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Spermatogenesis of the freshwater pearl mussel *Margaritifera laevis* (Bivalvia: Margaritiferidae): A histological and ultrastructural study

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ABSTRACT

Spermatogenesis in the freshwater pearl mussel *Margaritifera laevis* was investigated using light and electron microscopy. The testes of *M. laevis* are composed of numerous acini. We observed type A spermatogonia, large cells of irregular shape, solely near the acinus basal lamina. Type A spermatogonia proliferate and become type B spermatogonia, which are also irregular in shape and form clusters of germ cells of the same developmental stage. The numerous clusters differ with respect to developmental stage and are arranged randomly along the acinus periphery. The central region of the acinus was observed to contain only mature spermatozoa. This germ cell arrangement contrasts that of other bivalvians and may be characteristic of Margaritiferidae and Unionidae. We noted that each germ cell cluster is entirely covered throughout spermatogenesis by Sertoli cells that are loosely bound together. This report is the first to describe the involvement of Sertoli cells in Unionoidea spermatogenesis. Mature spermatozoa of *M. laevis* are of the primitive sperm type, having a cylindrical head with a discoidal acrosome and a midpiece with five spherical mitochondria.

1. Introduction

Light and electron microscopy studies of spermatogenesis and spermatozoa structure have been conducted in many bivalvian species (e.g., Coe and Turner, 1938; Heard, 1975; Franzén, 1983; Eckelbarger et al., 1990; Komaru et al., 1995; Healy et al., 2000; Franco et al., 2008). In mammals, germ cells lodged on the seminiferous tubule epithelium are accompanied by somatic cells called Sertoli cells. These cells are responsible for the development of germ cells and spermatogenesis, providing the germ cells with nutrition and structural support and engulfing and digesting residual sperm cells (Chemes, 1986; Griswold, 1995, 1998; Johnson et al., 2008; Cheng and Mruk, 2012). Bivalvian Sertoli cells, which are also known as follicle cells (Coe and Turner, 1938), nutritive cells (Coe, 1943), accessory cells (Eckelbarger and Davis, 1996), phagocytic cells (Rocha and Azevedo, 1990; Eckelbarger et al., 1990), and support cells (Sousa et al., 1989), have been described in many studies, but their structure and role in spermatogenesis have not been well investigated (Eckelbarger and Davis, 1996; Franco et al., 2011; Kim et al., 2013).

In marine bivalves, sperm maturation is known to follow a linear course, as in mammals, from the outer wall of the testicular acinus toward the lumen, with spermatogonia located nearest the wall, spermatocytes and spermatids closer to the acinus lumen, and mature sperm confined to the central lumen (Coe and Turner, 1938; Hodgson and Bernard, 1986; Eckelbarger and Davis, 1996; Franco et al., 2011). However, in a freshwater unionoid bivalve, *Anodonta cygnea*, Rocha and Azevedo (1990) reported that the germ cells do not exhibit this linear pattern of development toward the lumen. Thus, comprehensive ultrastructural studies of spermatogenesis and Sertoli cells in various bivalvian lineages, including freshwater bivalvian representatives, are needed.

Ultrastructural analyses of spermatozoa are useful for addressing phylogenetic issues in bivalves (e.g., Popham, 1979; Eckelbarger et al., 1990; Healy, 1996; Healy et al., 2000, 2008; Kim et al., 2013). Almost all species studied in the class Bivalvia have spermatozoa of the primitive type (Franzén, 1983; Hodgson and Bernard, 1986; Healy et al., 2008). These spermatozoa are characterized by their small size, a short midpiece with four or five rounded mitochondria, and a long flagellum. The least variable structures appear to be the flagella, centrioles, and mitochondria (Popham, 1979); the nuclei and acrosome show great morphological diversity (Franzén, 1983; Eckelbarger and Davis, 1996; Erkan and Sousa, 2002).

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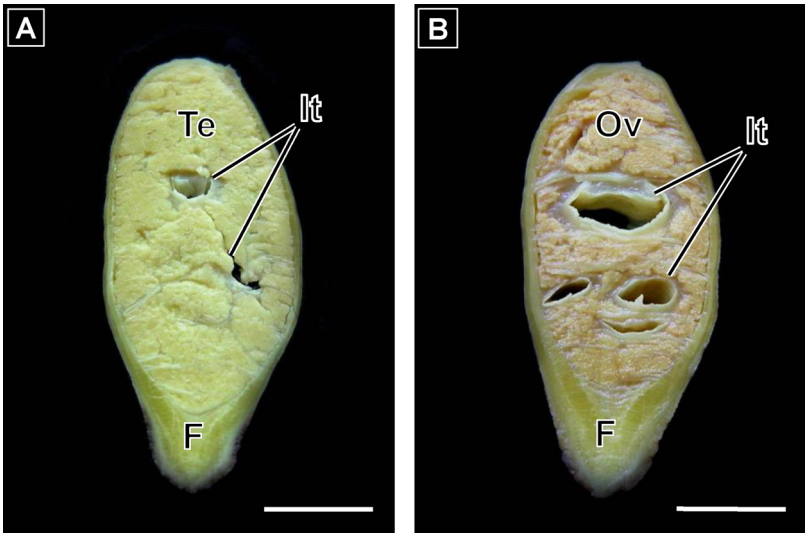

Fig. 1. Abdominal cross sections of male (A) and female (B) *Margaritifera laevis*. F, foot; It, intestine; Ov, ovary; Te, testis. Scale bars = 1 cm.

While many histological studies of Margaritiferidae describe the testes (Smith, 1978; Ross, 1992; Hanstén et al., 1997; Grande et al., 2001), spermatogenesis and the structure of the spermatozoa have not been clarified. In the present study, we provide detailed histological and ultrastructural descriptions of spermatogenesis in margaritiferids, one of the freshwater bivalvian representatives, using the Japanese freshwater pearl mussel *Margaritifera laevis* (cf. Kondo and Kobayashi, 2005; Kondo, 2008).

2. Material and methods

Specimens of the freshwater pearl mussel *Margaritifera laevis* were collected from 2011 to 2013 in the Chubu-Nogu River, Nagano Prefecture, Japan and stored in Bouin's fixative for several days. For histological observations, the testicular tissues were dissected from the fixed specimens, rinsed in 70% ethanol, and dehydrated through a graded ethanol series. Dehydrated specimens were embedded in a mixture of methacrylate resin Technovit 7100 (Külzer, Wehrheim, Germany) and styrene at a ratio of 4: 1 or 7: 3 and processed into 2-μm thick serial sections using a semi-thin microtome (H-1500, Bio-Rad, Hercules, California, USA) equipped with a tungsten carbide knife (Superhard Knife, Meiwafosis, Tokyo, Japan) as described by Machida et al. (1994a, b). Sections were stained with Delafield's hematoxylin, eosin, and fast green FCF. We observed the stained sections using a biological microscope (Optiphot-2, Nikon, Tokyo, Japan).

For observations using a transmission electron microscope (TEM), a 10-mm cube of tissue was excised from the gonads of living mussels and fixed in a modified Karnovsky's fixative (0.5% paraformaldehyde + 0.5% glutaraldehyde in 0.05 M HCl-sodium cacodylate buffer, pH 7.2) for 2 h at 4 °C. Intact acini that appeared on the surface of the cubes during processing were trimmed with a sharp razor, rinsed in buffer several times, and post-fixed in a 0.5% osmium tetroxide solution for 30 min at 4 °C. The fixing procedure described here yielded the best TEM images of *M. laevis* spermatogenesis, avoiding artifacts caused by trimming of the tissue. Postfixed acini were then dehydrated in a graded ethanol series, cleared in propylene oxide, and embedded in a water-miscible epoxy resin (Quetol 651, Nisshin EM, Tokyo, Japan). Embedded acini were processed into 80-nm thick sections using an ultramicrotome (MT-XL, RMC, Tucson, USA) equipped with a diamond knife (Histoknife Wet 8 mm, Drukker, Cuijk, Netherlands), doubly stained with uranyl acetate and lead citrate, and observed under a TEM (HT-7700, Hitachi, Tokyo, Japan) at 80 kV.

For scanning electron microscope (SEM) observations, mature spermatozoa were rinsed in phosphate-buffered saline and fixed in Karnovsky's fixative (2% paraformaldehyde + 2.5% glutaraldehyde in 0.1 M HCl-sodium cacodylate buffer solution, pH 7.2) for 24 h at 4 °C. Fixed spermatozoa were postfixed with 1% osmium tetroxide for 1 h, dehydrated in a graded ethanol series and acetone, and dried using a critical point dryer (Samdri-PVT-3D, tousimis, Rockville, MD, USA). Dried specimens were coated with gold using an ion sputter (JFC-1100, JEOL, Tokyo, Japan) and observed using an SEM (SM-300, TOPCON, Tokyo, Japan) at an accelerating voltage of 15 kV.

3. Results

3.1. Gross morphology: spermatogenesis and Sertoli cells

The breeding season of *Margaritifera laevis* in the Chubu-Nogu River is early spring (late March to early April) (Kobayashi and Kondo, 2005, 2009). The gonads of *M. laevis* are extraordinarily large, occupying most of the available space of the visceral mass (Fig. 1). *M. laevis* spermatogenesis begins in June, and spermatozoa first appear in the acini in July. Spermatogenesis continues to the breeding season of the next year (see Fig. 2A–E), actively occurring from July to January.

The testes of *M. laevis* are composed of numerous acini (Fig. 2A). Clusters of germ cells of the same developmental stage occupy the peripheral region of each acinus (Fig. 2A, B, and C), while mature spermatozoa are located in the central region (Fig. 2A). Germ cell clusters differ from each other with respect to developmental stage, and clusters of varying stages are arranged in a disorderly way along the periphery of the acinus (Fig. 2A). As in fish, each germ cell cluster is entirely covered by Sertoli cells throughout spermatogenesis (e.g., Billard, 1986; Leal et al., 2009) (Fig. 3A and B). This observation contrasts that of other bivalves, in which spermatogenesis is reported to show the linear pattern of development as seen in mammals (e.g., Franco et al., 2011). The Sertoli cells are loosely bound to each other as well as to germ cells (Fig. 3B). The cytoplasm of Sertoli cells includes large amounts of glycogen granules, lipid droplets, electron-dense granules, and sperm morulae (Figs. 2B, C, 3A, and B). Sperm morulae are spherical structures composed of chromatin-dense cells. The number of cells constituting a sperm morulae differs, ranging from 1 to 26 per section.

3.2. Ultrastructure of male germ cells

Type A spermatogonia are large, irregularly shaped cells that are only found near the basal lamina of the acinus. They have a large nucleus (ca. 10 μm in diameter) that is pleomorphic in shape and has one