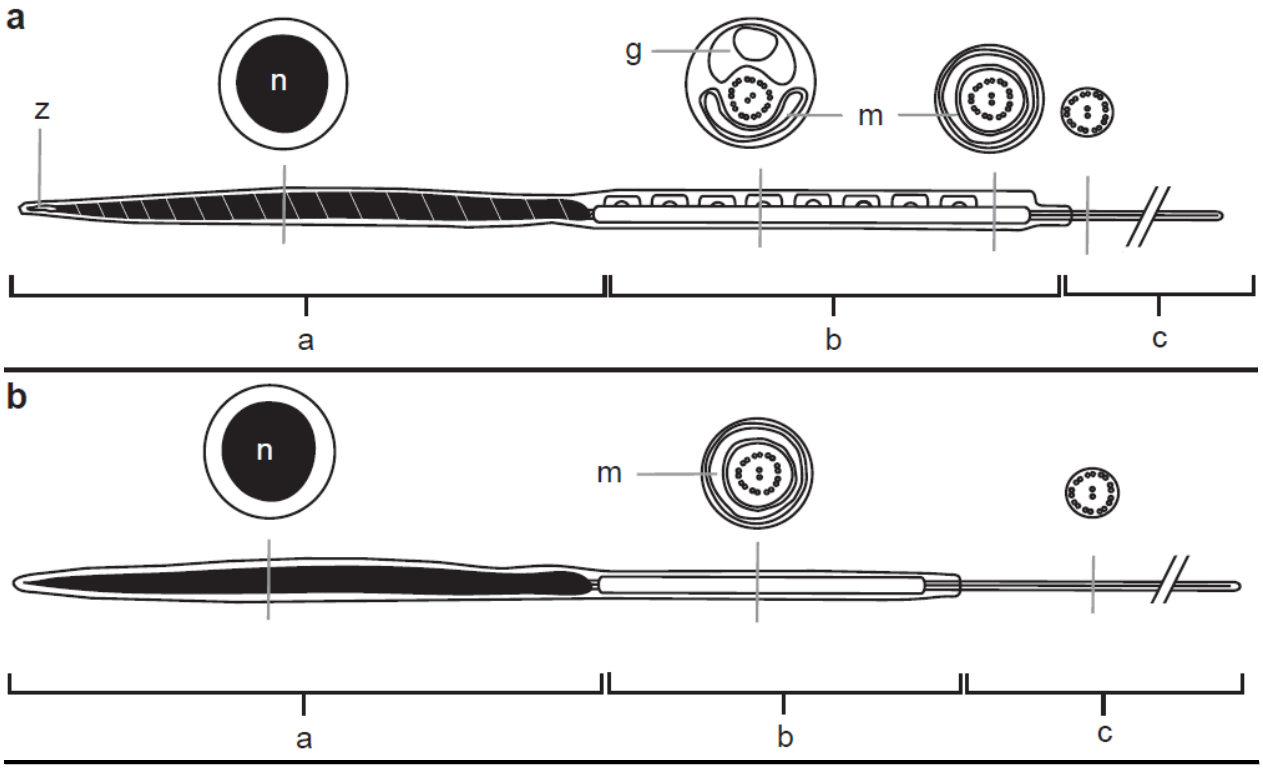

Fig. 5 Schematic representation of the mature sperm in *Flagellophora apelti* (**a**) and *Nemertoderma westbladi* (**b**). *a* = head, *b* = middle piece, *c* = tail, *g* = granular derivative, *m* = mitochondrial derivative, *n* = nucleus, *z* = acrosome

DISCUSSION

STRUCTURE OF THE TESTES

Gonads can be defined in different ways. According to the definition of Schmidt-Rhaesa (2007), gonads are organs bordered by epithelia and surrounding the gametes. This definition excludes cases in which gametes are present between epithelia or between parenchymal cells (Schmidt-Rhaesa 2007).

As is the case for most other Bilateria, Nemertodermatida are generally known to have lining cells around the gonads. Tyler and Rieger (1977) found a clear epithelium delimiting the gonads in *N. westbladi*, which they considered an advanced character. In the cladistical analysis by Lundin and Sterrer (2001), all studied species are considered to have testes lined by cells (for *Ascoparia neglecta* Sterrer 1998 and *Ascoparia secunda* Sterrer 1998 no data were available). The testes in *M. stichopi* consist of individual follicles surrounded by flattened follicle wall cells (Lundin and Hendelberg 1998). It is possible that these flattened follicle wall cells have microvilli-

like protrusions, which have a nutritive function (Lundin and Hendelberg 1998). The follicles in *M. stichopi* are found in two rows in the anterior part of the body.

However, in this study, we observed clear differences in the lining of the testes between the species studied. In *N. westbladi*, the testes are paired and clearly delimited by cells. Within each testis, the follicles are surrounded by presumable remnants of the testis lining cells. The origin of the lining cells of the testis in unknown, and therefore, it is not possible to state if this species has true gonads in the sense of Schmidt-Rhaesa (2007). *F. apelti* has an unpaired, follicular testis in the posterior part of the body; each follicle is densely packed and encompassed by surrounding gut and stromal cells, but no lining cells unite the individual follicles. Consequently, *F. apelti* lacks true testes. Nevertheless, for clarity's sake, we used the term ''testes'' in this paper.

In animals at a more basal phylogenetic position, such as cnidarians and ctenophores, gonads are not separate organs, and some authors prefer to call these concentrations of gametes ''gametogenic areas'' (Schmidt-Rhaesa 2007). The germ cells in cnidarians are generally found in interstitial positions of the body tissue which, without the germ cells or before they arise, exhibit no reproductive specialization (Campbell 1974). Ctenophora also lack true lining cells around their testes. The testicular compartments in this taxon are delineated by processes of surrounding cells, and each of the compartments contains cells differentiating synchronously (Pianka 1974; Hernandez-Nicaise 1991). In these aspects, the testes structure of Ctenophora is comparable to what we observed in *F. apelti*. But while the testicular follicles in Ctenophora are lying concentrated in the vicinity of the meridional canal, the follicles in F. apelti are lying scattered in the posterior part of the body.

A ''true'' gonad is known for most Bilateria, except some species of Catenulida (Platyhelminthes), Acoela and Xenoturbella (Schmidt-Rhaesa 2007). No delimited gonads or organs associated with germ cell development have been found so far in *Xenoturbella bocki* Westblad 1949. Instead, spermatid clusters occur in the parenchymal or gastrodermal tissues (Obst et al. 2011).

Detailed studies of the ultrastructure of the testes and the organization of the different stages of spermatogenesis within the testes of species of Acoela are scarce. The male gonad of *Isodiametra pulchra* Smith and Bush (1991) was studied in detail, and

this species has paired, compact, nonfollicular testes where the early stages of spermatogenesis occur on the outer, dorsal periphery and the later stages on the inner, ventral side (Boone et al. 2011). No clear lining cells surround the testes. The structure of the testes in species of Acoela varies greatly: testes can be saccular or asaccular, mixed with the ovary or separate, follicular or non-follicular, compact or diffuse (Rieger et al. 1991). Further ultrastructural research on Acoela is needed to determine whether there are similarities in the structure of the gonads in Acoela and Nemertodermatida.

SPERMATOGENESIS

The complete process of spermatogenesis in a species of Nemertodermatida has never been described. Lundin and Hendelberg (1998) did give a detailed description of spermiogenesis in *M. stichopi*; however, they did not cover the early stages of spermatogenesis nor mention the positions of developmental stages within the testes.

In both *M. stichopi* and *F. apelti*, the nucleus transforms from a large, heterogeneously electron-dense appearance, to an elongated and homogeneously electron-dense shape. In *M. stichopi*, two mitochondria start to elongate and coil around the axoneme during the spermatid stage. In *F. apelti*, we observed an elongation but no coiling. Lundin and Hendelberg (1998) noted the presence of vacuoles filled with granules or tiny vesicles of medium electron density in spermatids of *M. stichopi*, which disappear during spermiogenesis. In F. apelti, electron-dense granules appear during the secondary spermatocyte stage, and these granules group during the spermatid stage. In the mature spermatozoon, they are found as granular derivatives.

While spermatogonia and primary spermatocytes are similar in acoelans (Boone et al. 2011) and nemertodermatids, the first differences appear in secondary spermatocytes, with the appearance of species-specific granules in the cytoplasm. Spermatids also differ between the two taxa, as the major morphological changes toward the mature spermatozoon take place. In acoelans, accessory microtubules appear, the nucleus starts to elongate, different granules and ovoid-shaped mitochondria can be found (Raikova and Justine 1994, 1999; Raikova et al. 1997; Boone et al. 2011). The two flagella start their incorporation; their orientation is

inverted. So the overall morphology and morphogenesis of spermatids in Acoela is very different from those of *F. apelti*. In *Xenoturbella bocki*, no studies of spermatogenesis have yet been conducted.

MATURE SPERMATOZOON

Tyler and Rieger (1975, 1977) studied the sperm of *Nemertoderma* sp. A, which was later identified as *F. apelti* (Sterrer 1998). They described the general structure of the sperm cell: an elongated nucleus in the head, a middle piece containing an axial filament and mitochondria, and a flagellum with 9 x 2 + 2 arrangement of the microtubules extending as a tail. Tyler and Rieger (1975) described six to eight crescent-shaped bodies with vesicular structures inside the middle piece as ‘‘presumably mitochondrial derivatives’’. However, our study shows that the mitochondrial derivatives in the middle piece are not the crescent-shaped bodies, but thin and elongated structures in which the inner and outer membranes as well as the remnants of the cristae can be discerned. We observed the initiation of the elongation process of the mitochondria during the spermatid stage and found no indication that the mitochondria give rise to the crescent-shaped bodies that Tyler and Rieger (1975) call ‘‘mitochondrial derivatives’’. The structures that Tyler and Rieger (1975) presume to be mitochondrial derivatives are in fact granular derivatives. In the spermatid stages, several granules can be found within one lined vesicle and in some of these granules more electron-dense structures can be found. This is also the case in the crescent-shaped bodies in the mature spermatozoa, so most likely those structures are derived from the granule-filled vacuoles in the spermatid. We found eight to ten granular derivatives, while Tyler and Rieger (1975) counted six to eight; the dimensions of the mature spermatozoa also differ slightly. Sterrer (1998) studied sperm of *F. apelti* using light microscopy, and he detected that the number in the segments of the middle piece (granular derivatives) and the proportions of the mature sperm cells varied depending on the sampling site where the specimens were found; we join this view in order to clarify the differences between our measurement and those of Tyler and Rieger (1975).

The mature sperm of several species of Nemertodermatida has been studied ultrastructurally: *M. stichopi* (Hendelberg 1977; Lundin and Hendelberg 1998), *Nemertoderma bathycola* (Hendelberg 1977), *N. westbladi* (Hendelberg 1977) and *F.*

apelti (Tyler and Rieger 1975, 1977, this study). The general structure of the sperm cells is similar for all the species studied: nemertodermatids have filiform sperm with an elongated head containing the nucleus, a middle piece containing an axoneme and mitochondria, and a tail made up of a flagellum with 9 x 2 + 2 arrangement of the microtubules. However, the detailed morphology of each sperm part appears to be speciesspecific. The head in *F. apelti* contains an acrosome, while no acrosome was found in *M. stichopi* (Lundin and Hendelberg 1998) or *N. westbladi* (Tyler and Rieger 1977). In *N. bathycola*, the presence of an acrosome is unclear (Hendelberg 1977). The electron-dense and elongated nucleus has a spiral groove in *F. apelti* and a tapering corkshrew-like proximal end in *M. stichopi*. The middle piece contains an axoneme and mitochondria in all the studied species. In *M. stichopi*, the mitochondrial derivatives are elongated tubes, which are spirally coiled around the axial filament (Lundin and Hendelberg 1998). In *N. westbladi* and *N. bathycola*, the mitochondrial derivatives are tubes that surround the axial filament (Hendelberg 1977; Tyler and Rieger 1977). In *F. apelti*, the mitochondrial derivatives are flattened and elongated. We note that at the distal end of the middle piece in *F. apelti* (Fig. 4c), the mitochondrial derivative also has a similar shape to that of *N. westbladi* and *N. bathycola*: a tube surrounding the axial filament. In all species, the 9 9 2 ? 2 type axoneme extends as a flagellar tail. In both *F. apelti* and *M. stichopi*, the nucleus and mitochondria start to elongate during the spermatid stage. Granular vesicles can be found in the spermatids of both species, as well as in the mature spermatozoon of *F. apelti*, but not in the mature spermatozoon of *M. stichopi*.

We found some aberrant spermatids (Fig. 3c) in *F. apelti* and aberrant spermatozoa in *N. westbladi* (Fig. 4d) that had two axonemes. Two axonemes were also found in some spermatids of *M. stichopi* (Lundin and Hendelberg 1998) and some mature spermatozoa of *F. apelti* (Tyler and Rieger 1977). Lundin and Hendelberg (1998) attribute the occurrence of biflagellar spermatids and spermatozoa to folding of the sections. As the spermatid with two axonemes in our Fig. 3c is sectioned longitudinally, we consider this highly unlikely. Tyler and Rieger (1977) ascribed phylogenetic importance to the occurrence of biflagellate sperm: they remark the resemblance of the aberrant nemertodermatid sperm to acoel sperm with 9 + 2 axonemes, indicating a tie between Acoela and Nemertodermatida (Tyler and Rieger

1977). We assume that aberrant biflagellar sperm are the consequence of failure in the transfer of centrioles during the division of the secondary spermatocyte.

The sperm of acoelan species is considerably different from that of nemertodermatid species (see also Hendelberg 1977). Acoelan species have filiform spermatozoa with an elongated nucleus, but apart from that, their morphology is quite different. Acoel sperm always have two flagella, which are inverted and incorporated into the sperm cell (Hendelberg 1977), while nemertodermatid sperm only have one flagellum, which is neither incorporated nor inverted. Furthermore, the cytoplasmic region of sperm of acoelan species contains accessory microtubules and the mitochondria are not elongated or coiled, but keep their ovoid shape.

The mature sperm of *Xenoturbella* is round-headed, uniflagellate, and lacks a middle piece; it resembles the assumed bilaterian primary condition. However, as *Xenoturbella* is probably free-spawning (Obst et al. 2011), its sperm morphology is probably related to this method of fertilization (Franzén 1956), which makes it less useful for inferring phylogenetic relationships. Lundin and Hendelberg (1998) indicated that the mature sperm in Nemertodermatida resemble the type that Franzén(1956) denotes as ‘‘modified from the primitive metazoan sperm type’’, and their unique morphology probably reflects an internal fertilization.

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CHAPTER 4: SPERMATOGENESIS AND THE STRUCTURE OF THE TESTES IN *ISODIAMETRA PULCHRA* (ISODIAMETRIDAE, ACOELA)

Modified from:

Boone M., Willems M., Claeys M., Artois T. (2011). Spermatogenesis and the structure of the testes in *Isodiametra pulchra* (Isodiametridae, Acoela). Acta Zoologica 92 (2): 101-108.

BACKGROUND

Next to the Nemertodermatida, also Acoela were studied ultrastructurally. The acoel *Isodiametra pulchra* is a member of the family Isodiametridae, a rather derived family within Acoela. Despited its derived position, *I. pulchra* is often used in studies on the development, regeneration and stem cell dynamics (De Mulder et al. 2009, Moreno et al. 2010), due to the ease of culturing this species. The information on the spatial organization of the different cell types in the testes of *I. pulchra* is pivotal for the correct interpretation of immunohistochemical stainings and in situ hybridizations. We therefore studied the testes of *I. pulchra* by transmission electron microscopy, in order to describe the different stages of spermatogenesis and their spatial organization in this species.

ABSTRACT

Spermatogenesis and the structure of the testes were studied ultrastructurally in *Isodiametra pulchra* (Smith and Bush, Transactions of the American Microscopical Society 1991; 110: 12; Hooge and Tyler, Journal of zoological systematics and evolutionary research 2005; 43: 100). The testes are paired, compact, non-follicular and lie dorsally and dorso-laterally to the paired ovaries, partially enfolding them. All stages of spermatogenesis, including spermiogenesis, are described at the ultrastructural level and their spatial organization within the testes is discussed. The cells at the early stages of spermatogenesis (spermatogonia and spermatocytes) are located on the dorsal and dorso-lateral sides of the testes, while the late stages (spermatids and filiform spermatozoa with 9+2 axonemes) lie at the ventral and inner periphery of the testes, adjacent to ovaries. All the cell types can be found both at the anterior and the posterior end of the testes. The value of the structure of the testes as a phylogenetic marker is addressed.

INTRODUCTION

According to recent molecular phylogenetic studies, Acoela is the most basal taxon within the Bilateria (Ruiz-Trillo et al. 1999, 2002; Jondelius et al. 2002; Telford et al. 2003; Baguna and Riutort 2004; Philippe et al. 2007; Wallberg et al. 2007; Baguna et al. 2008; but see Dunn et al. 2008), which makes it very interesting for evolutionary studies.

Because acoels are soft-bodied worms that show only very few morphological clues that can help to identify them, the phylogeny of the Acoela was rarely studied in the past. Hooge et al. (2002) studied the phylogenetic relationships within the Acoela using molecular and morphological markers, but only a limited number of species was included, and a large-scale phylogenetic analysis is still lacking.

The ultrastructural morphology of spermatozoa could provide important characters to infer phylogenetic relationships within acoels (Raikova and Justine 1999; Raikova et al. 2001; Hooge et al. 2002; Petrov et al. 2004), as do the morphology of the penis (Hooge and Tyler 2005; Raikova et al. 2006), the body musculature (Hooge et al. 2002) and the presence and morphology of the bursal nozzles (Petrov et al. 2006). Mature spermatozoa have already been studied in detail in different taxa of acoels (Hendelberg 1969, 1977; Tyler et al. 1986; Justine et al. 1998; Raikova et al. 1998, 2001; Raikova and Justine 1999; Petrov et al. 2004; Tekle et al. 2007), and these studies have confirmed that all acoel spermatozoa are filiform and have two incorporated axonemes (Hendelberg 1969, 1983, 1986). These axonemes consist of microtubules that can be arranged in either a 9+2, a 9+0 or a 9+1 pattern, a feature considered an important phylogenetic marker (Raikova et al. 2001). The mature sperm of *Isodiametra pulchra* has been described by Petrov et al. 2004.

The process of forming mature spermatozoa out of primordial germ cells or spermatogonia is called spermatogenesis. The final stages of spermatogenesis, during which spermatids mature into spermatozoa, together constitute spermiogenesis. Whereas spermiogenesis has been described in several species of acoels (Raikova and Justine 1994, 1999; Raikova et al. 1997), the early stages of sperm formation have never been studied. These early stages of spermatogenesis (spermatogonia or germ cells) are especially interesting, given that acoels have

pluripotent stem cells (neoblasts), which are responsible for forming all types of differentiated somatic cells and the germ cells. Recently, there has been growing interest in the study of these neoblasts in acoels (Gschwentner et al. 2001; Egger et al. 2007; Gaerber et al. 2007). Given the exceptional fact that neoblasts are the sole source for every cell type, it is important to know whether neoblasts and primordial germ cells can be distinguished at the ultrastructural level.

Rieger et al. (1991) described the basic organization of the testes in Acoela. The known diversity in the structure of the testes in acoels seems to be large; testes can be asaccular or saccular, compact or diffuse, paired or unpaired, follicular or non-follicular, mixed or separated from the female gonad (Rieger et al. 1991). Some acoels have follicular testes, which means that the testes consist of packages of clusters of cells that develop together and that are spatially separated from other follicles. In other species, no follicles can be found. The testes can be compact or diffuse, depending on the degree of density to which the cells are packed together. It is still unclear how the testes are organized ultrastructurally in different species and how the different stages of spermatogenesis are organized within the testes or follicles. Nevertheless, it is essential to describe and understand the spatial organization of the different cell types in the testes of Acoela, to be able to interpret the results of further studies on the function of germ cells and neoblasts, as for example immunohistochemical stainings and in situ hybridization (De Mulder et al. 2009). Moreover, a detailed study of the testes in acoels will increase the knowledge on the morphology of this challenging taxon, which will enable us to infer the evolution of the testes in this group and to examine the possibility to use this characteristic in phylogenetic studies.

In this contribution, we present a detailed ultrastructural study of spermatogenesis and the spatial cellular organization within the testis of the acoel *Isodiametra pulchra* (Smith and Bush 1991) Hooge and Tyler 2005; a species that is recently used in studies on regeneration and stem cell dynamics (De Mulder et al. 2009), and for which a number of EST's have been sequenced (Philippe et al. 2007). It is the first time the complete spermatogenesis of an acoel is described.

MATERIALS AND METHODS

CULTURES

Isodiametra pulchra (Isodiametridae, Acoela) is a small (365 lm long; Smith and Bush 1991), unpigmented, tearshaped acoel with an isodiametric penis located within the seminal vesicle. Specimens of I. pulchra are kept in Petri dishes filled with artificial sea water, which is enriched with nutrients (Guillard's f／2 medium) (see Rieger et al. 1988), and are fed with the diatom *Nitzschia curvilineata*. To maintain constant conditions, they are held in an incubator at 20°C on a 14:10 day–night cycle. This culture of *I. pulchra* originated from a culture set up by Prof. Dr. Seth Tyler and Dr. Matthew Hooge of the University of Maine, the type location can be found in the species description (Smith and Bush 1991).

TRANSMISSION ELECTRON MICROSCOPY AND SECTIONING

Mature specimens (16 days post-hatching) were relaxed with 7.14% $MgCl_2$. Immediately after that, the animals were fixed in a cocktail of glutaraldehyde fixative and osmium fixative (Eisenmann and Alfert 1982) for approximately 10 min at 4°C. The glutaraldehyde fixative was 4% glutaraldehyde in the first buffer (100 mL: 0.2 M sodium cacodylate with 0.58 g NaCl and 11.97 g sucrose; pH 7.2), and the osmium fixative was 1% osmium tetroxide in the second buffer (100mL: 0.2 M sodium cacodylate with 3.48 g NaCl; pH 7.2). After fixation with the cocktail, the specimens were fixed in glutaraldehyde fixative for 1 h at 4°C and then post-fixed with osmium fixative for 1 h at 4°C. After rinsing in the second buffer for 10 min at 4°C and rinsing for 5 min in double distilled water, they were dehydrated, using acetone series of increasing concentrations. The specimens were subsequently infiltrated with a low-viscosity embedding medium (Spurr 1969), and polymerisation was performed at 70°C for 8 h. Semi-thin sections of 1 and 2 µm and ultra-thin sections of 60 nm were made on a Reichert-Jung Ultracut E or a Leica-Reichert Ultracut S (Leica, Vienna, Austria). The specimens of *I. pulchra* were sectioned either transversely or longitudinally. Specimens were sectioned semi-thin until the region of interest was reached, after which ultra-thin sections were made. The testes were completely sectioned from the anterior to the posterior tip. Semi-thin sections were studied using an Olympus BX 51 microscope equipped with an Olympus C5060 digital camera.

Ultra-thin sections were studied with a Jeol JEM-1010 transmission electron microscope (Jeol Ltd., Tokyo, Japan) operating at 60 kV and pictures were digitized using a Ditabis system (Pforzheim, Germany). A 3D-reconstruction of the reproductive structures of *I. pulchra* was made based on a series of serial sections of 2 µm, using Amira 3.1.1 software (TGS Europe, Bordeaux, France).

RESULTS

STRUCTURE OF THE TESTES

Sixteen days after hatching, the post-embryonic development in *I. pulchra* has been completed and the worm is able to reproduce. A 3-dimensional reconstruction of the reproductive structures in an adult specimen of *I. pulchra* is presented in Fig. 1A–C. In the adult worms, the testes lie dorso-laterally to the ovaries, with the anterior part of the testes slightly enfolding the ovaries. The bursa and the vesicula seminalis, including the penis, are situated centrally in the caudal part of the body. The testes as a whole are well-defined, but there is no proper delimitation of follicles within the testes. Therefore, the testes are clearly non-follicular. The cells are so densely packed that they build up a compact testes. In what follows we will describe the structure of the testes and each successive stage of spermatogenesis, from spermatogonium to mature spermatozoon. All the stages of spermatogenesis can be found all along the entire length of the testes, anteriorly as well as posteriorly, but they are organized in a way that the early stages (spermatogonia, primary and secondary spermatocytes) are lying on the outside of the testes (meaning at the side of the epidermis), while spermatids and mature sperm can be found at the centre and inner side of the testes (meaning at the side of the ovaries). This organization is presented in Fig. 1D. From our observations, we can conclude that the male germinative zone is found at the side of the testes adjacent to the epidermis. The spermatids and spermatozoa are found in fours, as they originate from a single spermatocyte. The ‘tails’, the end of the spermatozoon which is opposite to the nucleus and contains the two axonemes, can be found in every region of the testes in between the other cells, even though the shafts of the spermatozoa are found at the inner side of the testes.

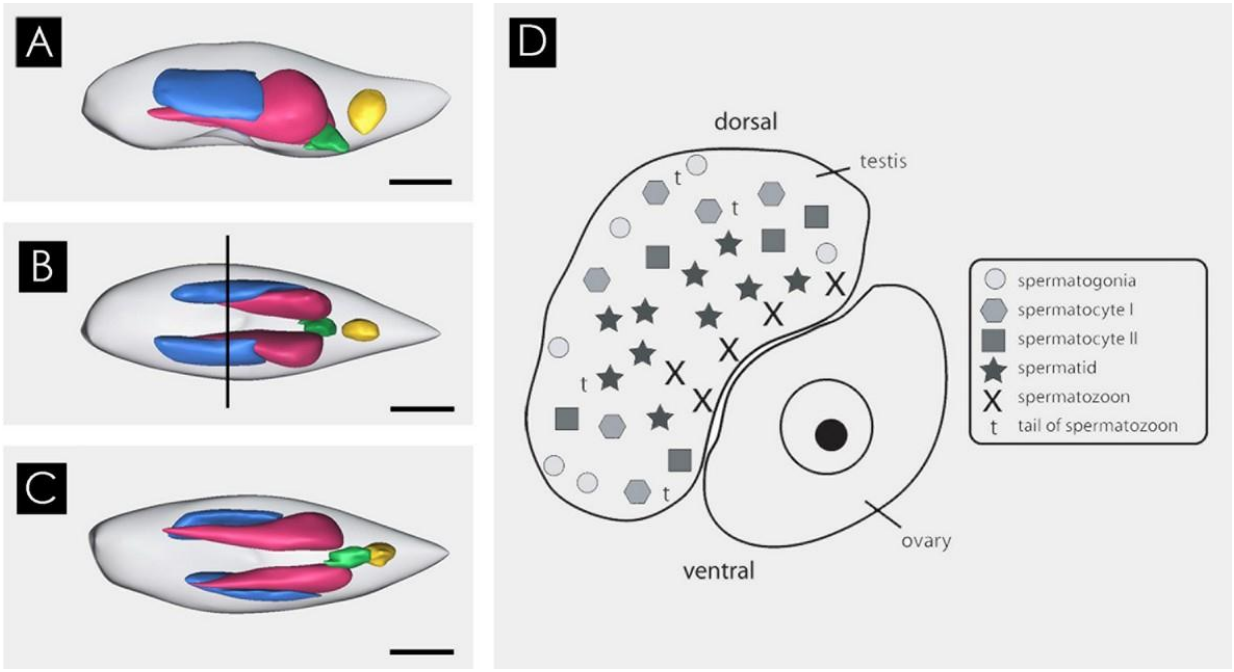

Fig. 1 – *Isodiametra pulchra*. A-C: 3D reconstruction based on serial semi-thin sections of the reproductive organs in the acoel I. pulchra. On each side, sperm ducts connect the testes and the vesicula seminalis (not in visible on 3D reconstruction). A: lateral view; B: dorsal view with the indication of the level of the cross-section presented in 1D ; C: ventral view. Color codes: blue: testes; pink: ovaries; green: bursa; yellow: vesicula seminalis. Left is anterior, right is posterior. Scale bar: 100 µm. – D: Diagram of a cross section of I. pulchra at 130 µm from the anterior tip, where the organization of the different cell types in the testes is shown. The early stages, spermatogonia, spermatocytes I and II, are lying at the outer periphery of each testis (dorso-lateral side), while spermatids are in the centre of each testis and mature spermatozoa are lying at the inner periphery of each testis, on the side adjacent to the ovary. Note that the “tails” (opposite end of the nucleus) of the spermatozoa are scattered in between all the cell types. This scheme is representative of the organization of each testis along its entire length. Left is the side of the body wall; right is the side of the central parenchyma.

SPERMATOGENESIS

THE SPERMATOGONIUM

The nucleus of a spermatogonium (Fig. 2A) contains small clumps of heterochromatin centrally, which are unconnected to each other. The nucleo-cytoplasmic ratio is high; the nucleus fills the larger part of the spermatogonium. The undifferentiated cytoplasm contains a few mitochondria and ribosomes. This ultrastructure is remarkably similar to that of a neoblast (somatic stem cell). Figure 2B shows a neoblast that is situated near the body wall. This cell has a high nucleo-cytoplasmic ratio, comparable to the one in the spermatogonia. Both the cytoplasm and the nucleus of the neoblast resemble those of the spermatogonium: the cytoplasm contains a few mitochondria and ribosomes, while clumps of heterochromatin are found in the nucleus.

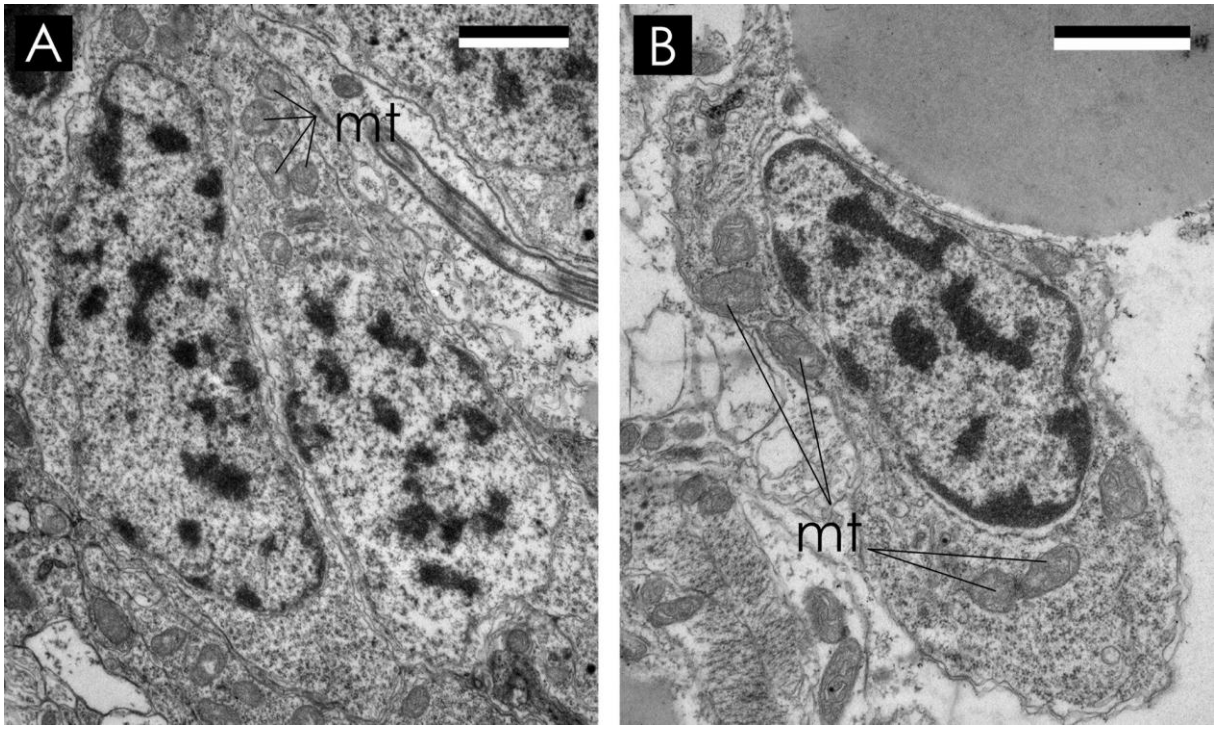

Fig. 2 – Spermatogonia and neoblast in *I. pulchra* – A. Spermatogonium with scattered clumps of heterochromatin in the nucleus. The undifferentiated cytoplasm contains mitochondria and ribosomes. – B. Neoblast that is located near the body wall (under the muscle layer). Note the undifferentiated cytoplasm with mitochondria and ribosomes, and the scattered clumps of heterochromatin in the nucleus. mt: mitochondria. Scale bars: 1 µm.

THE PRIMARY SPERMATOCYTE

The nucleus of the primary spermatocyte has a round to ovoid shape and is characterized by the presence of synaptonemal complexes, which are clearly visible (Fig. 3, arrowheads), and scattered clumps of heterochromatin. The nucleo-cytoplasmic ratio is still high compared to what is found in somatic cells, but lower than in spermatogonia. The cytoplasm contains some mitochondria and ribosomes, and sometimes rough endoplasmatic reticulum can be found. Intercellular bridges connect the cytoplasm of adjacent primary spermatocytes (Fig. 3B, arrow).

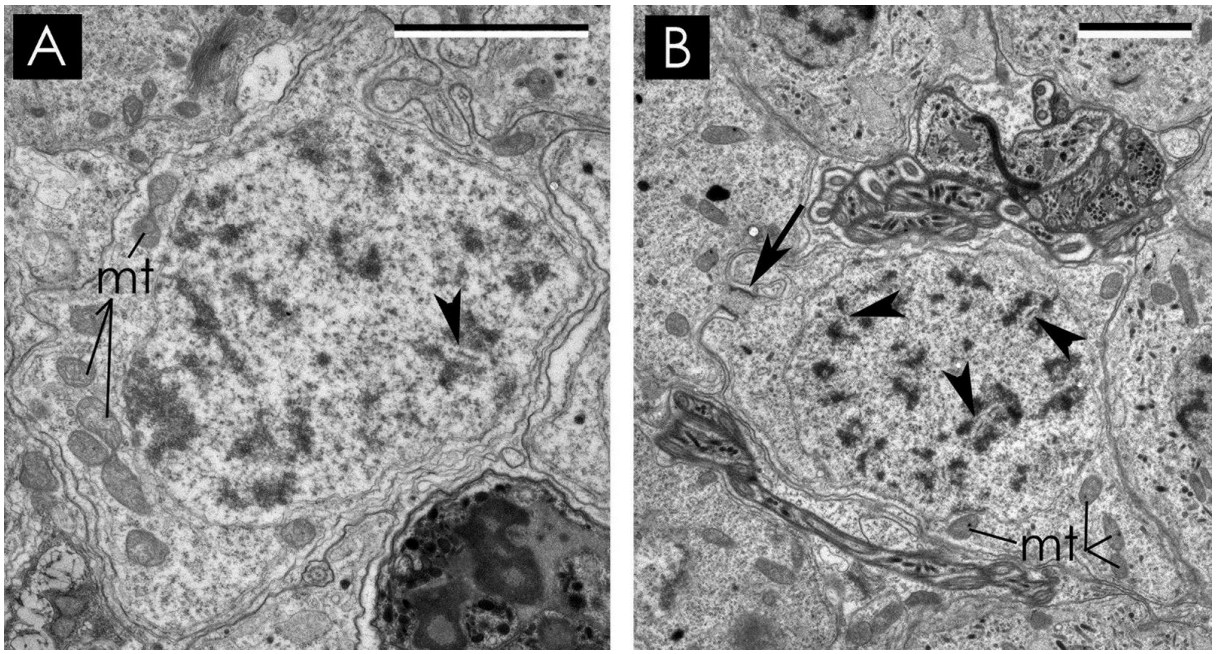

Fig. 3 – Primary spermatocytes in *I. pulchra* – A. Primary spermatocyte with scattered chromatin and a synaptonemal complex (arrowhead) in the nucleus. Note the undifferentiated cytoplasm which contains only a few mitochondria and ribosomes. – B. Two primary spermatocytes with several synaptonemal complexes (arrowheads) in the nucleus. Note the intercellular bridge between the two cells (arrow). mt: mitochondria. Scale bars: 2 µm.

THE SECONDARY SPERMATOCYTE

The nucleus of the secondary spermatocyte is round and contains more heterochromatin that is also more condensed than that of the primary spermatocytes. Apart from the mitochondria and ribosomes, also centrioles (Fig. 4, arrowheads), swollen endoplasmatic reticulum and electron-dense granules (Fig. 4, black arrows) can be noticed in the cytoplasm. The granules are not membranebound. As in primary spermatocytes, intercellular bridges connect the cytoplasm of adjacent secondary spermatocytes. Every secondary spermatocyte gives rise to two haploid spermatids after the second meiotic division, marked by the presence of centrioles (see Fig. 4B, arrowheads indicate the centrioles).

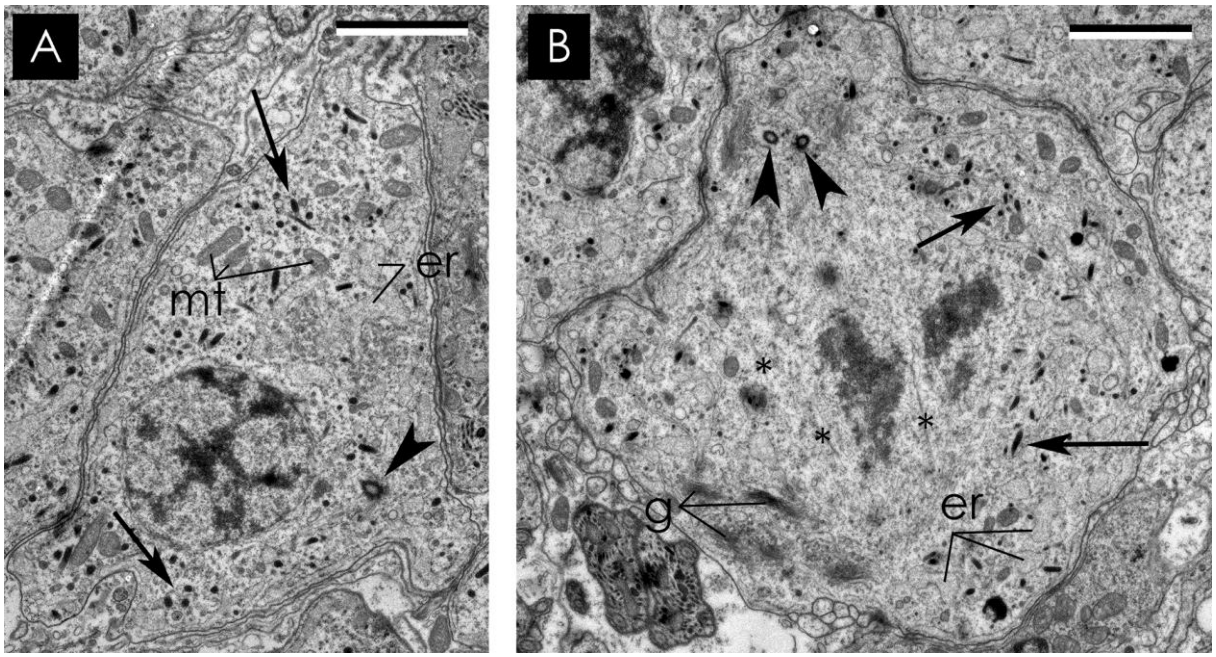

Fig. 4 – Secondary spermatocytes in *I. pulchra* – A. Secondary spermatocyte with a centriole (arrowhead), ribosomes, mitochondria, swollen endoplasmatic reticulum and granules in the cytoplasm (black arrows). The nucleus contains scattered chromatin, which is more abundant than in the primary spermatocytes. – B. Secondary spermatocyte with a meiotic spindle figure. The two centrioles (arrowheads) of one of the two centrosomes are visible, as well as the spindle fibres (microtubules; asterisks) that are pulling the sister chromatids apart. Two different types of granules in the cytoplasm (black arrows) are present. g: Golgi complex; mt: mitochondria; er: endoplasmatic reticulum. Scale bars: 2 µm.

THE SPERMATID

During the spermatid stage, the round to ovoid cell starts to elongate (Fig. 5). Spermatids are always found in clusters of four cells; each of these clusters originates from one primary spermatocyte. An early spermatid is somewhat square shaped, not yet fully elongated, but not ovoid anymore either. Cytoplasmic organelles are abundant: mitochondria, Golgi complexes, ribosomes and swollen endoplasmatic reticulum can be found in high numbers. The cytoplasm of the spermatids contains electron-dense granules, which may appear either in groups (arrowhead) or individually. These granules are not membrane-bound (Fig. 5D). The nucleus is elongating, while the shape in cross section is changing from a round-ovoid shape to an S-shape. Figure 5A,B clearly shows the flagella (arrowheads), which are still free and lie along the cell body at the beginning of spermiogenesis. They will be incorporated in the shaft of the spermatid during the elongation phase. From our observations of transverse sections, the flagella are surrounded by a single canal formed by the two membranes surrounding the axoneme (Fig. 5E). Numerous cortical microtubules are found near the cell membrane (Fig. 5C,F).

Fig. 5 (next page) – Spermatids in *I. pulchra* – A. Early spermatids sectioned transversally. The flagella that will later be incorporated as axonemes are indicated with arrowheads; note the granules in the cytoplasm (arrows). – B. Early spermatid sectioned longitudinally. The nucleus is elongating but the flagella are not incorporated as axonemes yet (arrowheads). The cytoplasm contains swollen ER, mitochondria, Golgi, polyribosomes and granules. – C. Incorporation of flagella as axonemes (arrowheads). The nucleus acquires an S-shape in cross sections. The cytoplasmic granules are indicated with black arrows. Note the cortical microtubules. – D. Detail of the incorporation of flagella as axonemes. The flagella are surrounded by two membranes, forming one canal (arrowhead) – E. Detail of the granules in the cytoplasm of spermatids, the granules being not membrane-bound. – F. Transverse section of late spermatids, sectioned at the nuclear level. The spermatids have elongated into a filiform shape. Note the cortical microtubules. The axonemes are already incorporated, but their membrane is still visible (arrowheads); the arrows point to the granules in the cytoplasm. cm: cortical microtubules; g: Golgi; mt: mitochondria; nu: nucleus; er: endoplasmatic reticulum. Scalebars: A, B, F: 2 µm; C: 1 µm; D: 200 nm; E: 300 nm.

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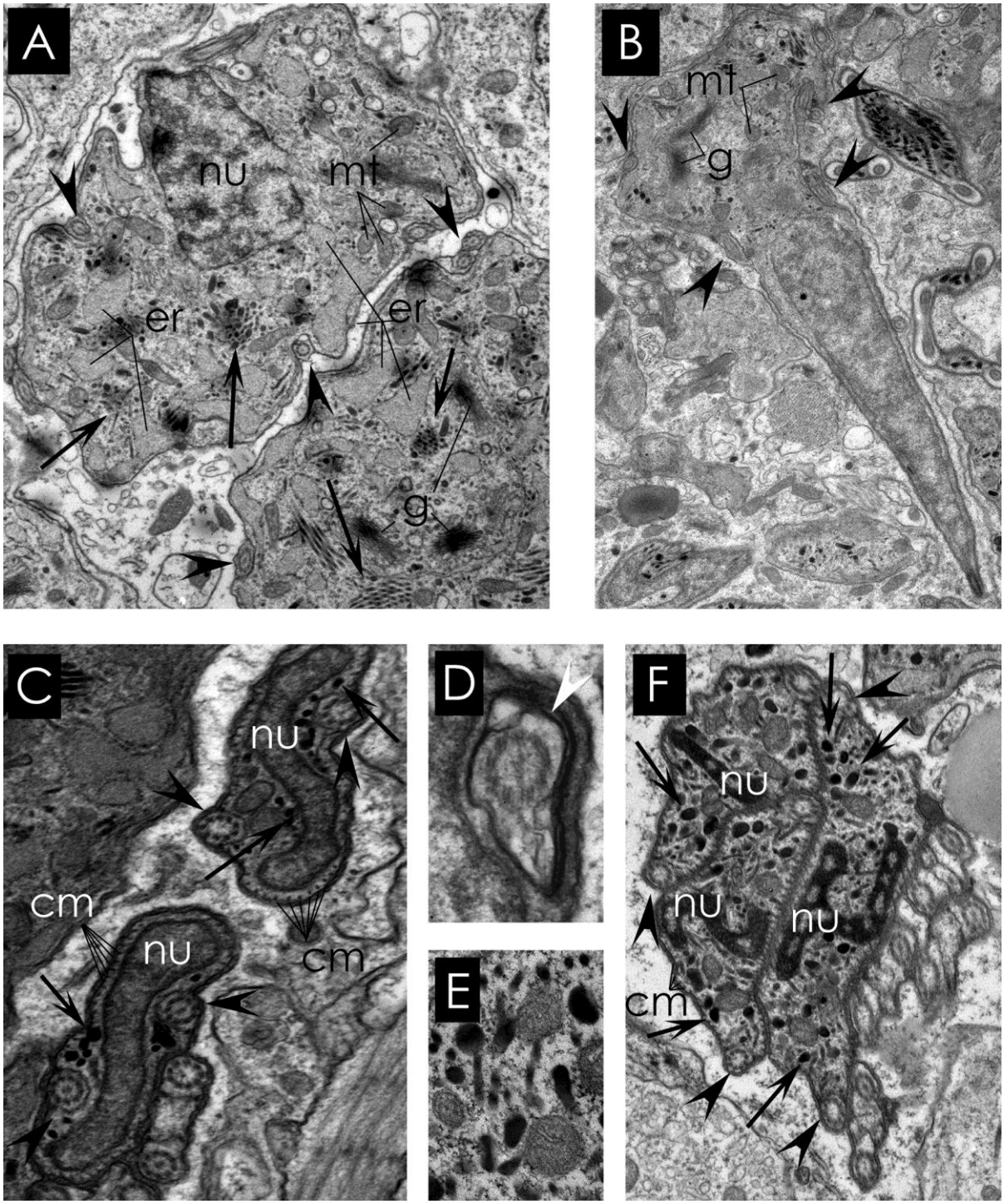

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THE MATURE SPERMATOZOON

Mature spermatozoa can best be studied in the seminal vesicle (Fig. 6). The mature, elongated sperm cells have a nuclear region on one end and a cytoplasmic region on the other end (Hendelberg 1969; Petrov et al. 2004). The nucleus is elongated, electron-dense and not fragmented. The cytoplasmic region is characterized by the presence of 9+2 axonemes (arrowheads in Fig. 6), mitochondria, granules and cortical microtubules. At the posterior (proximal) end of the axonemes, the two central microtubules are absent. The axonemes extend into the nuclear region. Mitochondria are abundant, as well as the electron-dense granules described earlier (black arrows in Fig. 6).

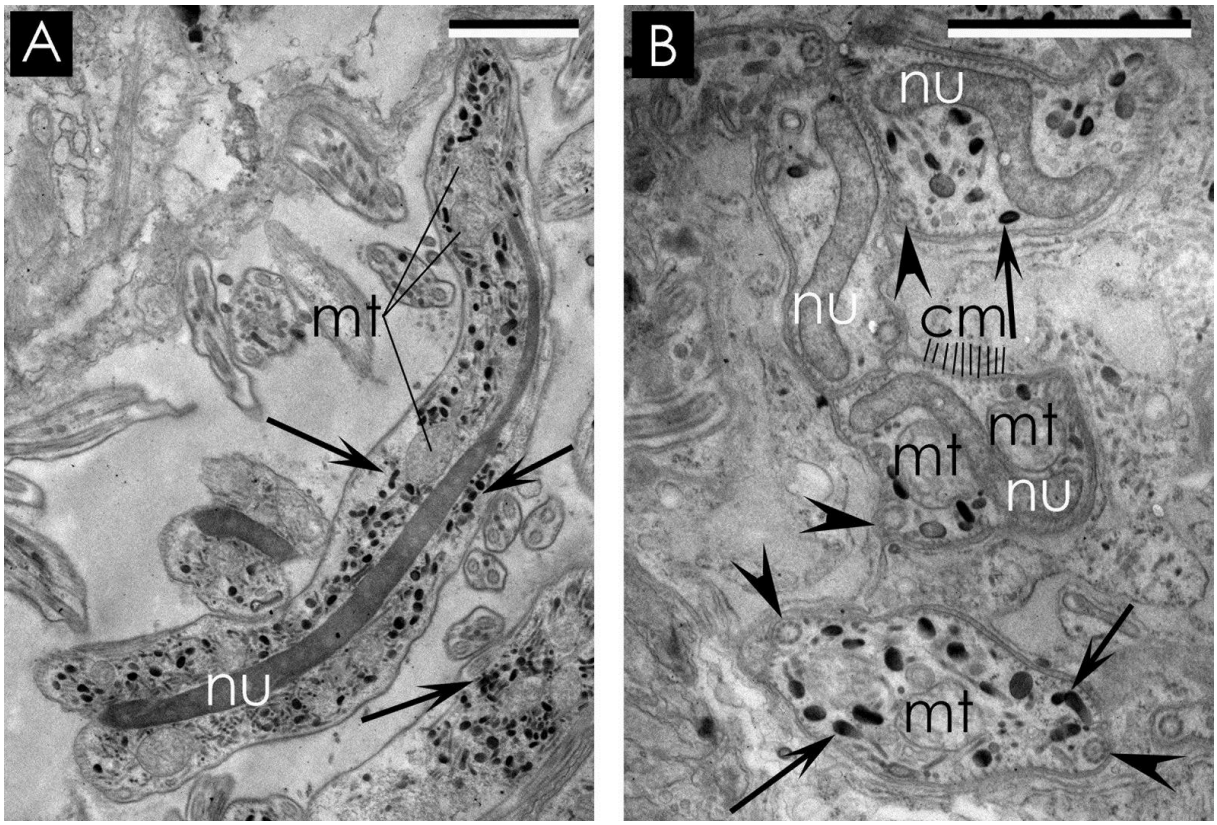

Fig. 6 – Mature spermatozoa as they appear in the seminal vesicle of *I. pulchra*. A – Longitudinal section of a mature spermatozoon with an elongated nucleus and granules (arrows) and a few mitochondria. B – Transverse sections of mature spermatozoa. The upper three spermatozoa are sectioned at the nuclear level, while the bottom one is sectioned in the cytoplasmic region. The nucleus is S-shaped when sectioned transversally. The 9+2 structure of the two axonemes (arrowheads) and the cortical microtubules can be clearly seen in this section. Note the granules (arrows) and the mitochondria in the cytoplasmic region. cm: cortical microtubules; mt: mitochondria; nu: nucleus. Scale bars: 2 µm

DISCUSSION

SPERMATOGENESIS

Spermiogenesis has been studied in detail in several species of acoels (Raikova and Justine 1994, 1999; Raikova et al. 1997), but the complete spermatogenesis and the detailed structure of the testes have never been published. The spermatogonium is an undifferentiated cell, which divides mitotically to produce two cells: a new spermatogonium and a cell that will differentiate into a primary spermatocyte. In *I. pulchra*, spermatogonia can only be distinguished from somatic neoblasts based on their location (they are in the testes); as to their ultrastructural morphology, they are identical to neoblasts. The function of spermatogonia and somatic stem cells is very similar: both divide to form a new spermatogonium or a stem cell, respectively, and a daughter cell that will differentiate into a germ cell or a somatic cell, respectively. A primary spermatocyte can easily be identified by the presence of the synaptonemal complexes. The synaptonemal complex is a protein structure, which mediates synapsis and crossing-over during the zygotene phase of the prophase of the first meiotic division (Alberts et al. 1994), and because of this function, it cannot be found in any other cell (except primary oocytes). Consequently, this feature is diagnostic of the primary spermatocytes. The fact that only few organelles are present in the cytoplasm is also indicative of this stage, but not exclusive, as it is also found in other stages. After the first meiotic division, each of the primary spermatocytes gives rise to two secondary spermatocytes. The secondary spermatocyte is the stage at which the second meiotic division starts. Consequently, it is more differentiated than the primary spermatocyte. It already contains the two types of granules that are seen in spermatozoa. The cells have not started to elongate (cf. spermatids), and their nucleus is still round-ovoid in shape, which makes them easily distinguishable from spermatids. Spermatids in *I. pulchra* are not connected to each other through a cytophore, as is the case in other acoels (see Raikova and Justine 1994). During their incorporation in the spermatids, the axonemes are bordered by a single canal, formed by the two membranes surrounding the axoneme. This mode of incorporation can also be found in other Euacoela, as e.g. *Actinoposthia beklemischevi* (Raikova et al. 2001), and is in contrast with the very complex flagellar incorporation involving complicated cytoplasmic canals in *Paratomella rubra* (Raikova et al. 1997, 2001).

Incorporation of flagella is also found in other taxa e.g. the Neodermata (Watson 1999) and Kalyptorhynchia (Watson 2001), although the mode of incorporation differs. As described by Petrov et al. (2004), mature spermatozoa in *I. pulchra* have 9+2 axonemes and cortical microtubules. As the other 'small-bodied convolutids', *I. pulchra* was transferred into the new family Isodiametridae, by Hooge and Tyler (2005) because of the presence of an isodiametric penis, with the 9+2 sperm axonemes as an additional character for that family. The electron-dense granules in mature spermatozoa can also be found in other Acoela (Raikova and Justine 1994) but it is difficult to identify homologies between them. Their function is also unclear; an acrosome function was proposed (Hendelberg 1983) but this cannot be assessed in a morphological study alone.

IMPLICATIONS OF THE STRUCTURE OF THE TESTES

The known diversity in the structure of the testes in acoels seems to be large; testes can differ in position in the body, lining, relation to ovaries, internal spatial organization, direction of maturation of the germ cells, density (Rieger et al. 1991), etc. Testes can be paired or unpaired, and mixed or separated fromthe female gonad. In I. pulchra, the testes are paired but separated from the ovaries, while in *Diopisthoporus longitubus* (Diopisthoporidae) and *Oligofilomorpha karlingi* (Solenofilomorphidae), the testes are mixed with the ovaries and unpaired (Dörjes 1971). A diffuse testis is a testis in which the germ cells are divided into groups by processes from parenchymal or gut cells, e.g. the testis in *Paratomella rubra* (Rieger et al. 1991).When the germ cells are densely packed, the testes are called compact, as is the case in *I. pulchra*. Follicular testes are composed of follicles of certain stages of spermatogenesis differentiated from a single germ cell, the follicles being spatially separated from each other. This is seen in Paratomellidae and some members of the Proporidae (*Proporus venenosus* and *P. brochi*; Dörjes 1971), but most testes in acoela are non-follicular, e.g. *I. pulchra*. Although the testes of *I. pulchra* as a whole are well-defined, it is difficult to detect separate regions within the testes. Even though early stages of spermatogenesis are found at the outside of the testes (germinative zone) and spermatids and mature sperm are found at the inside of the testes, a strict layered organization as in some acoels (e.g. *Oxyposthia praedator*; Rieger et al. 1991) and Macrostomorpha (*M. lignano*; Willems M., pers.

comm.) is lacking. Moreover, the ‘tails’ (end of the spermatozoon opposite to the nucleus) of the spermatozoa are lying in between the different cell types because of their elongated nature, which makes it even harder to define layers in the testes. It could be that the side of the testis neighbouring the ovarium, where the mature spermatozoa are lying, functions as a sperm duct, with the sperm moving in the direction of the seminal vesicle. Some of the characters of the testes provided by our analysis of the structure of the testes in *I. pulchra* provide very useful additional information that is impossible to get from the species description or deduce from literature. We believe that the same accounts for other species as well, hence further research is required. Examples of this additional information are the direction of maturation of male sex cells, the presence or absence of layering and the exact position of testes and ovaries. It would be very interesting to create an extended study on the testes of species of many putative unrelated taxa, to detect a pattern in the phylogenetic distribution of the various types of gonads and to be able to construct a datamatrix to infer the evolution of the testes within the Acoela. As illustrated above, the morphology of the male gonads can vary substantially between taxa. Because acoels do not have many other clear morphological characteristics, the structure of the testes surely could provide many additional features that could be important in our study of acoel phylogeny.

ACKNOWLEDGEMENTS

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CHAPTER 5: ACOELA

INTRODUCTION

Acoela is a taxon of marine, bilaterian, soft-bodied worms. Acoels lack protonephridia and an anus. Their phylogenetic position is heavily debated: at first they were considered to be members of the Platyhelminthes, forming the taxon Acoelomorpha together with the Nemertodermatida (for an overview and references, see Ehlers 1985). Phylogenetic studies based on SSU and LSU rRNA placed the Acoelomorpha at the base of the Bilateria (Ruiz-Trillo et al. 1999, Telford et al. 2003) and studies based on 18S small subunit rRNA and mitochondrial genes (Jondelius et al. 2002) and nuclear protein coding myosin heavy chain type II (Ruiz-Trillo et al. 2002), 18S rRNA and 28S rRNA genes (Wallberg et al. 2007), and on a combination of several molecular markers (Baguña et al. 2008) staggered the sistergroup relation of Acoela and Nemertodermatida. Both groups were established as separate early bilaterian clades. Recently, phylogenomic studies have shaken the tree again: Hejnol et al. (2009) reunite acoels and nemertodermatids, show a sistergroup relation of Acoelomorpha with Xenoturbellida and attribute a basal position within the Bilateria for this taxon. Complete mitochondrial genome data of an acoel also supports the view that a monophyletic Acoelomorpha is the earliest divergent extant lineage within Bilateria (Mwinyi et al. 2010). Philippe et al. (2011) suggest that the taxon Xenacoelomorpha is situated within the Deuterstomia, as a sister group to the Ambulacraria. Jondelius et al. (2011) made the first large-scale phylogenetic analysis of the internal relations within the Acoela. Although there is still an ongoing debate on the exact phylogenetic position of the Acoela, it is clear that they are one of the key taxa in order to understand the evolution of morphology within the Bilateria. Therefore, morphological studies of representatives of Acoela are of pivotal importance.

Several morphological studies have been performed on Acoela, especially on characters that are important for internal phylogenetic relationships: body musculature (Hooge et al. 2002), bursal nozzles (Petrov et al. 2006), penis morphology (Hooge & Tyler 2005; Raikova et al. 2006) and mature spermatozoa (Hendelberg 1969, Hendelberg 1977, Tyler et al. 1986, Justine et al. 1998, Raikova et al. 1998, Raikova and Justine 1999, Raikova et al. 2001, Petrov et al. 2004, Tekle

et al. 2007, Boone et al. 2011). Studies of the spermiogenesis (Raikova and Justine 1994, Raikova et al. 1997, Raikova and Justine 1999) have been conducted in several acoels, while the complete spermatogenesis and the ultrastructure and organization of the testes have been studied in *Isodiametra pulchra* (Boone et al. 2011).

The gonads in Acoela are known to be very diverse (Rieger et al. 1991), but it is still unclear how the testes are organized in detail in different species and how the structure and morphology of the testes evolved within the acoels. Moreover, clear definitions of the different types of testes are lacking.

In this study, we present the first detailed ultrastructural study of the spermatogenesis and the spatial cellular organization of the testes of the acoels *Philocelis karlingi* Westblad 1946, *Actinoposthia beklemishevi* Mamkaev 1965, *Childia macroposthium* Steinböck 1931 and *Paratomella unichaeta* Dörjes 1966, species which were sampled and successfully processed. An overview of the testes structure in Acoela is given, based on (type)material from the Swedish Museum of Natural History and data from literature, as well as a set of definitions concerning the (ultra)structure of the testes. The testes traits are analyzed within the phylogenetic framework presented by Jondelius et al. (2011). Finally, we compare the ultrastructure of the testes to what is known in other taxa.

MATERIAL AND METHODS

Specimens of *Childia macroposthium* were extracted from mud samples, which were collected around Essvik, Kristineberg, Sweden in 2008. They were fixed in a cocktail of glutaraldehyde and osmium fixative (Eisenmann & Alfert 1982); for details on the fixation and embedding procedure, see Boone *et al*. (2011). For details on collection and fixation procedure of *Actinoposthia beklemishevi* and *Philocelis karlingi*, see Raikova and Justine 1994. Semi-thin sections of 1 µm and 2 µm and ultra-thin sections of 60 nm were made on a Reichert-Jung Ultracut E or a Leica-Reichert Ultracut S (Leica, Vienna, Austria) equipped with a diamond knife. Specimens were sectioned semi-thin until the region of interest was reached, after which ultra-thin sections were made. Ultra-thin sections were post-stained with uranyl acetate (40 min at 20°C) and lead citrate (10 min at 20°C) and studied with a Jeol JEM-1010

transmission electron microscope (TEM) (Jeol Ltd., Tokyo, Japan) operating at 60 kV. Photomicrographs were digitized using a Ditabis system (Pforzheim, Germany). The quality of the sections of *P. unichaeta* does not allow a detailed ultrastructural study of all the stages of spermatogenesis and the structure of the testes.

Mounted sections of specimens of Acoela from the Swedish Museum of Natural History were studied using light microscopy (LM) and a camera in order to describe the different morphologies of the testes in Acoela. A list of the studied sections can be found in Appendix 1. We also retrieved information on the morphology of testes of other Acoela from literature, an overview can be found in Appendix 2. We scored the different morphologies of the testes according to the definitions that can be found below. Different authors tend to use different terminologies and no clear definitions on the terminology exist. Here we suggest following set of definitions for the description of the testes in Acoela.

term	definition
Paired	The testes are organised in two lateral bands that are spatially separated from each other.
Unpaired	The testes are not organised in two lateral bands that are spatially separated from each other.
Sacular	The testes or parts of the testes are bordered by true lining cells.
Pseudo-sacular	The testes or parts of the testes are bordered by a membrane but not by true lining cells.
Asacular	Neither testes nor parts of them are bordered by true lining cells or by a membrane.
Follicular	The testes comprise different primary follicles*, each in a different stage of spermatogenesis. The different follicles are spatially separated, either by a membrane or by parenchymal space.
Non-follicular	The primary follicles can not be individually discerned; they are not spatially separated from each other.
Compact	All the cells (whether or not grouped in follicles) are lying close together, hence forming a compact mass.
Diffuse	The individual sperm cells or follicles are separated from each other, thus not forming a compact mass.

*Primary follicles originate from a single spermatogonium or a cluster of spermatogonia. The occurrence of follicles is inherent to the process of spermatogenesis. The structure of the testes depends on how these primary follicles are organised and whether or not the cells from a primary follicle stay together.

Characterstates related to the structure of the testis were traced along the Acoela phylogeny as presented by Jondelius et al. (2011) using parsimony reconstructions (Cunningham et al., 1998) implemented in Mesquite v1.11 (Maddison and Maddison, 2006). The character states at the internal nodes were also reconstructed using parsimony as the optimality criterion with the 'reconstruct ancestral states' module implemented in Mesquite. Ancestral state reconstruction in a likelihood framework was not possible given the frequent presence of multiple states.

RESULTS OF ULTRASTRUCTURAL ANALYSIS

ORGANISATION OF THE TESTES

Philocelis karlingi has paired testes which are situated dorsally. The cells in different stages of spermatogenesis are lying very closely together; no separate follicles can be discerned, hence forming a compact, non-follicular testis. The testes are asacular, as they have no true lining cells. Early stages of spermatogenesis can be found on the outer, dorsal side of the testes, while spermatozoa are found on the ventral side.

The testes in *Actinoposthia beklemishevi* are paired and are situated dorsally. The cells are loosely arranged; hence forming a diffuse testes. Cells originating from one spermatogonium are found to be connected in clusters, joined by a cytophore, but the testes are not follicular. Early stages of spermatogenesis are found on the dorso-lateral side of the testes, maturation proceeds from this side to the ventro-lateral side.

The testes in *Childia macroposthium* are follicular. The follicles are situated in the parenchyma and are organized in two lateral strands. Nor the individual follicles, neither the entire testes is lined by specialized lining cells. Individual follicles are built of 8 to 16 cells of the same developmental stage. Usually, they are spatially separated from each other by parenchymal tissue, but sometimes 2 or 3 follicles cluster together.

SPERMATOGENESIS

PHILOCELIS KARLINGI

Spermatogonia in *P. karlingi* (fig. 1A) have a large nucleo-cytoplasmatic ratio. The nucleus contains a nucleolus and small clumps of heterochromatin. The cytoplasm contains only ribosomes and some mitochondria.

Primary spermatocytes (fig. 1B) are characterized by the presence of synaptonemal complexes in the nucleus, as well as small, unconnected clumps of heterochromatin. The cytoplasm is comparable to that of spermatogonia: only ribosomes and mitochondria can be found.

In the nucleus of secondary spermatocytes (fig. 1C), the clumps of heterochromatin are larger than those in primary spermatocytes and are connected to each other (not visible in fig. 1C). Differentiation takes place in the cytoplasm: next to ribosomes and mitochondria, centrioles, RER, rods and granules can be found.

Early spermatids (fig. 1D) are characterized by a nucleus containing connected clumps of heterochromatin and a cytoplasm containing mitochondria, ribosomes, centrioles, granules, rods, Golgi-complexes, and flagella that are being incorporated. Their shape is round to ovoid. Late spermatids are more elongated, and the incorporation of the flagella is completed (fig. 1E). Two kinds of granules can be found in the cytoplasm: smaller ones which are less dense, and larger, electrondense ones.

The axonemata in the mature spermatozoa (fig. 1F) have a 9x2+2 configuration of microtubules. A large mitochondrion is situated centrally in the cytoplasm, surrounded by axial microtubules which are organized in two half-moon structures. Each half-moon structure is composed of 15-16 microtubules.

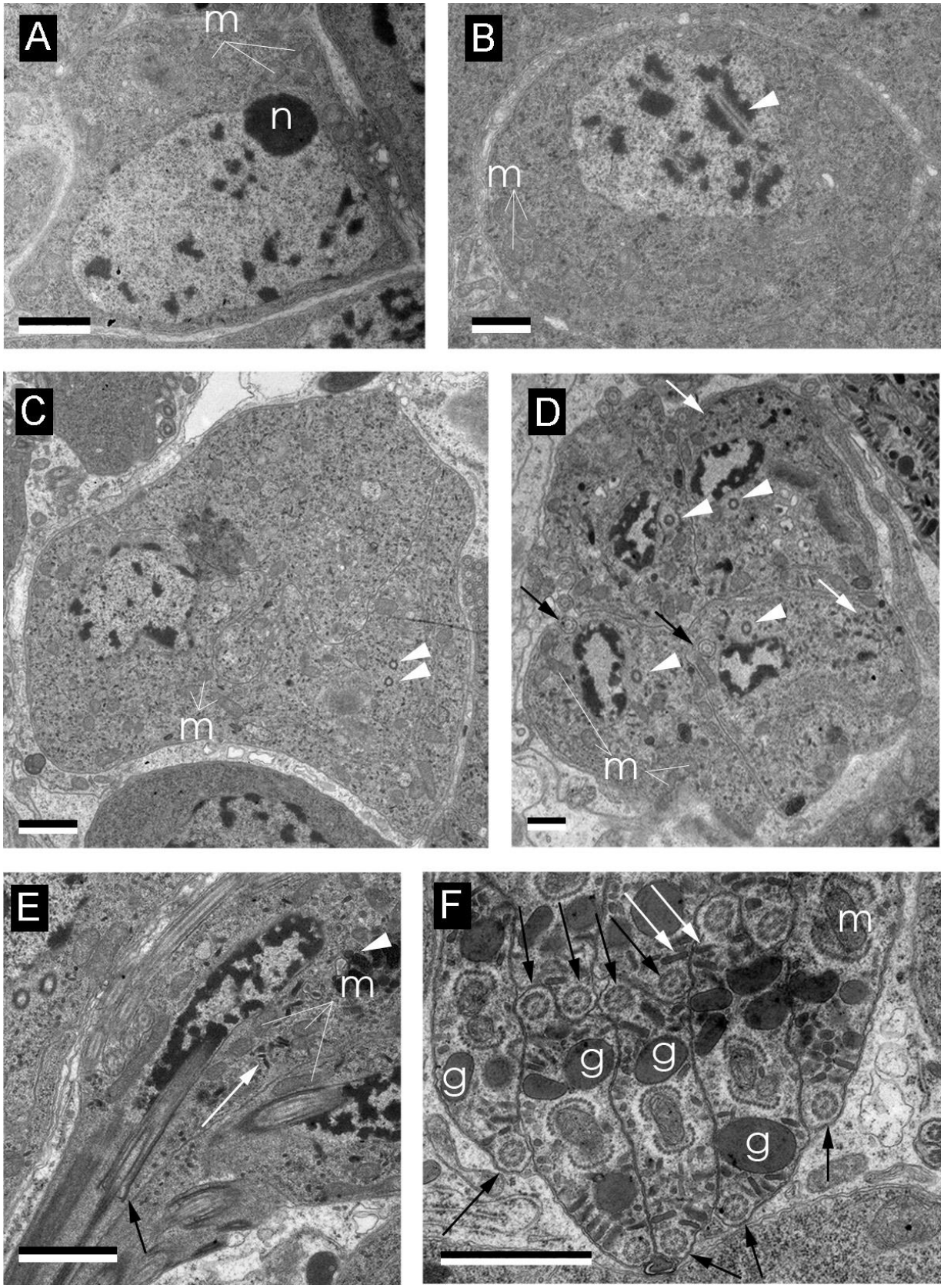

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Fig. 1 – Spermatogenesis in *Philocelis karlingi*. – **A**: spermatogonium with a large nucleo-cytoplasmic ratio, a nucleus containing a nucleolus (n) and cytoplasm containing mitochondria and ribosomes. – **B**: primary spermatocyte containing mitochondria and ribosomes in the cytoplasm and characterized by a synaptonemal complex (white arrowhead) in the nucleus – **C**: secondary spermatocytes containing mitochondria, ribosomes and a pair of centrioles – **D**: transverse section of spermatids with connected clumps of heterochromatin in the nucleus. The cytoplasm contains mitochondria, a centriole (arrowhead), electron dense granules (white arrows). The flagella (black arrows) are incorporated during this stadium. – **E**: longitudinal section of a spermatid with an incorporating flagellum (black arrow), mitochondria and small granules (white arrow) and larger granules (white arrowhead) – **F**: transverse section of spermatozoa in the cytoplasmic region. The cytoplasm contains small (white arrows) and large granules, two axonemes (black arrows) with a 9x2+2 configuration of microtubules, axial microtubules and a central mitochondrion. g: large, electrondense granules, m: mitochondria. Scalebars: 1 µm.

ACTINOPOSTHIA BEKLEMISHEVI

The nucleus of the primary spermatocytes is characterized by the presence of synaptonemal complexes, a nucleolus, and small, unconnected clumps of heterochromatin. The cytoplasm contains mitochondria, ribosomes, centrioles and rough endoplasmatic reticulum (fig. 2A).

Secondary spermatocytes (fig. 2B) are characterized by clumps of heterochromatin in the nucleus and a differentiated cytoplasm containing mitochondria, ribosomes, centrioles, rough endoplasmatic reticulum and Golgi complexes. Two types of granules are found: small, egg-shaped electron dense ones and round, less electron dense ones.

Spermatids of *A. beklemishevi* originating from one spermatogonium are grouped in clusters, joined by a central cytophore (fig. 2C). They have a nucleus in which the heterochromatin forms a network of interconnected clumps. During the spermatid stage, the nucleus flattens, elongates and becomes electron dense. The cytoplasm contains mitochondria, ribosomes, Golgi-complexes, centrioles, incorporating flagella, cortical microtubules and two types of granules. The overall shape of the cells shifts from round-ovoid to more elongated (fig. 2D).

Spermatozoa of *A. beklemishevi* have an elongated nucleus, which appears flattened and S-shaped in cross sections. Large, round granules and smaller, ovoid granules as well as mitochondria are found in the cytoplasm. Cortical microtubules are present, and the two axonemata have a 9x2+2 configuration of microtubules (fig. 2E).

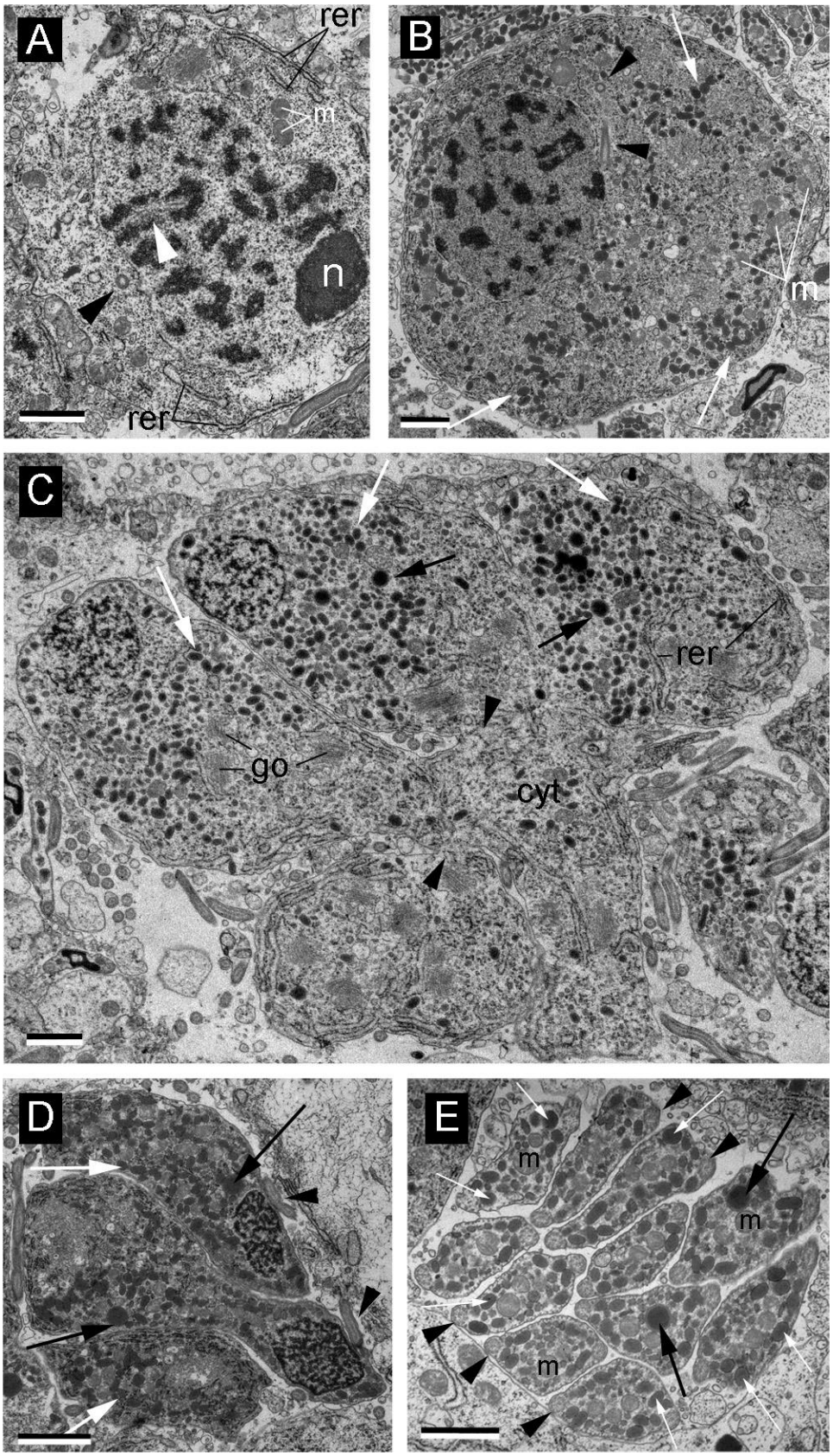


Fig. 2 – Spermatogenesis in *Actinoposthia beklemishevi*. – **A**: primary spermatocyte with a nucleus containing a synaptonemal complex (white arrowhead) and a nucleoleus (n). Rough endoplasmatic reticulum, mitochondria, ribosomes and centrioles (black arrowhead) are present in the cytoplasm. – **B**: secondary spermatocyte with granules (white arrows), a pair of centrioles (black arrowhead), ribosomes and mitochondria in the cytoplasm. – **C**: early spermatids connected by a cytophore (cyt)

through intercellular cytoplasmic bridges (black arrowheads). Ribosomes, mitochondria, rough endoplasmatic reticulum, and Golgi complexes are present in the cytoplasm, as well as two types of granules (black and white arrows). – **D**: late spermatid which transforms from a round-ovoid shape to an elongated shape. Large and small electron dense granules are found in the cytoplasm (black and white arrows), as well as mitochondria, Golgi complexes, ribosomes and endoplasmatic reticulum. The flagella (black arrowheads) are not yet incorporated in the cell. – **E**: transverse section of the cytoplasmic region of mature spermatozoa, containing large and small electrondense granules (black and white arrows), mitochondria, cortical microtubule and axonemes with 9x2+2 configuration of microtubules (black arrowheads). cyt: cytophore, go: golgi complex, m: mitochondria, rer: rough endoplasmatic reticulum. Scalebars: 1 µm.

CHILDIA MACROPOSTHIUM

The early stages in *C. macroposthium* (spermatogonia and spermatocytes) are characterized by a high nucleo-cytoplasmic ratio (fig. 3A). The shape of these cells is round to ovoid and the nucleus contains scattered clumps of heterochromatine.

During the spermatid stage (fig. 3B), cells undergo a transformation from a round shape to an elongated shape. This transformation is also reflected in the shape of the nucleus, which becomes elongated and has a network of heterochromatin. The cytoplasm contains ribosomes, mitochondria, Golgi-complexes, rough endoplasmatic reticulum and small, round granules which appear to be grouped. The incorporation of the two flagella takes place during this stage.

Mature spermatozoa in *C. macroposthium* have a round nucleus, electron dense, round granules and axonemata with a 9x2+1 configuration (fig. 3C, 3D). No accessory microtubules could be observed.

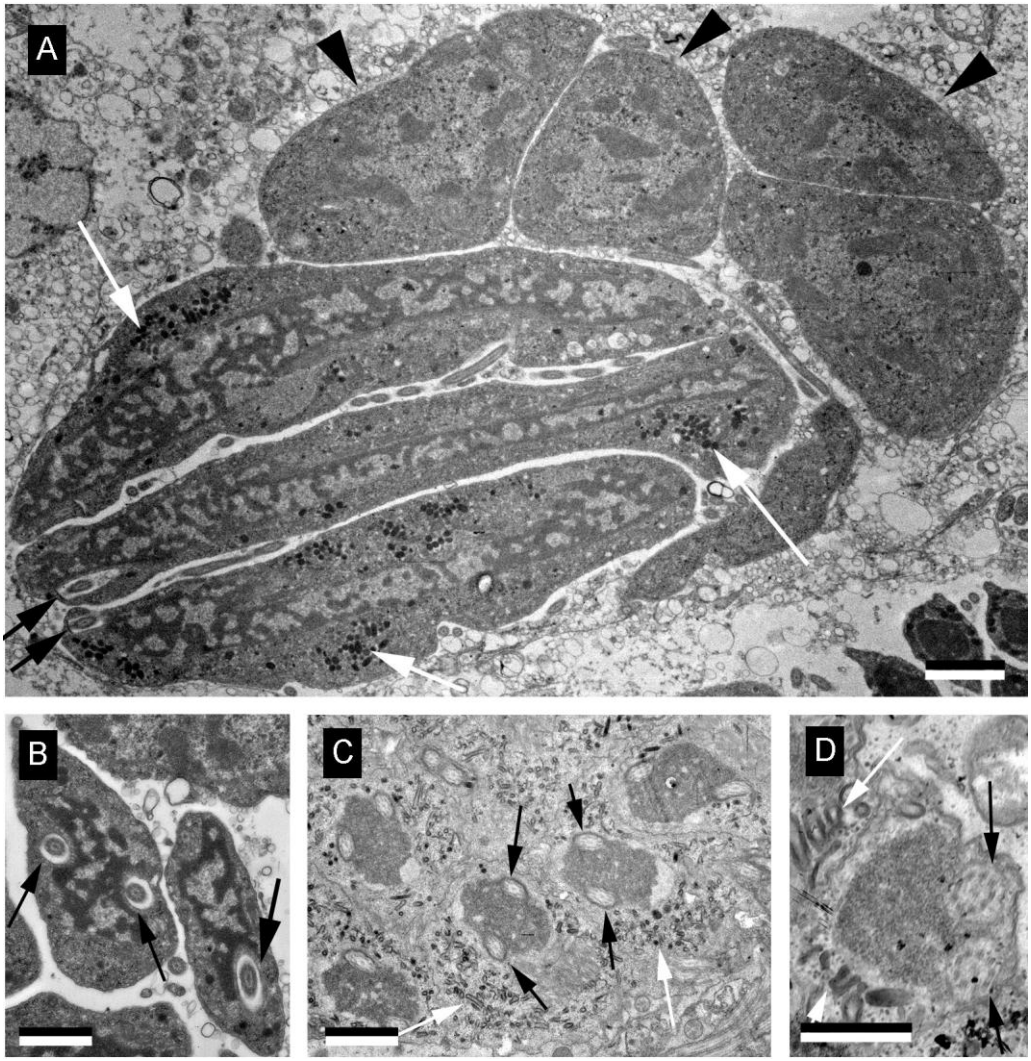


Fig. 3 – **A**: stages of spermatogenesis in *Childia macroposthium*. The early stages (black arrowheads) are characterized by a high nucleo-cytoplasmic ratio. Spermatids are elongated and their nucleus has a network of heterochromatin. Spermatid cytoplasm contains ribosomes, mitochondria, Golgi complexes, rough endoplasmatic reticulum and granules (white arrows). The two flagella (black arrows) are being incorporated during this stage. – **B**: transverse section of spermatids in *C. macroposthium*. The flagella with 9x2+1 configuration of microtubules are incorporated into the cytoplasm. – **C, D**: transverse sections through the nuclear region of mature sperm in *P. unichaeta*. The nucleus is bordered by the two 9x2+2 axonemata (black arrows) and the cytoplasm contains egg- and gastrula-shaped granules (white arrows). Scalebars: A, B, C: 1 µm, D: 0,5 µm

PARATOMELLA UNICHAETA

During the spermatid stage, the nucleus is electron-dense and round in cross-sections. The spermatid cells incorporate two axonemes with the 9x2+2 configuration of microtubules. Two types of granules are present: gastrula-shaped ones and egg-shaped ones.

TESTES MORPHOLOGY WITHIN AN ESTABLISHED PHYLOGENETIC FRAMEWORK

Our own observations (TEM + LM) of the structure of the testes in Acoela were combined with data from literature (for an overview, see Appendix 2), and the resulting set of character states was plotted onto the acoel phylogenetic tree (Jondelius et al. 2011). The results are summarized and discussed below.

The testes in Acoela are usually located dorsally in the body. Following species have testes which are found on the ventral side: *Polycanthus torosus, Pseudaphanostoma smithrii, Haplogonaria syltensis, Philactinoposthia coneyi, Praesagittifera naikaiensis, Symsagittifera corsicae, Childia vivipara* and *Actinoposthia haplovata. Haploposthia vandula* is the only studied species in which the testes extend from a ventral position anteriorly to a dorsal position posteriorly. *Pseudaphanostoma herringi*, *Proporus bronchii*, some representatives of Solenofilomorphidae, *Paratomella rubra* and *Diopisthoporus longitubus* have medially situated testes; i.e. they are found in a position between ventral and dorsal. The ancestral state of the position of the testes is either dorsal or median. Because of the limited number of data the exact state could not be appointed. Nevertheless, several early branching taxa, such as *Diopistoporus longitubus, Paratomella rubra* and some Solenofilomorphidae have medial testes, intuitively the most parsimonious position to evolve to both a ventral and a dorsal position (fig. 4).

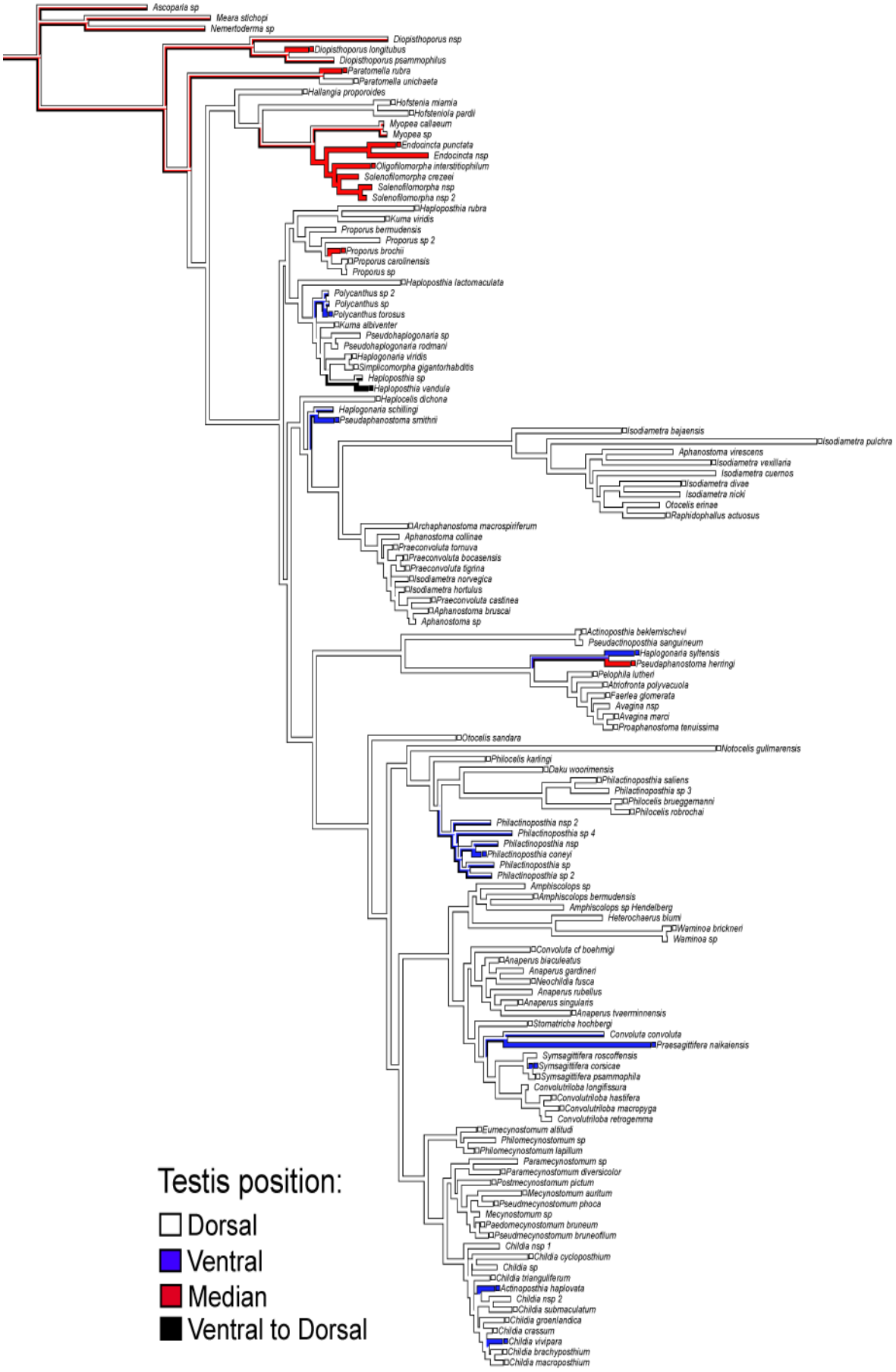
Most Acoela have paired testes (fig. 5). Only the following species have unpaired testes: *Diopisthoporus longitubus, Endocincta punctata, Oligofilomorpha interstitiophilum, Proporus bronchii, Haploposthia rubra, Anaperus biaculeatus* and *Pseudaphanostoma herrengi*. The ancestral state of this character within Acoela could not be determined but the alteration paired vs unpaired has happened at least four times independently.

Virtual all known Acoela have asacular testes, except one: the early branching *Diopisthoporus species* (*D. longitubus*), which has true lining cells around the mixed part of the gonad.

Non follicular testes represent the ancestral state within the Acoela (fig. 6) and the presence of follicular testes has evolved several times independentely. Species with follicular testes include all members of the Paratomellidae, Proporidae, Childiidae,

Haploposthia rubra and some members of Convolutidae. A spermatogonium gives rise to a primary follicle, in which sperm cells from one spermatogonium develop. Follicular testes may be formed when these primary follicles from different spermatogonia cluster together and become separated from other groups by interstitial cells. Follicular testes can be lost when the interstitial space between different follicles disappears, thus compacting the testes.

Most investigated Acoela have compact testes, the ancestral state within Acoela. Diffuse testes has evolved several times independently and were found in *Hallangia proporoides, Proporus carolinensis*, some members of Isodiametridae and Childiidae and in a taxon within the Convolutidae (fig. 7).



1 **Fig. 4 –** Parsimony testis position reconstruction onto the acoel phylogeny of Jondelius et al. (2011)

1 **Fig. 5** – Parsimony reconstruction of paired vs. unpaired testes onto the acoel phylogeny of Jondelius
2 et al. (2011)

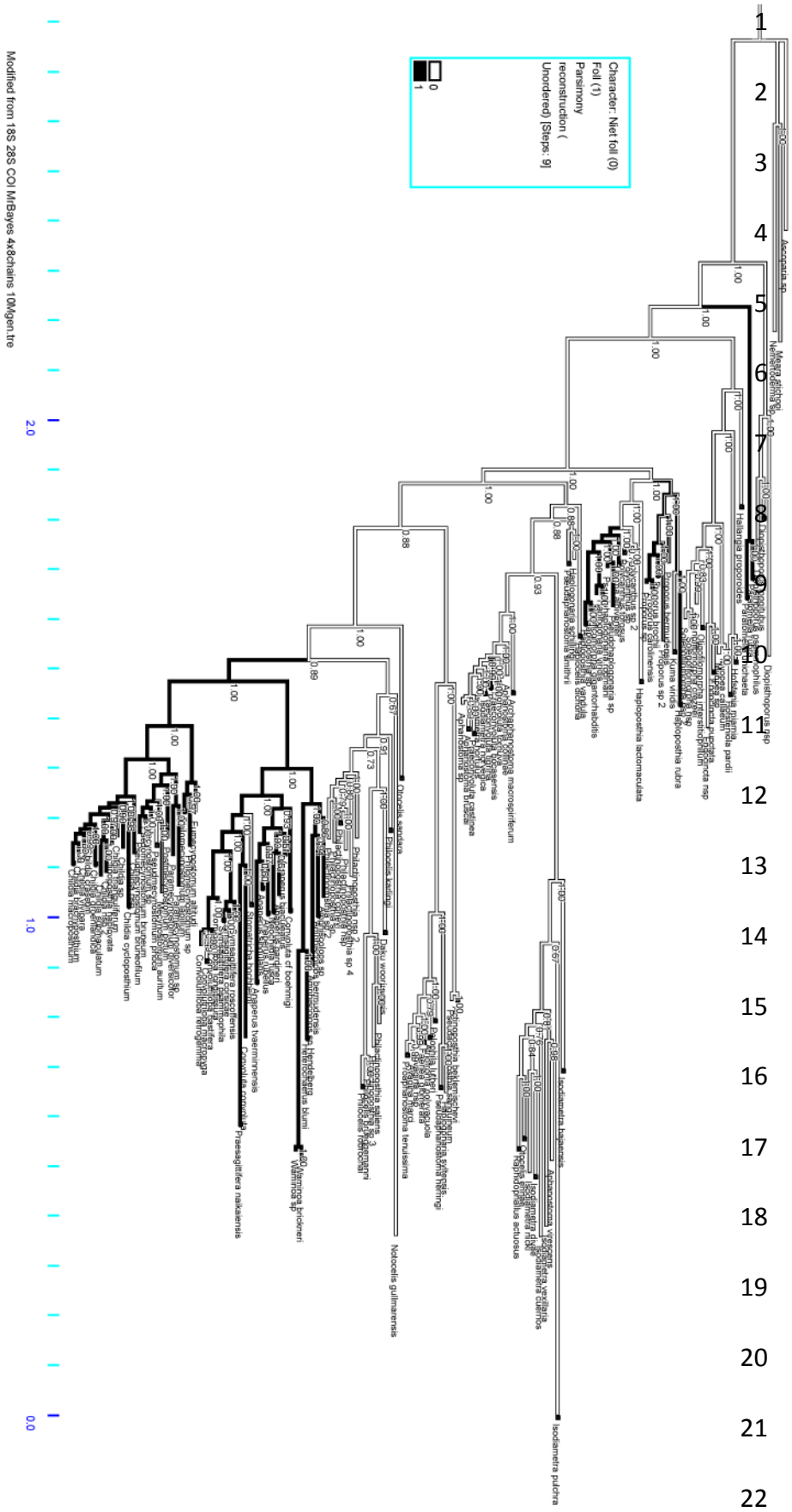
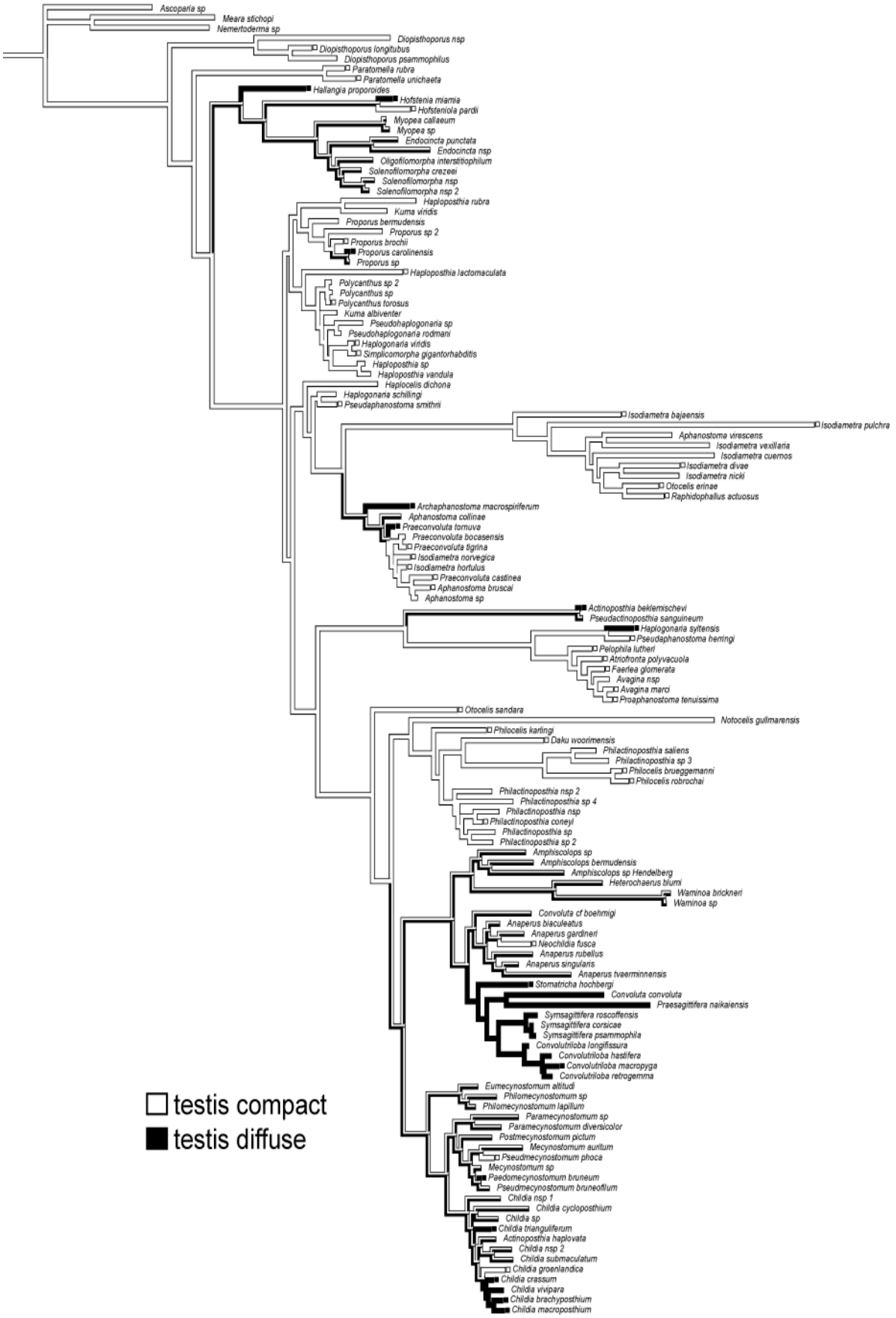


Fig. 6 – Parsimony reconstruction of follicular vs. non-follicular testes onto the acoel phylogeny of Jondelius et al. (2011)



1 **Fig. 7** – Parsimony reconstruction of compact vs. non-compact testes onto the acoel phylogeny of
2 Jondelius et al. (2011)

The testes and ovaries are separated, except for *D. longitubus*, *Haploposthia rubra* and *H. lactomaculata,* which have mixed gonads. The ancestral state for sacular *vs.* asacular testes and mixed *vs*. separated male and female gonads could not be univocally reconstructed because of the early branching in the phylogenetic tree (Jondelius et al. 2011) of the species having sacular and mixed gonads.

Most acoels with paired testes have two separated germinal zones. Only three species have paired testes in which the two testes originate from one germinal zone: *Raphidophallus actuosus, Symsagittigera psammophila* and *Pelophila lutheri*.

Ancestral state reconstruction appoints the direction of maturation of the subsequent stages of spermatogenesis from dorsolateral to the ventral side. However, this is only based on the limited number of species which we studied ultrastructurally.

GENERAL DISCUSSION

STRUCTURE OF THE TESTES

Gonads are epithelially lined cavities into which mature gametes are released (Smith & Tyler 1985). These gametes originate mostly from the germinative epithelium, but in some cases, this epithelium is discontinuous or even absent, leaving the extracellular matric (ECM) as the only border of the gonad (Schmidt-Rhaesa 2007). In Acoela, this ECM is also lacking.

The variability of the testis structure within Acoela is enormous, but two types of organization appear to be most common. The first type is compact, asacular, non-follicular and paired. These testes are situated dorsally in the body and are completely separated from the ovaries, as found in *I. pulchra* (Boone et al. 2011) or *P. karlingi* (fig. 1). In this type, the cells mature from the dorso-lateral side to the ventral side. In the second type, the testes are follicular, asacular and situated in two lateral strands on the dorsal side, as found in *C. macroposthium* (fig. 3) and *C. crassum. A. beklemishevi* represents another type of testis: spermatids originating from one spermatogonium are joined by a cytophore although the testes are non-follicular. They are diffuse, paired and asacular. Maturation proceeds comparable to *I. pulchra* and *P. karlingi*. It is remarkable that the species that branches the earliest, *Diopisthoporus longitubus* has a completely different testes: an unpaired, mixed male

and female germinative zone, after which a dorsal testicular part and a ventral ovarian part separate. The germinative zone is bordered by parenchymal remnants that form a membrane. Mamkaev (1986) remarked that the highest morphological diversity often is observed in groups at the base of large phylogenetic branches where a particular new pattern of body organization was formed. However, the presence of a remarkable different testes morphology for the early branching taxa is problematic to come to a reliable ancenstral state reconstruction.

In contrast to Acoela, Nemertodermatida do have lining cells surrounding developing sperm cells (Tyler & Rieger 1977, Lundin & Sterrer 2001, Boone et al. 2011c). *F. apelti* has unpaired, follicular testes in which each of the follicles is surrounded by lining cells (Boone et al. 2011c). *N. westbladi* has paired, sacular and follicular testes.

As is the case in Acoela, the structure of the testes in Platyhelminthes can also vary greatly between and within taxa (Rieger et al. 1991): representatives of Catenulida lack sacular gonads (Ehlers 1985), representatives of Macrostomida have compact, paired and sacular testes (Rieger et al. 1991). Some Polycladida & Prolecitophora have a mixed germinal zone, their testes can be diffuse or compact, follicular or non-follicular (Rieger et al. 1991). Representatives of Seriata, Lecitoepitheliata, Rhabdocoela and Temnocephalida have sacular testes, which are mostly paired and compact.

Most acoels resemble Cnidaria and Ctenophora in having asacular testes. Cnidaria and Ctenophora are hermaphroditic animals in which gonads are merely accumulations of sex cells in definite sites (Hyman 1940). Mature sex cells are discharged through the mouth except in *Coeloplana* and *Ctenoplana*, in which the testes open at the aboral surface via ducts (Hyman 1940). However, cnidarian gametogenic areas are bordered by unspecialized epithial cells, which is not the case in acoelan testes. Scyphozoans can have follicular testes, which are continuous with the genital epithelium, thus differing from acoels with follicular testes, which are mostly asacular (Pianka 1974). Diopisthoporidae, the most early branching taxon of Acoela, have mixed gonads, which give rise to both male and female germ cells. A mixed gonad is also present in some ctenophorans.

In contrast to Acoela, most other bilaterian animal taxa have “true” testes: epithelial bordered organs surrounding the gametes (Schmidt-Rhaesa 2007). However, the epithelium surrounding the gonads is found to be discontinuous in Tardigrada and absent in members of Rotifera and Acanthocephala.

SPERMATOGENESIS

The complete spermatogenesis and the ultrastructure and organization of the testes is described only for *Isodiametra pulchra* and the species presented here (Boone et al. 2011 and this study). Studies of the spermiogenesis have been conducted in several acoels (Raikova and Justine 1994, Raikova et al. 1997, Raikova and Justine 1999). Spermatogonia seem to have a quite uniform morphology in all species studied: a high nucleo-cytoplasmic ratio, small and isolated clumps of heterochromatin, and an undifferentiated cytoplasm containing ribosomes and mitochondria. The nucleus of primary spermatocytes is characterized by the presence of synaptonemal complexes, scattered clumps of heterechromatine, and a round-ovoid shape. The cytoplasm contains mitochondria, ribosomes and rough endoplasmatic reticulum. Secondary spermatocytes are morphologically more differentiated than primary spermatocytes: the chromatine in their nucleus is more condensed and the first cytoplasmic granules appear during this stage (fig. 1, 2, Boone et al. 2011a). Raikova *et al.* 1997 observed granules in primary spermatocytes, although it is not clear from their figures whether it is a primary or secondary spermatocyte. In Nemertodermatida, the first granules also appear in secondary spermatocytes (Boone et al. 2011b). During the spermatid stage of acoel spermatogenesis, both nucleus and cytoplasm undergo transformation in order to form filiform spermatozoa with an elongated electron-dense nucleus. In all species studied on the ultrastructural level, the free flagella are incorporated into the cytoplasm as axonemata during this stage and one or two types of cytoplasmic granules can be found (Raikova & Justine 1994, Raikova et al. 1997, Raikova & Justine 1999, Boone et al. 2011 a). All mature acoel spermatozoa are filiform and have two incorporated axonemes (Hendelberg 1969, 1983, 1986) in which the microtubules can be arranged in a 9x2+2, a 9x2+0 or a 9x2+1 pattern. The cytoplasm contains axial or cortical microtubules, mitochondria and one or two types of

electrondense granules (Raikova & Justine 1994, Raikova et al. 1997, Raikova & Justine 1999, Raikova et al. 2001, Tekle et al. 2007, Boone et al. 2011 a).

Spermatogonia and primary spermatocytes in Acoela have a similar morphology to those stages of spermatogenesis in Nemertodermatida. Morphological differentiation becomes visible in secondary spermatocytes. From that stage on, acoelan spermatogenesis differs from that in Nemertodermatida. Mitochondria in nemertodermatid sperm cells are elongated and in some cases coiled (Boone *et al.* 2011c); the transformation from ovoid mitochondria to this special shape is visible during spermiogenesis. Secondary spermatocytes and spermatids are characterized by electron-dense granules in both Acoela and Nemertodermatida. However, granules differ in shape, and they fuse into granular derivatives in Nemertodermatida, which is not the case in Acoela. Spermatids and mature sperm in Acoela have accessory microtubules (axial or cortical), but these are lacking in Nemertodermatida. Spermatozoa in Acoela have two inverted and incorporated flagella, while they only have one free flagellum in Nemertodermatida, although aberrant biflagellate sperm cells can be found (Tyler & Rieger 1977, Lundin & Hendelberg 1998, Boone et al. 2011c).

CONCLUSIONS

During acoel spermatogenesis, morphological differentiation between species is visible from the secondary spermatocyte stage onwards. Acoelan testes differ greatly in morphology; hence there is no single model for the acoelan testes. This should be taken into account when using representatives of Acoela in overviews or comparative studies in a phylogenetic context. Different traits of the testes show similarities to testes in other taxa: asacular testes as found in Acoela are comparable to the asacular gonads in Ctenophora, Cnidaria, and Catenulida (Platyhelminthes). Follicular testes are also found in Platyhelminthes and Nemertodermatida. Our data demonstrate the complexity of acoelan testes and illustrate that inferences about the evolution of morphological traits in general should be taken with great caution and sufficient taxonomic sampling.

CHAPTER 6: GENERAL DISCUSSION

The main goals of this research are 1) increasing the general morphological knowledge on Acoela and Nemertodermatida, 2) inferring the evolution of the morphology of the testes in Acoela, and 3) comparing the morphology of the testes and spermatogenesis of Acoela and Nemertodermatida.

In order to reach these goals, we collected species of Acoela and Nemertodermatida from different locations (Boone et al. 2011b), performed ultrastructural studies on species of both groups (Boone et al. 2011a, 2011c, chapter 5), traced the evolution of traits related to the testes along the acoel phylogeny (as presented by Jondelius et al. 2011), and compared the ultrastructure of the testes and spermatogenesis in Acoela and Nemertodermatida (Boone et al. 2011c, chapter 5).

1. STRUCTURE OF THE TESTES

Within Nemertodermatidae, *Meara stichopi, Sterreria psammicola and Nemertinoides elongatus* have paired and follicular testes and lining cells are present (Lundin 1999). *Nemertoderma westbladi* (and *N. bathycola*) have paired, compact and globular testes, with flattened lining cells. Within the globular testes, different follicles can be discerned. Representatives of Ascopariidae, the other nemertodermatid family, appear to have a single median, follicular testes. No specialized lining cells were observed, but the scattered follicles are surrounded by gut cells and parenchymal cells (Boone et al. 2011c). Although the two families within Nemertodermatida have different types of testes, the morphology of the testes is uniform within each group.

Within Acoela, *Diopisthoporus longitubus* has a compact gonad with a mixed germinal zone, where oocytes (ventrocaudal) and spermatocytes (dorsocaudal) are produced. More caudally, the gonad differentiates into two parts: a ventral ovary and a dorsal testis. This testis is connected with the copulatory organ through a genital canal (Westblad 1942). No true lining exist, but there is a compaction of surrounding parenchymal cells.

Haploposthia brunea (Proporidae) is a species with unpaired gonads and a common male and female germinative zone, but sperm cells travel through gaps in the parenchyma to the copulatory organ, not in a specialized canal (Westblad 1942).

Hofstenia miamia has numerous, dorsofrontal testes follicles, from where the sperm cells scatter to a ventrofrontal seminal vesicle. *Childia groenlandica* and *Convoluta convoluta* have paired, asacular, follicular testes.

Isodiametra pulchra has testes that are paired, compact, non-follicular and located dorsally. Maturation of sperm cells proceeds from the dorso-lateral to the ventral side (Boone et al. 2011a). *I. pulchra* is an acoel species that is often used in studies on development, regeneration and stem cells system (De Mulder et al. 2009, Egger et al. 2009, Moreno et al. 2010). Therefore, knowledge of its morphology on the ultrastructural level is important to be able to interpret the results of the developmental and regenerational studies. Using BrdU-labelling of cells in the S-phase of the cell cycle in *I. pulchra*, De Mulder et al. (2009) and Egger et al. (2009) showed that stem cells appear on the lateral sides of the body and this location corresponds with the position of the early stages of spermatogenesis in this species (Boone et al. 2011a). However, the exact spatio-temporal relation of stem cells and spermatogonia still remains to be disentangled. Are the neoblasts the progenitors of the germ line? This could be tested by labeling stem cells on ultrathin sections using immunogold stainings. As BrdU and piwi-like genes label both somatic and germ line stem cells in *I. pulchra,* another marker should be used to unravel the difference between both cell types. As *I. pulchra* does not branch early in the acoel phylogenetic tree, it would also be very interesting to perform these labeling methods on representatives of acoel families with a radically different testes morphology that do branch more early, such as Diopisthoporidae, Paratomellidae, Hallangidae and Hofsteniidae. Such an approach could clarify the origin of the germ line in this phylogenetically important taxon and our data can provide a foundation for such functional approach within this framework.

For the interpretation of the structure of the testes of Acoela and Nemertodermatida, both hermaphrodite taxa lacking a fossil record and to interprete the contemporary morphology within a phylogenetic framework, one should take into account the following points: 1) reproductive structures are subject to trade-offs caused by sexual conflict, especially in hermaphrodites; 2) morphological characters can easily be lost and potentially regained; 3) reproductive tissue (gametes and gonads) have different origins in different taxa (Schmidt-Rhaesa 2007) and this indicates that gonads evolved several times independently; 4) form reflects function but not necessarily

evolution. Furthermore, in Acoela (and probably also in Nemertodermatida) testes can degenerate during a period of starvation. Moreover, in other organisms, such as fish, it is known that testes morphology can change during the live cycle of the organism (Rey-Vazquez et al. 2012). From our studies, it becomes clear that morphology of the testes is much more complex than generally assumed. The acoelan ancestor probably had non-follicular and compact testes, but it remains unclear whether or not the testes in this ancestor were epithelialized, and whether or not they were paired, as the ancestral states of these characters could not be determined. Acoels that are often used in phylogenomic studies, are representatives of derived branches within the acoelan tree (e.g. *I. pulchra, Convolutriloba longifissura, Symsagittifera roscoffensis*). Our data on the testes of Acoela (chapter 5) shows that caution has to be taken when generalizing aspects of acoel morphology. A “model organism” such as *I. pulchra* (De Mulder et al. 2009) does not represent all acoels and current study expands our knowledge to increasingly distant relatives of this putative model organism.

Similar to some Acoela, gametes in Ctenophora and Cnidaria are generally concentrated in gametogenic areas without specialized lining. Nevertheless, Pianka (1974) mentions a thin testicular membrane surrounding the outer part of the gametogenic tissues. However, in their degree of specialization, organization, and compaction, gonadal tissues in some cases may merit consideration as organs, as ovarian and testicular tissues are separated by thin interstitial processes. In Ctenophora, spermatogonia occur in a localized part of the testis and give rise to patches of developing spermatocytes, all in the same stage of development (Pianka 1974). Near the onset of spermiogenesis, packets that are more mature are separated from less mature ones by interstitial processes of the digestive epithelium or small cells, a possible patway to form follicular testes.

The structure of the testes in Platyhelminthes differs greatly among different taxa: they can be sacular or asacular, compact or diffuse, paired or unpaired, follicular or non-follicular. Within several parallel evolutionary lines, the formation of a tunica-bounded, compact testis is evident. The principal unit within the male gonad appears to be primary follicles, which usually include a cytophore (Rieger 1991). The formation of these primary follicles is inherent to the process of spermatogenesis.

Although the variety in structure is large, the structure of the testes in a particular taxon of Platyhelminthes often seems uniform.

2. SPERMATOGENESIS AND SPERM MORPHOLOGY

Ultrastructure of spermatozoa is a useful character to unravel phylogenetic relations and several authors have studied mature sperm morphology in Acoela and Nemertodermatida (Hendelberg 1969, Tyler & Rieger 1975, Tyler & Rieger 1977, Raikova & Justine 1999, Raikova et al. 2001, Hooge et al. 2002, Petrov et al. 2004, Tekle et al. 2007).

Several authors have intensely studied mature sperm morphology. Sperm types can be correlated with the mode of fertilization (Franzén 1956): animals with external fertilization have round-headed sperm cells, while species with internal fertilization have many different morphologies. In fact, these spermatozoa are often so derived, they can hardly be compared between taxa (Schmidt-Rhaesa 2007). Despite their diverse morphologies in different taxa, sperm cells also show unity: most sperm cells contain a cilium to ensure motility, and one or more mitochondria to provide energy for the cilium. Furthermore, parallel trends are present among different animal taxa: the loss of acrosomal vesicles, spiralization, and elongation. We observed these trends also in Acoela en Nemertodermatida (Boone et al. 2001a, 2011c, chapter 5).

Most Acoela are hermaphrodites, hence subject to sexual conflict. It is known that actin-reinforced parts of the female organs form a bottle-neck through which sperm has to pass in order to fertilize eggs and to avoid digestion by the seminal bursa (Achatz et al. 2010), leading to competition between sperm cells. This competition results in thinning of mature sperm cells, either through the loss of axonemes, the reduction of the number of cytoplasmic microtubules or the replacement of cortical microtubules by axial microtubules. A co-evolution of female accessory organs and sperm ultrastructure was observed in Acoela (Achatz et al. 2010).

Spermatogenesis describes the complete process of forming mature, haploid spermatozoa from diploid spermatogonia through meiotic division. Although spermiogenesis only studies the differentiation of haploid spermatids into mature haploid spermatozoa, studies on spermiogenesis in animal taxa are more common than studies on spermatogenesis. In current study, the complete process of

spermatogenesis was analysed in Acoela and Nemertodermatida. We observed that cytoplasmic morphological differentiation of the cells in these taxa start in secondary spermatocytes or early spermatids (Boone et al. 2011a, 2011c, chapter 5), as they contain granules that are also found in mature spermatozoa. It is pivotal to recognize these early (and later) stages of spermatogenesis as their location determines the architecture of the testes.

3. PHYLOGENETIC POSITION OF ACOELA AND NEMERTODERMATIDA

Acoela and Nemertodermatida share a neoblast system that is responsible for development, homeostasis, growth and regeneration with Platyhelminthes (Egger et al. 2009, De Mulder et al. 2009). However, this neoblast system could be an example of convergent evolution, as the general morphology of Acoela and Nemertodermatida shows clear differences from Platyhelminthes on the ultrastructural level. Acoela and Nemertodermatida have the following characters in common which differ from Platyhelminthes: the ciliary rootlet system, the presence of a frontal organ, the absence of protonephridia and the presence of pulsatile bodies. Testicular morphology in Platyhelminthes is as diverse (Rieger et al. 1991) as in Acoela and the historical placement of Acoela and Nemertodermatida within Platyhelminthes is not surprising. Despite the uncertainty about the exact phylogenetic position of Acoela and Nemertodermatida, the molecular and morphological evidence clearly separate these taxa from the Platyhelminthes.

Acoela differ from Nemertodermatida in gut morphology, structure of the statocyst, ultrastructure of the sperm and lining of the male gonads. Mixed gonads are found in Acoela but not in Nemertodermatida. The cleavage of early embryos of *N. westbladi* initially show radial cleavage, after which the micromeres are shifted clockwise generating a spiral pattern. Only in the four-cell-stage, the cleavage resembles that of Acoela (Jondelius et al. 2004). Comparison of Acoela and Nemertodermatida with other animals is hampered by the fact that several structures are lacking and it is not clear whether this absence in different taxa implies homology or not. This is the case when comparing testes of Acoela and Nemertodermatida, where most representatives lack lining cells (Boone et al. 2001a, 2011c, chapter 5).

Many features of bilaterians that are absent in Acoela are also missing in Cnidaria and Ctenophora: a through gut, a centralized nervous system (although Bery et al. (2010) describe a central brain in a juvenile acoel), protonephridia. Therefore, the lack of these features may be ancestral for Acoela (Bourlat & Hejnol 2009). Extracellular matrix (ECM), which lacks in Acoela, is present in sponges; and it can therefore be assumed that the absence or scarcity of ECM in acoels is a derived state. According to Smith and Tyler (1985), the body wall, parenchyma and digestive tract of Acoela are also derived and are thus not concordant with a model of primitiveness. However, derived features (of Acoela) do not necessarily conflict with an early branching phylogenetic position. All extant animals feature a mix of ancestral and derived characters, as they have been adapting to their environment (Schmidt-Rhaesa 2007). An undifferentiated morphology can be both basal and derived (through secondary loss). Mamkaev (1986) stated that Acoela have this mix of derived (ciliary axonemal and rootlet structures) and primitive features (gut, gonads, nervous system) and that they exhibit many stages in the formation of fundamental organ systems of Metazoa.

Although Hyman (1940) calls her diagram depicting the relationships of the animal phyla merely suggestive, it is remarkable that she places acoel flatworms at the base of the Bilateria, based on her morphological observations of Acoela.

A possible synapomorphy linking Acoela and Nemertodermatida with *Xenoturbella* is the shelf-like termination of the epidermal cilia (Ax 1996, Ehlers and Sopott-Ehlers 1997). In *Xenoturbella bocki* no delimited gonads or organs associated with germ cell development have been found, diffuse spermatid clusters occur in the parenchymal or gastrodermal tissue (Obst et al. 2011). There are no reports on the early stages of spermatogenesis. Given these fragmental data, it is not possible to compare testicular morphology of Xenoturbellida and Acoelomorpha (Acoela and Nemertodermatida).

Philippe et al. (2011) used 3 data sets (mitochondrial genes, a phylogenomic set of amino-acid positions and microRNA) to support a position within the Deuterostomia for acoelomorpha and *Xenoturbella*. This implies that Xenacoelomorpha have lost characters present in the common ancestor of deuterostomes: a through gut, protonephridia and a central nervous system (Maxmen 2011). Edgecombe et al.

(2011) recognize a sister-group relationship between Acoelomorpha and Nemertodermatida, but acknowledge the debated position of Acoelomorpha and Xenoturbellida, which can be either as a sister group of the Nephrozoa or within the Deuterostomia. As no clear morphological characters are shared between Deuterostomia and Acoelomorpha and Xenoturbellida, a position within Deuterostomia would be imply secondary loss of different structures within Acoelomorpha and Xenoturbellida. Testes in Echinodermata and Enteropneusta are always epithelialized, sac-like structures, in which the gametes originate from germinal epithelium (Schmidt-Rhaesa 2007 and references there). Hence their morphology differs from what we observed in Acoela and Nemertodermatida.

Our data on the structure of the testes and spermatogenesis in Acoela and Nemertodermatida are not sufficient to clarify the position of Acoela and Nemertodermatida within the phylogeny of Metazoa.

4. IMPORTANCE OF MORPHOLOGICAL STUDIES

Hypotheses on phylogenetic relationships are currently largely based on molecular analyses, while morphological data are usually mapped on molecular trees, in order to infer the evolution of character states. Nevertheless, the use of morphology in a molecular millennium can still be essential to obtain a powerful phylogenetic hypothesis (Jenner 2004). Both molecular and morphological data are prone to weaknesses, leading to incongruent results and this is especially true for the groups studied here, given their unsettled position.

Sufficient taxon sampling is a crucial step. Collection of material is an evident prerequisite to perform both morphological and molecular studies, as an insufficient number of sampled taxa will lead to biased conclusions. However this is not straightforward for Acoela and Nemertodermatida (Wallberg 2009). In our studies, species of Acoela and Nemertodermatida were collected by using the Van Veen grab on different sites in Belgian marine waters (Boone et al. 2011b) and by dredging in the fjord near Kristineberg, Sweden (Boone et al. 2011c and chapter 5). Although the Belgian marine waters are an intensely studied habitat, Nemertodermatida were never observed before (Boone et al. 2011b). Curini-Galletti et al. (2012) also note that for these taxa, a high number of new, undescribed species can be found, even in

well-studied areas. This shows that sampling technique is pivotal for the retrieval of a given group. Smith and Turner (2005) have put it this way: even a cursory review of many current morphological and phylogenetic based research programs and journals would reveal that a great deal of the useful morphological diversity of the earth's biota, present and past, has not been scrutinized. Although several species new to the Belgian marine waters could be found using appropriate techniques, not all of the collected specimens were adults, which not only implied the absence of testes (and ovaries) but also made morphological identification impossible as the reproductive organs are pivotal for the identification of specimens of Acoela and Nemertodermatida (see also Curini-Galletti et al. 2012). Even if adults can be retrieved, a few specimens are insufficient to perform a comprehensive ultrastructural study. Only for *I. pulchra*, an acoel that can be cultured in the lab, different fixation methods for transmission electron microscopy could be tested, resulting in a more optimal protocol for fixing, preparing and sectioning. This was not possible for sampled individuals which resulted in a non-optimal fixation of some specimens collected, making them inadequate for detailed ultrastructural studies. Additional embedded material of acoel species was sent by colleagues, but specimens often appeared to be either immature, or not ideally fixed to enable detailed ultrastructural research of the testes and the different stages of spermatogenesis. Based on their phylogenetic position in combination of what is already known, it would be valuable to perform also ultrastructural studies on representatives of following families: Diopisthoporidae, Paratomellidae, Hallangidae and Hofsteniidae. However, as it is unknown if these species can be cultured, it will be challenging to collect them in sufficient numbers.

Nevertheless, detailed morphological studies are very time-consuming; material must be fixed, embedded and sectioned before morphological descriptions and comparisons can be made. When morphological characters are used in a phylogentic framework, character coding is a challenging step. Defining characters is crucial, but morphological characters are often ambiguous (Jenner 2004, Schmidt-Rhaesa 2007). As form reflects function, convergent evolution leads to similar structures in different taxa. Characters that are absent can be the result of a primary absence or a secondary loss. Especially for Acoela, which lack numerous structures, this is important to keep in mind. However, convergent evolution is present in both

morphological and molecular data, and molecular studies can be biased by contamination of the studied specimens, or by several artefacts, including long-branch attraction.

However, despite problems in both molecular and morphological studies, it is important to perform both and to combine data generated by both approaches. A combined approach can provide an improved insight into the evolution of animals, at least by reciprocal illumination. A backbone of molecular data can be used to map morphological information (Schmidt-Rhaesa 2003). Our data show that architecture of testes in Acoela (and Nemertodermatida) is composed of several characteristics (paired-unpaired; diffuse-compact; follicular-non-follicular; sacular-asacular; direction of maturation; Boone et al. 2011a and chapter 5). Mapping these character states onto a phylogenetic framework revealed that several of these characters evolved several times independently and that a “standard acoel testis” is an oversimplification. The testes in the ancestral acoel were probably non-follicular and compact, but concerning the position in the body, the paired vs. unpaired condition, and the presence or absence of lining cells, no inferences about the ancestral state could be made (chapter 5). This is due to the fact that representatives of families that branch early in the acoelan phylogenetic tree (Diopisthoporidae, Paratomellidae and Hofsteniidae) are radically different from the other taxa.

When morphological and molecular data conflict, it might be interesting to use a functional approach when it comes to comparative morphology, as function is key to the conservation of morphological traits. In the case of the testes morphology, this would mean studying fecundity and reproduction, potentially combined with silencing of germ line genes. Jenner (2006) puts it this way: it is the challenge of the new microscopy to find the unity underneath the diversity.

5. General conclusion

The testicular morphology in Acoela and Nemertodermatida is very diverse and complex. Our data show that caution should be taken when generalizing aspects of the morphology of these phylogenetically intriguing taxa.

1

CHAPTER 7: SUMMARY

The goals of this thesis are 1) increasing the general and detailed morphological knowledge on Acoela and Nemertodermatida, 2) inferring the evolution of the morphology of the testes in Acoela, and 3) comparing the morphology of the testes of Acoela and Nemertodermatida.

In order to reach the first goal, we sampled for representatives of Acoela and Nemertodermatida, fixed and prepared them for ultrastructural research. One of the sampling campaigns took place in the Belgian part of the North Sea, where we found following species of Nemertodermatida: *Flagellophora apelti*, *Sterreria psammicola*, *Nemertinoides elongates* and several specimens that could not be identified.

The ultrastructure of the testes in two representatives of Nemertodermatida was studied using transmission electron microscopy. *Nemertoderma westbladi* has testes which are delineated by lining cells. The testes are paired and different follicles surrounded by a membrane-like structure are found in each testis. *F. apelti* has several testes follicles, each containing a certain stage of spermatogenesis. A true gonad lacks in this species, as the gametes are not enclosed by a membrane or cell layer. Each stage of spermatogenesis in *F. apelti* is described, enabling the inference of the origin of the structures seen in mature spermatozoa. The overall structure of the mature spermatozoa is similar in all nemertodermatids and unique within the Metazoa: an elongated head containing the nucleus; a middle piece containing an axoneme, mitochondrial derivatives and in *F. apelti* granular derivatives; and a flagellar tail.

Spermatogenesis and the structure of the testes were studied ultrastructurally in following acoels: *Isodiametra pulchra*, *Childia macroposthium, Philocelis karlingi, Actinoposthia beklemishevi* and *Paratomella unichaeta*. All stages of spermatogenesis, including spermiogenesis, are described at the ultrastructural level (except for *P. unichaeta*) and their spatial organization within the testes is discussed.

Furthermore, 122 species of Acoela were studied using light microscopy and literature data. The resulting dataset was analysed within an established phylogenetic framework (as presented by Jondelius et al. 2011). We hypothesize that the acoelan ancestors had non-follicular and compact testes. Follicular testes and

diffuse testes have evolved several times independently troughout acoelan evolution. It remains unclear if the testes in the ancestral acoel were paired or unpaired, sacular or asacular and what their location was within the body (ventral, dorsal or median).

Finally, the male germ line in Acoela and Nemertodermatida was compared with related taxa. Testes and spermatogenesis in Acoela and Nemertodermatida show similarities but also distinct differences. Our detailed ultrastructural studies reveals part of the complexity of testicular morphology, which is more diverse than has been thought and illustrates that inferences about the evolution of morphological traits should be taken with great caution and sufficient taxonomic sampling.

CHAPTER 7: SAMENVATTING

De doelstellingen van deze thesis zijn 1) het vergroten van de algemene en de gedetailleerde morfologische kennis over Acoela en Nemertodermatida, 2) de evolutie nagaan van de bouw van de testes in Acoela, en 3) de morfologie van de testes van Acoela en Nemertodermatida vergelijken.

Om de eerste doelstelling te bereiken, gingen we via staalnames op zoek naar vertegenwoordigers van Acoela en Nemertodermatida, om deze te fixeren en klaar te maken voor ultrastructureel onderzoek. Eén van de staalnamecampagnes vond plaats in het Belgische deel van de Noordzee, waar we volgende soorten Nemertodermatida vonden: *Flagellophora apelti*, *Sterreria psammicola*, *Nemertinoides elongates* en verschillende specimens die niet konden worden geïdentificeerd.

De ultrastructuur van de testes in twee vertegenwoordigers van Nemertodermatida werd bestudeerd met behulp van een transmissie electronenmicroscoop. *Nemertoderma westbladi* heeft testes die zijn afgelijnd door cellen. De testes zijn parig en in elke testis zitten verschillende follikels die omgeven worden door een membraanachtige structuur. *F. apelti* heeft verschillende testesfollikels, die elk een bepaald stadium van spermatogenese bevatten. Een echte gonade ontbreekt bij deze soort, omdat de gameten niet ingesloten worden door een membraan of een cellaag. Elk stadium van spermatogenese in *F. apelti* wordt beschreven, waardoor het mogelijk wordt de oorsprong van de structuren uit de volwassen spermatozoa te achterhalen. De algemene structuur van de volwassen spermatozoa is gelijkaardig voor alle nemertodermatiden en uniek binnen de Metazoa: ze hebben een verlengd kopstuk dat de kern bevat, een middenstuk met daarin een axoneem, structuren afgeleid van mitochondria en structuren afgeleid van granules (deze laatste enkel bij *F. apelti*), en een staartflagel.

Spermatogenese en de structuur van de testes werden ultrastructureel bestudeerd in volgende acoelensoorten: *Isodiametra pulchra*, *Childia macroposthium, Philocelis karlingi, Actinoposthia beklemishevi en Paratomella unichaeta*. Alle stadia van spermatogenese, inclusief spermiogenese, werden beschreven op ultrastructureel niveau en hun ruimtelijke organisatie binnen de testes werd bediscussieerd.

Om de tweede doelstelling te bereiken, werden 122 soorten Acoela bestudeerd aan de hand van lichtmicroscopie, en werd heel wat wetenschappelijke literatuur geraadpleegd. Dit alles om testeskenmerken te kunnen plotten op een bestaand fylogenetisch kader (fylogenetische boom opsteld door Jondelius et al. 2011). We stellen als hypothese dat de stamvader van de Acoela niet-follikulaire en compacte testes had. Follikulaire en diffuse testes zijn verschillende keren onafhankelijk in de evolutie van acoelen ontstaan. Het blijft onduidelijk of de testes in de vroegste acoel parig of onpaar waren, en waar in het lichaam ze zich bevonden (ventraal, dorsaal of mediaan).

De derde doelstelling is de vergelijking van de mannelijke germinale lijn in Acoela en Nemertodermatida en aanverwante taxa. Testes in Acoela en Nemertodermatida tonen zowel gelijkenissen als verschillen. Onze gedetailleerde ultrastructurele studies ontrafelen een deel van de complexiteit van de testesbouw, en tonen dat het niet vanzelfsprekend is om conclusies te trekken over de evolutie van morfologische kenmerken.

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APPENDIX 1: LIST OF SECTIONS STUDIED BY LIGHT MICROSCOPY AT THE SWEDISH MUSEUM OF NATURAL HISTORY

Species	Slide number (SMNH)
Avagina marci	2732
Avagina marci	92890
Childia groenlandica	52235
Childia groenlandica	52236
Childia groenlandica	52240
Childia groenlandica	92554
Conaperta divae	2733
Convoluta convoluta	90311
Convoluta convoluta	90313
Convoluta convoluta	90316
Convoluta vexillaria	2735
Diopisthoporus gymnopharyngeus	3121
Diopisthoporus gymnopharyngeus	3121
Diopisthoporus longitubus	2502
Diopisthoporus longitubus	90377
Diopisthoporus longitubus	90427
Diopisthoporus longitubus	90377
Diopisthoporus longitubus	90378
Diopisthoporus longitubus	90367
Diopisthoporus longitubus	90407
Diopisthoporus longitubus	90409
Diopisthoporus longitubus	2502
Faerlea fragilis	90444
Faerlea fragilis	91828
Faerlea glomerata	90448
Faerlea glomerata	90450
Faerlea glomerata	90452
Faerlea glomerata	90454
Faerlea glomerata	90455
Halangia proporoides	2503
Halangia proporoides	90471
Halangia proporoides	90472
Halangia proporoides	90473
Halangia proporoides	90474
Halangia proporoides	90476
Haplocelis dichona	2739
Haplodiscus bocki	90701
Haplodiscus bocki	90705
Haploposthia albiventer	2742
Haploposthia albiventer	49203
Haploposthia albiventer	49204

Haploposthia lactomaculata	5880
Haploposthia lactomaculata	5881
Haploposthia microphoca	2743
Haploposthia monogonophora	2505
Haploposthia monogonophora	94269
Haploposthia monogonophora	94262
Haploposthia monogonophora	94263
Haploposthia rubra	49207
Haploposthia rubra	49208
Haploposthia rubra	49213
Haploposthia rubra	49206
Haploposthia rubra	49207
Haploposthia rubra	49211
Haploposthia rubra	49213
Haploposthia rubra	49210
Haploposthia rubropunctata	2506
Haploposthia rubropunctata	49255
Haploposthia rubropunctata	49270
Haploposthia rubropunctata	49267
Haploposthia rubropunctata	49278
Haploposthia rubropunctata	49256
Haploposthia rubropunctata	49257
Haploposthia rubropunctata	49259
Haploposthia viridis	49302
Haploposthia viridis	49312
Haploposthia viridis	49310
Hesiolicium inops	3057
Hofstenia atroviridis	2805; 2805a
Hofstenia atroviridis	74909
Hofstenia atroviridis	74912
Hofstenia miamia	89913
Hofstenia miamia	89913
Hofstenia miamia	2744; 2744a
Marcusiola tinga	2747; 2747a
Notocelis gullmarensis	2511
Notocelis gullmarensis	74931
Oligofilomorpha karlingi	2516
Oligofilomorpha karlingi	2516
Otocelis dichura	74968
Otocelis dichura	74969
Otocelis dichura	74971
Otocelis dichura	74974
Otocelis rubropunctata	74916
Otocelis rubropunctata	74917
Otocelis westbladi	2512
Otocelis westbladi	74920
Otocelis westbladi	74921

Otocelis westbladi	74923
Otocelis westbladi	74925
Otocelis westbladi	74928
Paraproporus rubescens	2489
Paraproporus rubescens	91729
Paraproporus rubescens	91737
Paraproporus rubescens	91730
Paraproporus rubescens	91732
Paraproporus rubescens	91738
Pelophila lutheri	91789
Pelophila lutheri	91793
Pelophila lutheri	91801
Pelophila lutheri	91802
Philocelis karlingi	2513
Philocelis karlingi	74941
Philocelis karlingi	74942
Philocelis karlingi	74943
Proporus brochii	69722
Proporus brochii	69725
Proporus brochii	2514
Proporus brochii	69721
Proporus brochii	69730
Proporus brochii	69722
Proporus brochii	69728
Proporus brochii	69727
Proporus lonchitis	2515
Proporus lonchitis	69731
Proporus lonchitis	69733
Proporus lonchitis	69732
Proporus minimus	69736
Proporus venenosus	69737
Proporus venenosus	69738
Pseudohaplogonaria viridipunctata	2507
Pseudokuma orphinum	2755

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APPENDIX 2: LIST OF PUBLICATIONS CONSULTED CONCERNING THE STRUCTURE OF THE TESTES

Species	Reference
Actinoposthia haplovata	Dörjes 1968
Amphiscolops bermudensis	Dörjes 1968
Anaperus biaculeatus	Boguta 1970
Anaperus gardineri	Dörjes 1968
Anaperus rubellus	Westblad 1945
Anaperus singularis	Hooge & Smith 2004
Anaperus tvaerminnensis	Westblad 1945
Aphanostoma bruscai	Hooge & Tyler 2003
Aphanostoma collinae	Hooge & Tyler 2008
Aphanostoma virescens	Dörjes 1968
Archaphanostoma macrospiriferum	Dörjes 1968
Atriofronta polyvacuola	Dörjes 1968
Avagina marci	Westblad 1946, 1948
Childia brachyposthium	Westblad 1942, Dörjes 1968
Childia cycloposthium	Westblad 1942, Dörjes 1968
Childia groenlandica	Dörjes 1968
Childia submaculatum	Westblad 1942, Dörjes 1968
Childia trianguliferum	Westblad 1942, Dörjes 1968
Childia vivipara	Tekle et al. 2006
Convoluta convoluta	Dörjes 1968
Convolutriloba hastifera	Winsor 1990
Convolutriloba longifissura	Bartolomaeus & Balzer 1997
Convolutriloba macropyga	Shannon & Achatz 2007
Convolutriloba retrogemma	Hendelberg & Akesson 1988
Daku woorimensis	Hooge 2003
Diopisthoporus longitubus	Westblad 1940
Diopisthoporus psammophilus	Dörjes 1968
Endocincta punctata	Crezee 1975
Eumecynostomum altitudi	Faubel & Regier 1983
Faerlea glomerata	Westblad 1945
Hallangia proporoides	Westblad 1946, Dörjes 1968
Haplocelis dichona	Dörjes 1968
Haploposthia lactomaculata	Tekle 2004
Haploposthia rubra	Westblad 1946, 1948
Haploposthia vandula	Hooge & Tyler 2001
Heterochaerus blumi	Achatz et al. 2007
Hofstenia miamia	Correa 1960
Hofsteniola pardii	Dörjes 1968
Isodiametra bajaensis	Hooge & Eppinger 2005
Isodiametra cuernos	Hooge & Tyler 2008
Isodiametra divae	Marcus 1950
Isodiametra hortulus	Hooge & Tyler 2003

Isodiametra nicki	Hooge & Tyler 2008
Isodiametra norvegica	Westblad 1946
Isodiametra vexillaria	Dörjes 1968
Kuma albiventer	Marcus 1954
Kuma viridis	Westblad 1948
Mecynostomum auritum	Westblad 1946, Dörjes 1968
Neochildia fusca	Bush 1975
Notocelis gullmarensis	Westblad 1946, Dörjes 1968
Oligofilomorpha interstitiophilum	Faubel 1974
Otocelis erinae	Hooge & Rocha 2006
Otocelis sandara	Hooge & Tyler 2003
Paedomecynostomum bruneum	Dörjes 1968
Paramecynostomum diversicolor	Dörjes 1968
Paratomella rubra	Crezee 1978
Paratomella unichaeta	Dörjes 1966
Pelophila lutheri	Westblad 1946
Philactinoposthia coneyi	Hooge & Rocha 2006
Philactinoposthia saliens	Westblad 1946, Dörjes 1968
Philomecynostomum tapillum	Dörjes 1968
Philocelis brueggemanni	Hooge & Tyler 2003
Philocelis robrochai	Hooge & Rocha 2006
Polycanthus torosus	Hooge 2003
Postmecynostomum pictum	Dörjes 1968
Praeconvoluta bocasensis	Hooge & Tyler 2008
Praeconvoluta castinea	Hooge & Tyler 2003
Praeconvoluta tigrina	Hooge & Tyler 2003
Praeconvoluta tornuva	Tyler & Hooge 1999
Praesagittifera naikaiensis	Yamasu 1982
Proaphanostoma tenuissima	Westblad 1946
Proporus bermudensis	Hooge & Tyler 2001
Proporus brochii	Westblad 1946
Proporus carolinensis	Hooge & Smith 2004
Pseudactinoposthia sanguineum	Dörjes 1968
Pseudaphanostoma herringi	Hooge & Rocha 2006
Pseudaphanostoma smithrii	Hooge & Tyler 2003
Pseudaphanostoma syltensis	Dörjes 1968
Pseudmecynostomum bruneofilum	Faubel 1974
Pseudmecynostomum phoca	Hooge & Tyler 2003
Pseudohaplogonaria rodmani	Hooge & Tyler 2007
Raphidophallus actuosus	Kozloff 1965
Simplicomorpha gigantorhabditis	Dörjes 1968
Simplicomorpha viridis	Dörjes 1968
Stomatricha hochbergi	Hooge 2003
Symsagittifera corsicae	Gschwentner et al. 2002
Symsagittifera psammophila	Beklemishev 1957
Symsagittifera roscoffensis	Graff 1891
Waminoa brickneri	Ogunlana et al. 2005

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APPENDIX 3: LIST OF PUBLICATIONS

1. A1 PUBLICATIONS

- Willems M., Leroux F., Claeys M., **Boone M**., Mouton S., Artois T., Borgonie G. (2009). Ontogeny of the complex sperm in the macrostomid flatworm *Macrostomum lignano* (Macrostomorpha, Rhabditophora). Journal of Morphology 270 (2): 162-174.

- Willems M., **Boone M**., Couvreur M., De Mulder K., Van Ranst J., Artois T., Borgonie G. (2009). Use of freeze-cracking in ontogenetic research in *Macrostomum lignano* (Macrostomida, Rhabditophora). Development Genes and Evolution 219 (5): 273-279.

- Willems M., Couvreur M., **Boone M**., Houthoofd W., Artois T. (2010). Distribution of proliferation cells and vasa-positive cells in the embryo of *Macrostomum lignano* (Rhabditophora, Platyhelminthes). Belgian Journal of Zoology 140: 149-153.

- **Boone M**., Willems M., Claeys M., Artois T. (2011). Spermatogenesis and the structure of the testes in *Isodiametra pulchra* (Isodiametridae, Acoela). Acta Zoologica 92 (2): 101-108.

- **Boone M**., Houthoofd W., Bert W., Artois T (2011). First record of Nemertodermatida from Belgian marine waters. Belgian Journal of Zoology 141 (1): 62-64.

- **Boone M**., Bert W., Claeys M., Houthoofd W., Artois T. (2011). Spermatogenesis and the structure of the testes in Nemertodermatida. Zoomorphology 130 (4): 273-282.

2. SCIENTIFIC SERVICES

- Taxonomic validation of the species of the taxon Nemertodermatida for "Belgian Register of Marine Species", compiled and validated by the VLIZ Belgian Marine Species Consortium (2010). Leen Vandepitte, Wim Decock & Jan Mees (eds) VLIZ Special Publication, 46, Vlaams Instituut voor de Zee (VLIZ): Oostende, Belgium. 78 pp. ISBN 978- 90- 812900- 8- 1.

- Editor of the taxon Nemertodermatida for the World Register of Marine Species (http://www.marinespecies.org/)