Bull. Fac. Fish. Hokkaido Univ.
33(1), 1-8. 1982.

Ultrastructure and Histochemistry of Granulosa and Micropylar Cells in the Ovary of the Loach, *Misgurnus anguillicaudatus* (Cantor)

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Abstract

In the loach, *Misgurnus anguillicaudatus*, granulosa cells of ovarian follicles at the yolk globule stage of development showed a weak but distinct histochemical response for Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD) only during the periods from pre-maturation to spawning of the fish. However, the granulosa cells did not show any ultrastructural characteristics of typical steroid-producing cells: they possessed mitochondria with parallel cristae and poorly developed endoplasmic reticulum with granulated cisternae in their cytoplasm.

In each ovarian follicle of the loach as early as the yolk vesicle stage of development, a micropylar cell was easily demarcated from granulosa cells by its enormous size, conical shape, and low affinity to dyes. By the histochemical test for 3β-HSD activity, micropylar cells of ovarian follicles at the yolk globule stage showed an intense reaction throughout the year, being in sharp contrast to the granulosa cells which lost the activity during the post-spawning and sexually quiescent periods. Cytoplasmic organelles such as mitochondria and endoplasmic reticulum of the micropylar cells were essentially similar in feature to those of the granulosa cells, and no ultrastructural signs denoting steroidogenesis were detectable in the micorpylar cells.

Micropylar cells of the loach had a thick cytoplasmic process which reached to the ooplasm through a micropylar canal. In the process, many microtubules of about 25 nm in diameter were arranged in parallel with the long axis of the process. Together with an extensive development of microfilaments in the cytoplasm of the cells, the microtubules seemed to play an important role in the development and structural maintenance of the cytoplasmic process during the formation of the micropyle.

It is well known that, in teleost fishes, spermatozoa can penetrate into eggs exclusively through the micropyle located in the egg membrane at the animal pole of the egg. Until the time of ovulation, the micropyle is covered with a micropylar cell which may have differentiated from one of the granulosa cells surrounding the oocyte. It is generally agreed that the micropylar cell has its primary function in forming the micropyle by extending its thick cytoplasmic process to ooplasm through developing egg membrane (see Laale, 1980[1], for review).

During the course of a series of studies on the development and maturation of oocytes in the loach, *Misgurnus anguillicaudatus*, the present writers could observe that micropylar cells of vitellogenic follicles in the ovary displayed a positive histochemical response for Δ^5-3β-hydroxysteroid dehydrogenase, an enzyme which is necessary for biosynthesis of steroid hormones. In the present study, histochemical and ultrastructural characteristics of micropylar cells are described

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and compared with those of granulosa cells of ovarian follicles of the loach.

We are deeply grateful to Professor, H. Takahashi, Faculty of Fisheries, Hokkaido University, who supervised this study and criticized the manuscript, and to Associate Professor, K. Takano, Faculty of Fisheries, Hokkaido University, for his helpful discussion and criticism of the work. We wish to thank also Mr. Y. Sugimoto, Faculty of Fisheries, Hokkaido University, for his expert technical help.

Material and Methods

Adult loaches, *Misgurnus anguillicaudatus*, ranging from 8.5 to 13.2 cm in body length and from 3.1 to 18.4 g in body weight, were collected from rivers in the suburbs of Hakodate once a month from October 1979 to September 1980. Their ovaries were fixed in Bouin's fluid, cut at 7–10 μm in thickness and stained with Delafield's hematoxlin and eosin for histological observations. Some of the sections were treated with periodic acid-Schiff (PAS) reagent for the demonstration of glycogen.

In the histochemical test for Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD), pieces of the ovaries were quickly frozen by dipping the material in acetone with solid CO_2, and sections of 15–20 μm in thickness were cut on a cryostat at −20°C. After the removal of free droplets by the procedure described by Takikawa and Matsuzawa[2], sections were incubated in a medium prepared according to the method of Rubin *et al.*[3] for 2 hours at 37°C using dehydroepiandrosterone as substrate. Some other sections were incubated in a control medium which lacked the steroid substrate.

Ultrastructural observations on ovaries were carried out on loaches collected in December 1979 and April 1980, when the fish were at the post-spawning and the pre-maturation period, respectively. Small pieces of ovaries were fixed in Karnovsky's fixative for 3 hours at room temperature, postfixed in osmium tetroxide in 0.2 M cacodylate buffer (pH 7.4) for 2 hours at 4°C, and embedded in Epon. Ultrathin sections stained double with uranyl acetate and lead citrate were observed with a Hitachi HU-12 electron microscope. Sections of about 1 μm thick were also cut for light microscopic comparison of the material after staining with methylene blue.

Results

As reported previously[4], the gonadosomatic index (gonad weight/body weight $\times$100) of female loaches used in the present study began to increase in April, reaching a maximum in June, and spawning occurred in the months from July to September. During these periods, ovaries of the fish were loaded with many oocytes at various phases of vitellogenesis and maturation. During the post-spawning and sexually quiescent period from October to March, most of the female still retained a small number of normal yolk-laden oocytes in their ovaries.

In ovaries of the fish collected in the months from March to September, a weak but distinct histochemical reaction for 3β-HSD was observed to occur around the oocytes at the yolk globule stage. The reaction was seen to be in granulosa cells of ovarian follicles since it occurred as a thin, continuous ring closely adhering

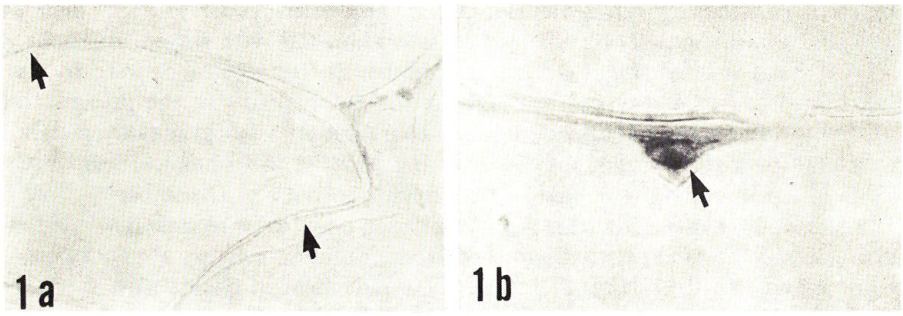


Fig. 1. Histochemical demonstration of 3β-HSD activity in ovarian follicles of loaches collected in April (a) and May (b), showing a weak activity in granulosa cells (arrows in a), and an intense activity in a micropylar cell (arrow in b). a, ×90; b, × 190.

to the zona radiata (Fig. 1–a). In the subsequent months from October to February, no histochemical response for 3β-HSD was detectable in any of the ovarian follicles of the fish examined.

Granulosa cells of ovarian follicles with a positive 3β-HSD activity had a flattened and large sized nucleus with an occasionally indented contour. In their cytoplasm, round or rod shaped mitochondria mostly had parallel cristae, though some of them appeared to have a few tubular cristae (Fig. 2). Endoplasmic reticulum was poorly developed and consisted of narrow and parallel arrays of

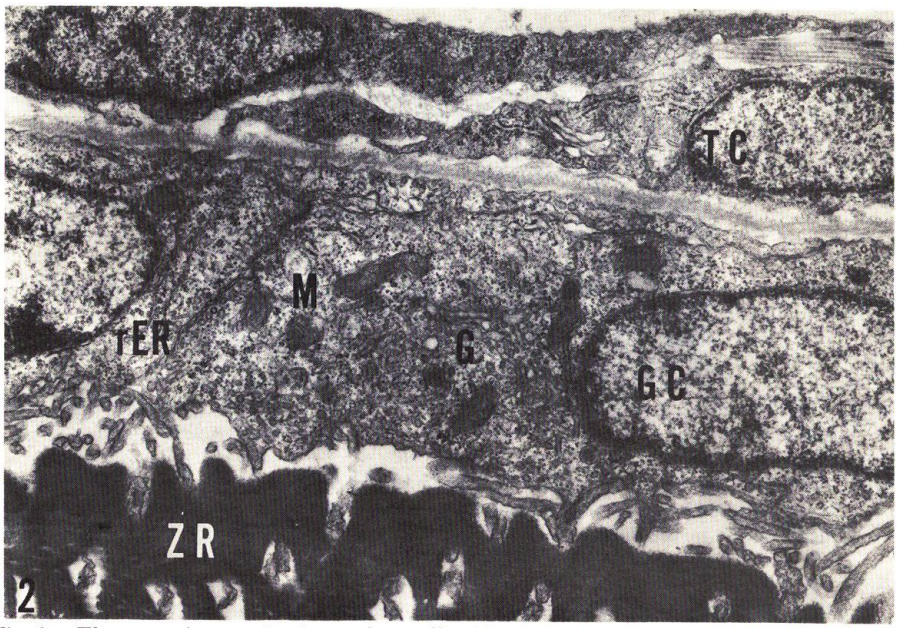


Fig. 2. Electron micrograph of granulosa cells (GC) of an oocyte of the yolk globule stage of a loach collected in April. G, Golgi apparatus; M, mitochondria; rER, rough endoplasmic reticulum; TC, thecal cell; ZR, zona radiata. × 13,500.

granulated cisternae. Well-developed Golgi apparatus was located near the nucleus and was composed of a stack of flattened lamellae with several dilations and associated small vesicles (Fig. 2). A large number of free ribosomes were scattered, in small clusters in some places, throughout the cytoplasm. In the present study, no distinct differences in ultrastructural characteristics of granulosa cells were detected between the ovarian follicles showing positive histochemical response for 3β-HSD in April and those devoid of the enzyme activity in December.

In the loach, ovarian follicles with yolk-laden oocytes were each provided with a clear cell which was easily distinguished from granulosa cells by its enormous size and low affinity to dyes (Fig. 3). The cell was conical in shape with its tapered apex facing the oocyte and with its proximal surface abutting mostly on granulosa cells overlaying it and partly on the basement membrane bordering the thecal layer (Fig. 4). The cell had in its proximal region a large and flattened nucleus with a prominent nucleolus. The apex of the cone-shaped cell was observed to be extended inward the ovarian follicles, thus giving rise to a funnel-like concave, or the micropyle, in the zona radiata. The apical extrusion of the micropylar cell reached the surface of ooplasm perforating through the zona radiata and formed a micropylar canal of a diameter of 3–4 μm at its innermost opening (Fig. 4).

Micropylar cells of the loach were light-microscopically detectable to be present in ovarian follicles with oocytes as early as the yolk vesicle stage of their development. By the present histochemical study,a distinct 3β-HSD activity was observed to occur in micropylar cells of oocytes at the yolk globule stage (Fig. 1–b). The histochemical response of micropylar cells was more intense than that of

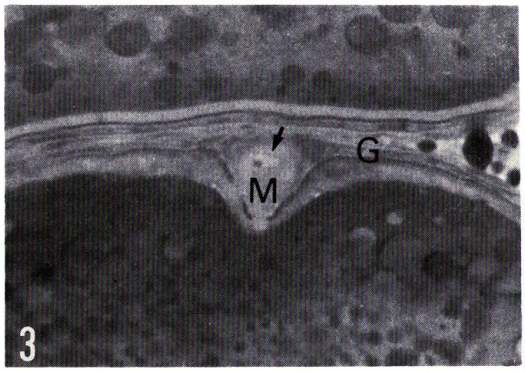
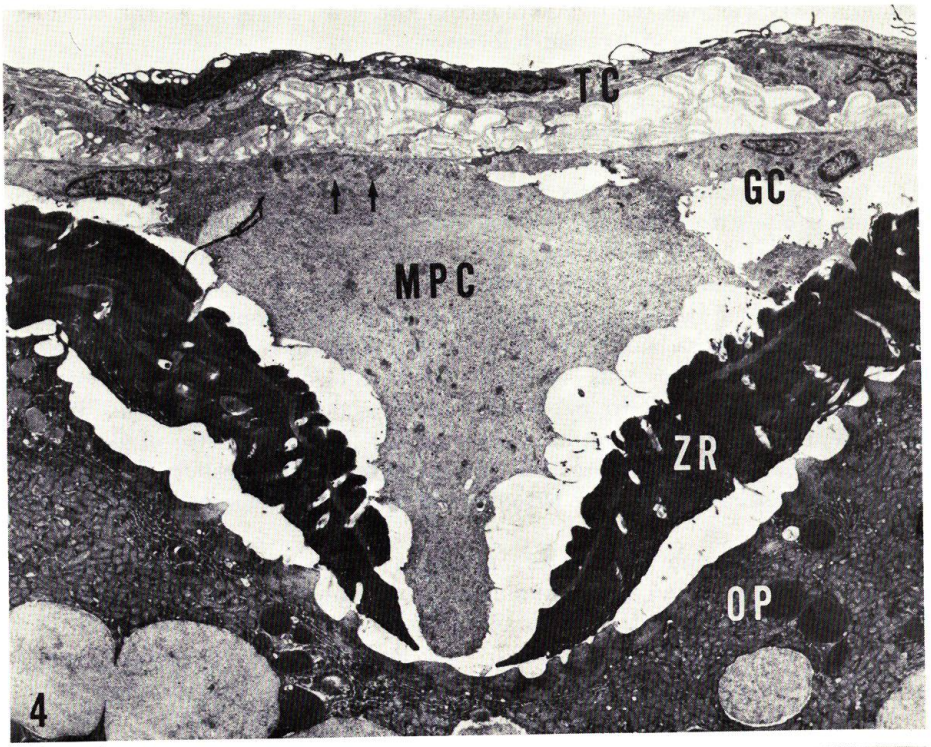


Fig. 3. Epon section (1 μm) of a micropylar cell (M) of a loach collected in December. Arrow indicates the nucleus of micropylar cell. G, granulosa cell. × 500.

Fig. 4. Electron micrograph of a micropylar cell (MPC) of the loach. Note a thick process of the micropylar cell penetrating micropylar opening. Arrows indicate mitochondria accumulated in the periphery of the cell bordering on the thecal layer. GC, granulosa cell; OP, ooplasm; TC, thecal cell; ZR, zona radiata. × 2,700.

Fig. 5. Electron micrograph of a portion of a micropylar cell, showing well-developed Golgi apparatus and mitochondria with parallel cristae. × 12,200.

Fig. 6. Electron micrograph of the cytoplasmic process of a micropylar cell. Note centriollar satellites (large arrows) and a large number of microtubules (small arrows) arranged parallel to the long axis of the process. × 11,000.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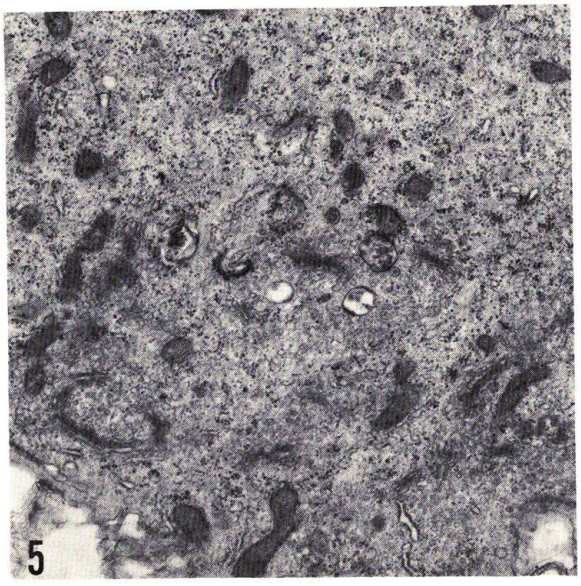
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granulosa cells and was positive throughout the year, being in sharp contrast to that of granulosa cells of ovarian follicles.

In micropylar cells, electron microscopically, round or rod shaped mitochondria were scattered in the cytoplasm, and some of them were frequently seen to be accumulated in the peripheral cytoplasm abutting on granulosa cells (Fig. 4). They were apparently provided with parallel cristae, and had one or more spherical dense granules within the matrix (Fig. 5). A small amount of rough endoplasmic reticulum composed of lamellar cisternae was distributed around the nucleus and in the peripheral region of the cell. Well-developed Golgi apparatus consisting of several lamellae and many small vesicles were observed most frequently in the proximal region of the cytoplasmic process (Fig. 5). Moreover, lysosomes and membrane-bound dense bodies were also found. The above mentioned cytoplasmic organelles of micropylar cells substantially resembled those of granulosa cells. Microfilaments were seen to be distributed throughout the cytoplasm, and were organized into some bundles around the nucleus. A large number of glycogen-like granules, about 30 nm in diameter, were scattered throughout the cytoplasm (Figs. 5, 6); histochemically, the cytoplasm of micropylar cells showed PAS positive reaction.

In the cytoplasmic process of micropylar cells, centriolar satellites were present near the apex, and a large number of microtubules, about 25 nm in diameter, were arranged parallel to the long axis of the process (Fig. 6). Insertion of the process into the ooplasm, which was the case in *Hypomesus transpacificus nipponensis*[5] and in *Clupea pallasi* (Ohta and Takano, unpublished), did not occur in the loach. Also, specialized attachment devices were not found between the process and the ooplasm. In addition to the cytoplasmic process, micropylar cells extended many microvilli to the ooplasm through pore canals of the zona radiata, and the ooplasm also extended microvilli to micropylar cells.

Discussion

In spite of the positive histochemical reaction for 3β-HSD, both micropylar cells and granulosa cells of ovarian follicles of the loach did not show any typical ultrastructural features of steroid-producing cells such as well-developed smooth endoplasmic reticulum, lipid droplets, and mitochondria with tubular cristae[6]. Ultrastructure of the granulosa cells of the loach was essentially similar to that of the medaka[7] which also showed a positive reaction for 3β-HSD.

The location of steroidogenic cells in teleost ovaries still remains in dispute (see Guraya, 1976,[8], for review), but many histochemical studies have indicated that the granulosa cell of the follicular envelope of oocyte is one of the positive sources of steroid hormones in teleost ovaries[7) 9)–11)]. On the other hand, there has been no report demonstrating enzyme activities for steroidogenesis in micropylar cells of ovarian follicles of teleost fishes so far as the present writers know.

In the pond smelt, *Hypomesus transpacificus nipponensis*, and the bitterling, *Rhodeus ocellatus ocellatus*, micropylar cells and granulosa cells of ovarian follicles were both negative to the histochemical test for 3β-HSD activity (Takano, personal communication). These findings and the results obtained in the present histochemical study suggest that the occurrence of 3β-HSD activity in

micropylar cells may be concerned with the presence of the same enzyme in granulosa cells of ovarian follicles. The functional coincidence of micropylar cells with granulosa cells is quite likely to occur because of the fact that the former has originated from the latter in early phase of development of ovarian follicles[12]. On the other hand, it is an interesting to note that, in the present study, 3β-HSD activity appeared in micropylar cells of loach oocytes even in the post-spawning period when no activity of the enzyme was detectable in granulosa cells. As reported previously[4], vitellogenin, the precursor yolk protein synthesized in the liver under the influence of estrogen, was detectable in the serum of female loaches throughout the year even during the post-spawning and subsequent sexually quiescent periods. It may be doubtful, however, whether there is any exact parallelism between the two phenomena. Further comparative studies on micropylar cells of various teleosts, particularly of those which show positive reaction for 3β-HSD in their granulosa cells, are necessary for settling the problem.

Micropylar cells of the loach have several ultrastructural features which are common to those of other teleosts: a characteristic arrangement of microtubules in the cytoplasmic process extending to the ooplasm (*Hypomesus transpacificus nipponensis*[5]; *Rhodeus ocellatus ocellatus*[13]; *Clupea pallasi* and *Plecoglossus altivelis*, Ohta and Takano, unpublished), and an extensive distribution of microfilaments throughout the cytoplasm (*Hypomesus transpacificus nipponensis*[5]; *Plecoglossus altivelis*, Ohta and Takano, unpublished). These structures may be implicated in the structural maintenance of large and conical micropylar cells. Especially, the development of microtubules may be indispensable for keeping the thick process stable during the formation of a micopyle.

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