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The ultrastructural characteristics of spermatogenesis in *Onchidium struma* (Pulmonata: Onchidiidae) and its functional adaptation

S.-H. CHEN¹, L.-P. XIA¹, H.-U. DAHMS²,³, X. PENG¹, & X.-P. YING¹*

¹College of Life and Environmental Sciences, Wenzhou University, Wenzhou, Zhejiang, China, ²Department of Biomedical Science and Environmental Biology, Kaohsiung Medical University, Kaohsiung, R.O.C., and ³Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung, Taiwan, R.O.C.

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Abstract

The ultrastructural characteristics of spermatogenesis of the mollusc *Onchidium struma* (Pulmonata: Onchidiidae) were studied by transmission electron microscopy. The spermatogenesis of *O. struma* is divided into five stages based on morphological changes of the nucleus, mitochondria and other organelles that include the spermatogonium, primary spermatocyte, secondary spermatocyte, spermatid and sperm. During sperm cell differentiation, the nucleus content is condensed from asymmetrical granules to flocculent-shape, filament-shape, long-filament-shape, uniform and finally dense granules. The shape of the nucleus is transformed from round to olive shaped, wing shaped and finally long cone shaped. There is a posterior nuclear fossa in the nucleus of the mature spermatozoon. During spermatogenesis, there are rich Golgi vesicles, endoplasmic reticula, endoplasmic reticulum vesicles and a great deal of mitochondria. The number of mitochondria increases at first and then decreases while the volume of mitochondria increases. The mitochondria crista increase in number and are fused. The distribution of mitochondria develops from random to polar, and the mitochondrial complex encloses around the axoneme at the final stage. The changes of nucleus and organelles during spermatogenesis are compared between *O. struma* and other gastropods, and its peculiarities for gastropod reproduction as well as physiological adaptations are discussed.

Keywords: *Onchidium struma*, spermatogenesis, spermatid, mollusc, ultrastructure

Introduction

*Onchidium struma* (Qiu Liyan, 1991) belongs to the Mollusca, Gastropoda, Pulmonata, Stylommatophora and Onchidiidae (Chen & Zhang 1999). *O. struma* is an endemic species of China and is distributed in the coastal and estuarine zone of the East China Sea and the South China Sea coast. They are usually found in mud flats of the intertidal and higher tidal zone, feeding on sediment and algae, and surviving when they fall dry for extended times (Wang et al. 2005a). Recently, the natural habitats of *O. struma* have been disturbed because of environmental pollution, and over-exploitation by human beings. These led to a decline of natural populations and even their vanishing from some coastal stretches of the Zhejiang south. There is a need to develop aquaculture methods for *O. struma*, but they should be accompanied by basic research in areas such as reproductive biology. Although investigators have studied basic reproductive biological aspects of *O. struma* which include its reproductive behaviour (Wang et al. 2005a), gonad development (Wang et al. 2006; Chen et al. 2010), sperm ultrastructure (Ying et al. 2008) and embryonic development (Wang et al. 2005b), the ultrastructural characteristics of spermatogenesis in *O. struma* have not been described.

Spermatogenesis and the morphology of sperm are often used in phylogenetic and evolutionary reconstructions because their characters are often taxon-specific and provide constitutive characters for species (Hodgson & Bernard 1988; Franco et al. 2008). Presently, there are several studies on the ultrastructure of spermatogenesis in gastropods, but these mainly focus on the prosobranchia (West 1978; Shiroya 1984; Jaramillo et al. 1986; Ke & Li
The particular aims of this study are to help clarify some aspects of spermatogenesis which is at the basis of reproductive biology of *O. struma*, and to find ultrastructural characters that can be used for phylogenetic analyses among the Pulmonata.

### Materials and methods

Fresh and mature adult specimens of *O. struma* used in this experiment were collected twice monthly from Long-gang culturing farm of Cangnan, Zhejiang province, China, from April to November in 2012 and April to July in 2013, according to the reproductive cycle of *O. struma* (Chen et al. 2010). *O. struma* is a hermaphrodite, and the reproductive system consists of three parts: hermaphroditic organs, the reproductive ducts and subsidiary glands (Wang et al. 2006; Chen et al. 2010). We collected 10 *O. struma* individuals each time and dissected them to isolate the hermaphroditic organs, which were dissected into 1 mm³ pieces immediately. For light microscopy, specimens were fixed in Bouin’s fluid and embedded in paraffin wax. Sections (7 μm) were stained with haematoxylin-eosin. Following staining, sections were dehydrated in increasing concentrations of ethanol and toluene, then coverslipped and analysed with an Olympus BX53 light microscope (Olympus, Japan). For transmission electron microscopy (TEM), the tissues were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.4) at 4°C and were post-fixed in 1% osmium tetroxide. After this procedure, the material was washed 3 times in distilled water for 5 min each and then dehydrated in a series of increasingly concentrated acetone solutions (30%, 50%, 70% and 90% acetone), with 15 min per solution, followed by dehydration in 95% and 100% acetone 3 times for 15 min each. After dehydration, the specimens were embedded in 618 Spurr’s resin for TEM analysis. Ultrathin sections were obtained by a Sweden LKB 2088 ultramicrotome, and then double stained with uranyl acetate and lead citrate. Thin sections were studied and photographed using a Hitachi-6500 transmission electron microscope.

### Results

The spermatogenesis in *O. struma* can be classified, according to shape, characteristic features and chromatin organisation, into five developmental stages, including spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and mature sperm (Figure 1A–C). They are organised in a way that the early stages (spermatogonia and primary spermatocytes) are lying on the outside of the testes (meaning at the side of the epidermis), while mature sperm can be found at the centre and inner side of the testes (Figure 1A).

**Spermatogonium**

The spermatogonium is more or less round, about 12–13 μm in diameter. The nucleus is ovoid and contains a nucleolus about 1 μm in diameter. Some heterochromatin is homogeneously dispersed throughout the nucleus, with scattered nucleoplasm and a clearly visible nuclear membrane. A small number of mitochondria and Golgi vesicles are also found in the cytoplasm here (Figure 2A).

**Primary spermatocyte**

The more or less round primary spermatocyte becomes slightly enlarged, about 15–17 μm in diameter, with an enlarging nucleus about 5–6.5 μm in diameter. The nucleus has already moved to one side of the cell and the nuclear membrane is clear, in which some of the chromatin becomes condensed to lumps. The organelles have a certain degree of polar distribution in the cytoplasm. The number of mitochondria increases. And the mitochondria are distributed on one side while the endoplasmic reticulum is distributed on the other side, indicating that the primary spermatocyte shows polarity (Figure 2B).

**Secondary spermatocyte**

After the first meiotic division, with its chromosomes halving, the primary spermatocyte turns into a secondary spermatocyte. The nucleus is divided into...
two parts, and then the cytoplasm becomes separated. Consequently, bi-nuclear cells or two cells connected with intercellular bridges appear (Figure 2C). Compared to the primary spermatocytes, the secondary spermatocytes are characterised by a smaller size (10~14 μm in diameter), and have a bigger semicircular nucleus (6.5~8 μm in diameter). Chromatin is heterogeneously distributed in clusters and the electron density is higher than that of the primary spermatocyte (Figure 2D). The structures of the organelles resemble those of the primary spermatocyte, and the organelles concentrate on one side of the cell, presenting a more polar distribution. There are many mitochondria in the cytoplasm; some mitochondria fuse and are in flat sacular shape whereas some mitochondria are still oval sacular (Figure 2D and E). Golgi bodies and a large number of Golgi vesicles are also found in the cytoplasm (Figure 2F). As we know, the Golgi bodies are responsible for protein modification. Golgi vesicles may contain modified proteins from the Golgi bodies.

**Spermatid**

Spermatids form when the secondary spermatocyte finishes the second meiotic division. During the process of differentiation from spermatid to mature sperm, the spermatid and nucleus begin to deform, the chromosome condenses, the base of the nucleus invaginates and the acrosome forms. The spermatid of *O. struma* can be divided into three stages: early, middle and late.

**Early-stage spermatid**

At early stage the spermatid is commonly of cylindrical shape with distinct membranes. The nucleus is rugby-ball shaped and smaller (3.3 μm × 1.2 μm to 4.2 μm × 1.5 μm in diameter) than that of the secondary spermatocyte. Chromosomes are filamenteous or flocculent and are distributed throughout the nucleus. The vesiculation phenomenon appears in certain parts of the nuclear envelope (Figure 3A). One side of the nucleus invaginates at the base, forming the posterior nuclear fossa. The bulk of Golgi vesicles get located at the other side. The majority of chromosomes in the nucleus attach to the inner side of the nuclear envelope, and the rest are in the central part. The centriole gradually moves to the base of the nuclear depression, and the axoneme is formed (Figure 3B). Abundant Golgi vesicles and endoplasmic reticulum vesicles appear on top of the nucleus, and at that time the proacrosomal vesicle is formed (Figure 3C). Many mitochondria with rich cristae concentrate on both sides of the axoneme, which locates at one side of the nucleus (Figure 3B and C).
Middle-stage spermatid

The shape of spermatids at the middle stage is irregular, and chromatin shows a filamentous distribution in the nucleus (Figure 3D–F). Proacrosomal granules appear in the various Golgi vesicles. These granules concentrate to form regular, round, fine outer membranes and the homogeneous electron-dense contents of proacrosomal granules (Figure 3D), forming...
Figure 3. The ultrastructure of early (A–C) and middle stage spermatid (D–F) of *Onchidium struma*. A, at the early spermatid stage, showing the nucleus, axoneme, centriole and mitochondria. B, at the early spermatid stage, showing the nucleus, Golgi vesicle, mitochondria, centriole, posterior nucleus fossa and axoneme. C, the oval-shaped nucleus of the early spermatid, showing the Golgi apparatus, Golgi vesicle and proacrosome vesicle. D, in the middle stage of spermatids, the nucleus, proacrosome granule, mitochondria and endoplasmic reticulum. E, showing the nucleus, proacrosome cap, Golgi vesicle and mitochondria of middle stage spermatid. F, the proacrosome cap, Golgi vesicle and mitochondria in the middle stage spermatid. Scale bars: A, 2 μm; B, 0.5 μm; C–F, 1 μm. Ax, axoneme; C, centriole; Er, endoplasmic reticulum; G, Golgi apparatus; Gv, Golgi vesicle; M, mitochondria; N, nucleus; Ne, nuclear envelope; Pac, proacrosome cap; Pag, proacrosome granule; Pav, proacrosome vesicle; Pnf, posterior nucleus fossa.
obvious proacrosomes as the number of proacrosomal granules apparently increases. At this time the posterior nucleus fossa is developed (Figures 3F and 4A).

Late-stage spermatid

As spermatids continue to differentiate, the nucleus transforms from kidney shape into long tubular shape. The proacrosome differentiates further to form horseshoe-shaped acrosomal vesicles buckling at one side of the nucleus (Figure 4B). The nucleus gets extended and thinner along the proacrosomal vesicle–centriole–mitochondrial direction as chromatin condenses to long filament shape, along the long-axis direction. As the differentiation advances, the nucleus continues its elongation, and long-filament-shaped chromatin swirls are observed from the cross sections. This transforms into highly electron-dense homogeneous material (Figure 4B–D). The posterior nuclear fossa becomes distinct (Figure 4C and E) and the nuclear vacuole appears (Figure 4F). With lasting differentiation, acrosomal vesicles turn into the acrosome, and the spermatids turn into sperm after abandoning redundant cytoplasm (Figure 4F).

Light microscopy observation showed that the sperm of *O. struma* exhibits a long, wave-shaped head and a long mid-piece forming the “spermatozoan tail”, and the average length of the mature sperm is about 437 µm (Figure 4C). TEM showed that the mature sperm consists of three parts: head, mid-piece and end-piece (Figure 4F–H). The head comprises a nipple-shaped acrosome and a long pillar-shaped nucleus (Figure 4F). The mid-piece is more complex. It is composed of a pair of axonemes, plasma membrane, mitochondria and mitochondrial derivative which including glycogen-filled helices, matrix materials and paracrystalline material (Figure 4G). The end-piece consists of a “9 + 2” axoneme and a plasma membrane (Figure 4H).

Discussion

During gastropod spermatogenesis, the morphology of the spermatogonium and spermatocyte shows little changes but the morphology of the spermatid exhibits considerable changes (Jaramillo et al. 1986; Ying et al. 2002; Chiva et al. 2011). Based on the observed results, different scholars have applied different methods to divide the different periods of spermatids in gastropods. Three ways are common: (1) according to the characteristics of acrosome evolution, spermatids can be divided into three stages, that is early-, middle- and late-stage spermatids, such as shown in *Bullacta exarata* (Philippi, 1849) (Ying et al. 2002), *Haliotis asinina* (Linnaeus, 1758) (Huang et al. 2006), *Neptunea cumingii* (Crosse, 1862) (Hou et al. 2006); (2) combining the changes of nucleus, chromatin and mitochondria, spermatids can be divided into five stages, such as in *Chorus gigarreus* (Lesson, 1879) (Jaramillo et al. 1986), *Haliotis diversicolor superstes* (Reeve, 1846) (Yan et al. 2006), *Trochus pyramis* (Born, 1778) (Wu et al. 2010); (3) based on the five stages and the morphological characteristics of spermatids, the spermatid differentiation process can be divided into six stages, such as in *Babylonia formosae* (G.B. Sowerby II, 1870) (Ke & Li 1992) and in *Cipangopaludina chinensis* (Gray, 1834) (Yan et al. 2004). We propose that the spermatid development be divided into three stages – early-, middle- and late-stage spermatid – according to the formation characteristics of the acrosome and posterior nuclear pocket, and changes in the shape and contents of the nucleus during spermatogenesis of *O. struma*.

The changes of nucleus and acrosome of gastropods and their functional adaptation

Significant changes of nuclear morphology and nuclear contents have been observed in spermatid differentiation of *O. struma*. The nucleus transforms from round to oval shape, wing shape and long cone shape at the final stage. This is definitely different from other gastropods (Table I). Chiva et al. (2011) suggested that the shape changes of the spermatid nucleus are related to the type of fertilisation. This means that the nucleus of gastropods is species-specific and it may be useful in classification. *O. struma* shows internal fertilisation, and the sperm is of a modified type (Ying et al. 2008). In the spermigenic chromatin condensation patterns, it appears that species with complex condensation patterns also exhibit internal fertilisation and modified nuclear sperm shapes (Chiva et al. 2011). Although nuclear morphology is critical for fertilisation, we know little about the molecular differences such as in histone and protamine which are key proteins for chromatin condensation. Further investigation should be carried out.

There are two mechanisms to determine nuclear morphology: one is the chromatin condensation, and the other is the role of microtubules surrounding the nucleus (Walker 1970; Buckland-Nicks & Hadfield 2005). The microtubular structure “manchette” is responsible for nuclear shaping during mammalian spermiogenesis (Yang et al. 2006). For instance, no microtubules are visible when the nucleus of *C. chinensis* (Yan et al. 2004) is
changing. In contrast, De Jong-Brink et al. (1977) found that in *Biomphalaria glabrata* (Say, 1818), there are many microtubules surrounding the nucleus when the nucleus is transforming, and microtubules disappear when the transformation is completed. Ying et al. (2008) found that

Figure 4. The ultrastructure of middle (A) and late stage spermatid (B–E) and sperm (F–H) of *Onchidium struma*. A, cross section of middle spermatid, showing posterior nucleus fossa. B, at the late stage of spermatid, showing the elongated nucleus in the anterior–posterior axis, acrosome vesicle and endoplasmic reticulum. C, the cylindric nucleus, mitochondria of late stage spermatid. D, at the late stage of spermatids, showing a cross section of the nucleus. E, at the late stage of spermatids, showing the nucleus and posterior nucleus fossa. F, mature sperm, showing the acrosomal cap, nucleus, nucleus vesicle and plasma membrane. G, Cross section of the mid-piece of sperm, showing axoneme, plasma membrane, mitochondrial and paracrystalline material of mitochondrial derivative. H, cross section of the end-piece of sperm, showing axoneme. Scale bars: A, F, 0.5 μm; B, 0.5 μm; C, D, 1 μm; E, 2 μm; G, H, 200 nm. Ac, acrosomal cap; Av, acrosome vesicle; Ax, axoneme; C, centriole; Er, endoplasmic reticulum; Gv, Golgi vesicle; M, mitochondria; N, nucleus; Nv, nucleus vesicle; P, paracrystalline material of mitochondrial derivative; Pm, plasma membrane; Pnf, posterior nucleus fossa.
Table I. The changes of nucleus shape during spermatogenesis of gastropods.

<table>
<thead>
<tr>
<th>The changes of spermatid nucleus</th>
<th>Species and references</th>
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<tbody>
<tr>
<td>Round to oval, wing and long cone shape</td>
<td>Onchidium struma (Qiu Liyan, 1991) (this paper)</td>
</tr>
<tr>
<td>Ellipse shape to flat shape, and eventually to an elongate shape</td>
<td>Bulbina formosa (G.B. Sowerby II, 1870) (Ke &amp; Li 1992)</td>
</tr>
<tr>
<td>Oval to rugby ball shape, to kidney shape and finally long cylindrical shape</td>
<td>Patellid lingpatsui Sowerby (Hodgson &amp; Bernard 1988)</td>
</tr>
<tr>
<td>Decreases, stretches, finally spiral</td>
<td>Cipangopaludina chinensis (Gray, 1834) (Yan et al. 2004)</td>
</tr>
<tr>
<td>Round or oval to middle-depressed kidney shape</td>
<td>Neptunea cunningii Crosse, 1862 (Hou et al. 2006)</td>
</tr>
<tr>
<td>Cone to filament</td>
<td>Pomacea maculata Perry, 1810 (Zheng &amp; Wu 2000)</td>
</tr>
<tr>
<td>Approximately round to long barrel along with the spermatid axis, with a nucleus invagination</td>
<td>Halotis asina Linnaeus, 1758 (Huang et al. 2006), H. diversicolor aquatilis Reeve, 1846 (Yan et al. 2006), Trochoidea pyramis Born, 1778 (Wu et al. 2010)</td>
</tr>
<tr>
<td>Round to a short cylindrical rod</td>
<td>Monodonta turbireata (Born, 1780) (Chiva et al. 2011)</td>
</tr>
</tbody>
</table>

Microtubules surround the nucleus of sperm in O. struma. In the present study, chromatin condensation was observed during nuclear morphological transformation, and the nucleus shortens when the chromatin is granular while the nucleus elongates when the chromatin is fibrous. Therefore, the mechanism determining nuclear morphology of spermatids of O. struma may be closely related to both the condensation of nuclear chromatin and the role of microtubules.

Walker (1970) classified the morphology of concentrating nuclear chromatin of spermatids into three categories: granular, fibrous and lamellar. The changes of chromatin during spermatogenesis of O. struma are different from the three patterns that Walker (1970) described, but similar to those of N. cunningii (Hou et al. 2006) and B. exarata (Ying et al. 2002). In O. struma, the nuclear chromatin changes from granular to fibril-like then to long fibers, finally concentrating into highly electron-dense homologous chromosomes. Maxwell (1983) thought that the chromatin of primitive sperm nuclei of molluscs is mostly granular, and less fibrous. Granular chromatin is the simplest type and occurs in sperm nuclei that are short and squat, while fibrous patterns can be observed in more elongated nuclei. Hodgson et al. (1997) described the pattern of chromatin condensation of patellogastropods and vetigastropods with only granular and fibrous phases. In caenogastropods and heterobranchs, chromatin condensation undergoes a third, lamellar phase. It can be concluded that chromatin differs significantly amongst species, which can provide a basis for species classification. The evolutionary clue may be found at the molecular level, and we are planning to study this in the near future.

Sperm of most gastropod species has an acrosome, but there are a few gastropod species without an acrosome of the sperm head (Roosen-Runge 1976; Yan et al. 2004) or consisting only of a few acrosomal vesicles (Hou et al. 2006). Parivar (1981) thought that no acrosome complex was related to sperm dimorphism. While Franzen (1979) and Yan et al. (2004) suggested that the structural change of the acrosomal complex and the presence of the acrosomal complex are related to the morphological structure of sperm, and these reflect functional requirements for fertilisation.

There are two origins of the acrosome in gastropod sperm: (1) it is derived from numerous proacrosomal granules in the cytoplasm (Hodgson & Bernard 1988). In this kind of spermatogenesis, the number of Golgi apparatus is less, and proacrosomal vesicles and vesicular bodies cannot be observed even at the spermatid stage. However, proacrosomal granules are observed in the cytoplasm. The acrosome formation of Halotis asina (Linnaeus, 1758) (Huang et al. 2006), H. diversicolor aquatilis (Reeve, 1846) (Shioya 1984), T. pyramis (Wu et al. 2010) and H. diversicolor aquatilis (Yan et al. 2006) is representative of this type of spermatogenesis. (2) The acrosome is derived from the Golgi apparatus— that is, the acrosomal complex consisting of small-volume vesicular bodies and a large-volume proacrosomal vesicle is secreted by the Golgi apparatus (Hodgson & Bernard 1988). This process is observed in the formation of the acrosomal complex of B. exarata (Ying et al. 2002), B. formosa (Ke & Li 1992) and Pomacea maculata (Lamarck, 1819) (Zheng & Wu 2000). The formation of the acrosome in O. struma is similar to that in these species. In the sperm differentiation of O. struma, the secretion of Golgi and endoplasmic reticulum is quite active. During the stage of spermatogonium and spermatoocyte, there is only a small number of Golgi vesicles, while the number increases and proacrosomal vesicles are beginning to develop in the apex of the nucleus during the early stage of the spermatid. During the middle stage of the spermatid, proacrosomal vesicles can clearly be observed. Proacrosomal granules appear while Golgi vesicles decrease and the acrosome develops afterwards.
Mitochondrial variation and physiological adaptation of gastropods

During gastropod spermatogenesis, the number of mitochondria increases first of all and then decreases, the volume changes from small to large, and concentrates to fuse in the nuclear base at a certain time. Mitochondria of primitive sperm are vesicular while the mitochondria of modified sperm are elongate and some are even spiral in shape (Maxwell 1983; Hodgson & Bernard 1988). Yan et al. (2004) suggested that the mitochondrial spiralisation represents the formation process of the mitochondrial sheath of the sperm, and the latter provides energy for sperm movement and prepares for sperm–egg fusion.

The number, morphology, volume and distribution of mitochondria changes greatly in different cells, even within one cell with different physiological states (Zhai et al. 2000). Generally speaking, within the spermatogonium there are few mitochondria that are scattered throughout the cell, while at the spermatocyte stage, the number of mitochondria increases significantly, which is relevant for further differentiation, and the mitochondria tend to be located at one end of the cell (Parivar 1981; Jaramillo et al. 1986). During the sperm differentiation of O. struna, the mitochondria are distributed at one side of the cell, and the number of mitochondria increases at first and decreases finally, whereas the volume increases throughout the process. The mitochondrial cristae increase and merge, eventually forming a mitochondrial complex that surrounds the axoneme. This is similar to the changes of mitochondria during the spermatid differentiation of B. exarata (Ying et al. 2002) and N. arthritica cumingii (Hou et al. 2006). In conclusion, mitochondria provide energy for sperm maturation, in order to meet the energy needs of spermatogenesis. In addition, the number of mitochondria increases during the process of spermatogenesis and finally decreases again. This reflects substantial physiological activities at each stage of spermatogenesis.

Acknowledgments

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