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Histochemical Detection of Δ^5 - 3β -Hydroxysteroid Dehydrogenase in the Ovary of Medaka, *Oryzias latipes*, During Annual Reproductive Cycle*

Yoshinori IWASAKI**

Abstract

Actual cellular sites of the occurrence of Δ^5 - 3β -hydroxysteroid dehydrogenase (3β -HSD) in the ovary of adult medaka, *Oryzias latipes*, were examined histochemically in relation to the annual reproductive cycle of the fish.

During the spawning period, 3β -HSD was detected, though rather weak in histochemical response, only in granulosa cells of follicles of the advanced oocytes beyond the secondary yolk stage. Enzyme was also found in these cells of the post-ovulatory follicles. In the ovary during the resting period of the reproductive cycle, in which most of the advanced oocytes were in the yolk vesicle stage, a positive histochemical response of the enzyme was noticed but only in some of the cells scattering singly or in clusters in the interfollicular areas of the ovaries. The enzyme activity found in the interfollicular cells was more intense and distinct than that formerly seen in the granulosa cells, but occurred rather infrequently when compared with the latter.

It has been well established that the ovary of teleostean fishes is capable of producing several steroid hormones (for recent reviews, see Barr¹⁾ and Reinboth²⁾). Along with some biochemical approaches, enzyme-histochemistry has been effectively adopted to show the steroidogenic activity and its cellular site in the ovary³⁾⁴⁾⁵⁾⁶⁾⁷⁾. By surveying the literature, it is conceivable that there are differences in the cellular locality of steroidogenic activity of the ovary among teleost fishes studied so far. That activity was observed to be localized in the granulosa cell of *Poecilia reticulata*⁸⁾ and *Acanthobrama terrae-sanctae*⁷⁾, in the theca cell of *Brachydanio rerio*⁴⁾, and in the granulosa and theca cells of *Scomber scomber*³⁾ and *Tilapia nilotica*⁷⁾. Moreover, it is accepted that the steroidogenic activity in these cells may vary in relation to oocyte maturation: in most cases, the histochemical evidence of steroidogenesis was recognized in the follicles of vitellogenic oocytes³⁾⁴⁾⁶⁾⁷⁾. Physiological significance of these phenomena still remains to be clarified, however, by further studies on various developmental stages in a given species.

The aim of the present study is to determine histochemically the actual site of the occurrence of Δ^5 - 3β -hydroxysteroid dehydrogenase, one of the enzymes essential for steroid biosynthesis⁸⁾⁹⁾, in the ovary of the medaka, *Oryzias latipes*. In addition, observations are extended to know whether there are any fluctuations

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in the activity of the enzyme during the course of annual reproductive cycle of the medaka.

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Material and Methods

Adult females of wild type medaka, *Oryzias latipes*, were employed as a material in the present study. They were raised in an outdoor pond in the campus of the Faculty of Fisheries under natural conditions of light and temperature. Histochemical detection of 4^5 - 3β -hydroxysteroid dehydrogenase (3β -HSD) in the ovary was carried out from November 1970 to June 1972, using 2 to 6 fish in each month.

Immediately after killing the fish, ovaries were rapidly frozen in a mixture of acetone and solid carbon dioxide. They were cut usually at a thickness of $20\ \mu$ in a cryostat maintained at about -20°C . Prior to incubation, free lipids were extracted from the sections according to the method recommended by Takikawa and Matsuzawa¹⁰): some of the fresh frozen sections were treated with cold pure acetone for 15–20 hours and then with cold pure ethyl ether for 1 hour followed by a rinse in 85% cold acetone for 2–5 minutes; others were treated only with 85% cold acetone for 2–5 minutes as a comparison. Incubation mixture used in the present study consisted of NADP, nitro-BT and dehydroepiandrosterone (DHA) in 0.057 M phosphate buffer⁴). In some cases, DHA in the incubation mixture was replaced by pregnenolone as another substrate of the enzyme. Control sections were prepared by the same procedure as mentioned above except that the steroid substrate was omitted from the incubation mixture. After being incubated for 1 hour at 37°C , all the sections were finally fixed in neutral formalin, dehydrated by graded alcohol series and mounted in balsam for microscopic observations. It was remarked in the present study that DHA was a useful substrate to elicit a positive histochemical response of 3β -HSD but pregnenolone was ineffective in demonstrating the reaction just as was the control incubation.

In parallel with the above histochemical procedure, ovaries of some females were fixed with Bouin's fluid, serially sectioned at $8\ \mu$ in thickness, and stained with Delafield's hematoxylin and eosin for histological observations.

Observations

The ovary of adult medaka is a single median organ which is constructed from many ovigerous folds in which oogonia and oocytes of various developmental stages are found buried in a loose connective tissue stroma. As already described by Yamamoto and Yoshioka¹¹), oocytes in the peri-nucleolus and yolk vesicle stages

IWASAKI: Ovarian 3 β -HSD of medaka

Table 1. Changes of Δ^5 -3 β -hydroxysteroid dehydrogenase activity during the annual reproductive cycle of female medaka.

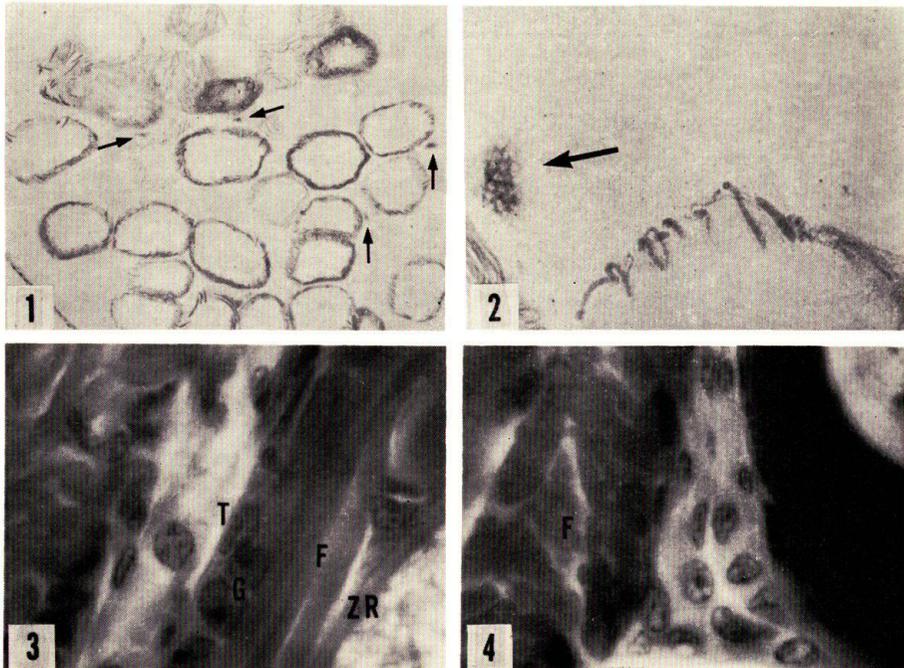
Date of observation	Developmental stage of most advanced oocyte	Locality of enzyme activity	No. of ovaries with positive reaction (No. of ovaries examined)
Nov. 1970	Yolk vesicle stage	Interfollicular cells	4 (6)
Dec. 1971	"	"	2 (5)
Feb. 1971	"	"	1 (4)
Mar.	"	"	1 (4)
Apr.	Primary yolk stage	"	2 (4)
May	Maturation stage	Granulosa cells	5 (5)
Jun.	Ripe stage	"	3 (3)
Jul.	"	"	4 (4)
Aug.	"	"	4 (4)
Sep.	Yolk vesicle stage	"	1 (4)*
Oct.	"	Interfollicular cells	1 (4)
Nov.	"	"	2 (4)
Jan. 1972	"	"	1 (4)
Feb.	"	"	1 (4)
Mar.	"	"	1 (4)
Apr.	Primary yolk stage	—	0 (4)
May	Maturation stage	Granulosa cells	4 (4)
Jun.	Ripe stage	"	2 (2)

* One out of four ovaries examined in this month contained oocytes of the ripe stage and showed enzyme activity in the granulosa cell.

are always present in the ovary of the medaka throughout the year. During the spawning period lasting from late April to early September, active vitellogenesis toward complete maturation of oocytes occurs unceasingly, and the oocytes advancing their development beyond the primary yolk stage are found as prominent elements in the ovary. In the post-spawning period of September and October, the cessation of vitellogenic activity is followed by frequent appearances of degeneration of the yolk-laden oocytes. From those months till the next spawning period, ovarian oocytes showing most advanced development remain in the yolk vesicle stage.

In the present study, possible seasonal changes in the activity of ovarian 3 β -HSD were examined histochemically. The histochemical response of 3 β -HSD was recognized to occur in two different patterns in association with the developmental cycle of ovarian oocytes, as shown in Table 1.

During the months from October to April, when the most advanced oocytes encountered in the ovary were in majority in the yolk vesicle stage except for a few ones in the primary yolk stage appearing in April, the occurrence of 3 β -HSD activity was demonstrated only in some cells which were distributed quite sparsely in the interfollicular area of the ovary (Fig. 1). The enzyme reaction was very distinct in most cases. Although the enzyme activity was not always detectable



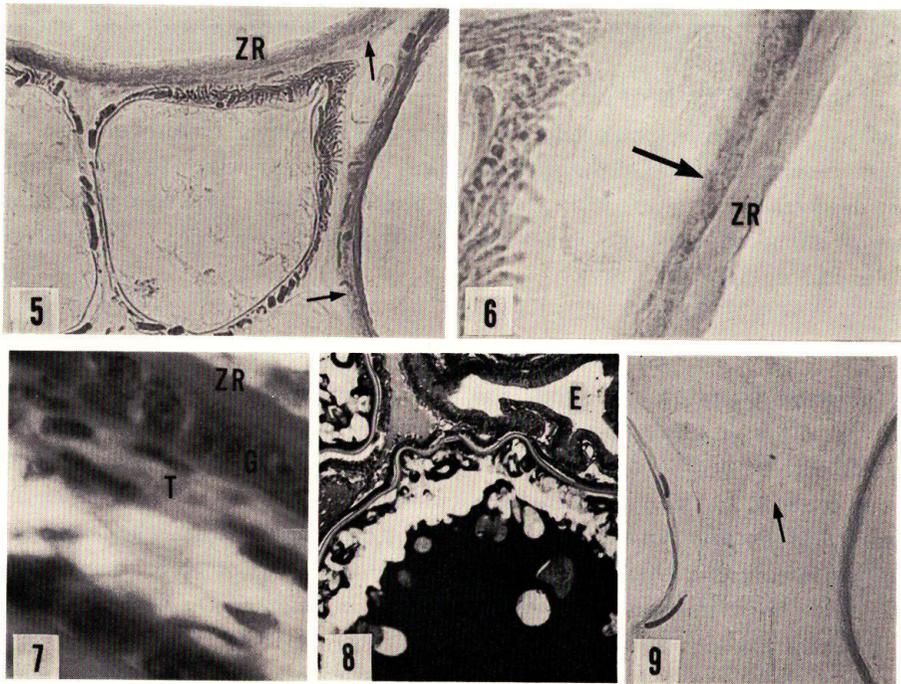
Figs. 1 and 2. Sections through the ovary of the medaka subjected to 3β -HSD histochemistry, examined in the resting period (November) of the annual reproductive cycle. The distinct activity of the enzyme is detectable in interfollicular cells (arrows). Villi and attaching filaments of ovarian oocytes are also shown as dark shades in the picture, but these structures are entirely negative in histochemical response. Fig. 1, $\times 50$; Fig. 2, $\times 270$.

Figs. 3 and 4. Sections through the ovary examined in the resting period (November), revealing interfollicular cells found singly (Fig. 3) or in clusters (Fig. 4) in the proximity of oocytes of the yolk vesicle stage. *F*, attaching filament; *G*, granulosa cell; *T*, theca cell; *ZR*, zona radiata. $\times 1300$

in all the ovaries in these months, it occurred more frequently in November and December than in the other months (Table 1). The reacting cells, which existed on many occasions as clusters of more than ten cells each appeared to be rather large in size, with a cell nucleus of 3 to 4 μ in diameter (Fig. 2). In histochemical sections, the villi and attaching filaments of ovarian oocytes appeared to have a pinkish brown color that was apparently distinguishable from the bluish color of diformazan deposits of the histochemical reaction. By histological observations, some cells situated singly or in clusters in the vicinity of ovarian follicles of the yolk vesicle stage were found to have a resemblance to those with positive 3β -HSD activity in their size and localization (Figs. 3 and 4). Moreover, some cells with similar histological features were present also in the vicinity of atretic follicles

and were presumed to be also the sites of the enzyme activity, since the enzyme was noticed to occur in the cells lying near the masses of the cells which were pigmented yellow in frozen sections. On the other hand, 3β -HSD activity was discovered neither in the granulosa cells nor in the theca ones of the oocytes of the yolk vesicle and primary yolk stages.

In May, just at the start of the spawning period, evident changes were noticed both in the ovarian structure and in the ovarian 3β -HSD response. Many ovarian oocytes were developing into the ripe stage, and the aforementioned enzyme reaction in the interfollicular cells could not be detected in any of the ovaries examined in this month. Instead, a positive enzyme reaction became to appear in the granulosa cell layer of the oocytes which were in or beyond the secondary yolk stage (Figs. 5 and 6). The reaction was considerably different in intensity and in appearance from that found in the interfollicular cells. It was noticed as



Figs. 5, 6 and 9. Sections through ovaries of the medaka subjected to 3β -HSD histochemistry, examined in the spawning period (May and June). The enzyme response is found as a pale blue band on granulosa layer (arrows) of oocytes advancing beyond the secondary yolk stage (Figs. 5 and 6) and post-ovulatory follicles (Fig. 9). ZR, zona radiata. Fig. 5, $\times 135$; Fig. 6, $\times 475$; Fig. 9, $\times 90$.

Figs. 7 and 8. Sections through ovaries of the medaka sampled in the spawning period. In Fig. 7, structures of a follicle of an oocyte of the tertiary yolk stage are exhibited. E, post-ovulatory empty follicle; G, granulosa cell; T, theca cell; ZR, zona radiata. Fig. 7, $\times 1300$; Fig. 8, $\times 100$.

a very weak, blue-colored band covering the granulosa layer. The nucleus of each reacting cell was very obscure in contour (Fig. 6).

Granulosa cells of the oocytes beyond the secondary yolk stage did scarcely show any remarkable change in their size during the oocyte maturation from the yolk vesicle stage to the tertiary yolk stage (Figs. 3 and 7), although they became to occupy the region closely adjacent to the zona radiata invading the villi and attaching filaments. Theca cells of these ovarian follicles never showed the sign of 3β -HSD activity. Interfollicular cell elements were still present but were considerably dispersed possibly as a result of a rapid enlargement of the oocyte in vitellogenesis. During the succeeding months of the spawning period, distinct reaction of 3β -HSD was always noticed in the granulosa layer of oocytes of the secondary yolk, tertiary yolk, and maturation stages in all the ovaries examined (Table 1). Moreover, in these months, some post-ovulatory follicles were always encountered together with these large oocytes in the ovary (Fig. 8). Granulosa cells composing these empty follicles also exhibited a weak but evident reaction of the enzyme (Fig. 9).

In September, 3β -HSD activity in the granulosa cells was detected in only one out of the four ovaries examined (Table 1). The remaining three ovaries showed no histochemical response of the enzyme in any constituent cells. In these ovaries, many degenerating oocytes, or the so-called "atretic follicles", were encountered, and the most advanced, healthy oocytes were in the yolk vesicle stage. These atretic follicles had no 3β -HSD activity.

Discussion

In recent literature concerning the steroidogenesis in the gonads of teleost fishes, there are several reports which deal with a histochemical demonstration of 3β -HSD activity in the ovary. Most of them showed a distinct activity of the enzyme confined to the constituent cells of ovarian follicle such as granulosa cells (Lambert, in *Poecilia reticulata*⁶⁾; Yaron, in *Acanthobrama terrae-sanctae*⁷⁾), theca cells (Yamamoto and Onozato, in *Brachydanio rerio*⁴⁾), or in both granulosa and theca cells (Bara, in *Scomber scomber*⁸⁾; Yaron, in *Tilapia nilotica*⁷⁾).

It seems common to all the cases cited above that the enzyme confined to the ovarian follicle is histochemically detectable only when the associated oocytes are in active vitellogenesis or in complete maturation. The results of the present study in *Oryzias latipes* also offered evidence of the presence of 3β -HSD activity in the granulosa cells, but not in the theca ones, of yolk-laden oocytes in the stages beyond the secondary yolk stage of maturity. A weaker reaction was observable also in the granulosa cells of some post-ovulatory follicles. No reaction of the enzyme was, however, noticed in the follicles of oocytes younger than the primary yolk stage as well as in interstitial cells of those ovaries in the spawning period.

It has been well established that the vitellogenesis in fish ovaries is under the control of pituitary gonadotropin¹²⁾. Using the medaka reared under the nearly same conditions as in the present study, Kasuga and Takahashi¹³⁾ suggested the promoted release of gonadotropic hormone(s) from the pituitary gland during the spawning period. The present study showed that a weak but constant activity was maintained throughout the spawning period in the granulosa cells in association with advanced vitellogenesis. These facts may imply that the activity of the enzyme in these cells is also dependent on pituitary gonadotropic influences just as is the enzyme in the testicular interstitial cells of *Carassius auratus*¹⁴⁾. It seems likely that certain steroid hormones are produced in the granulosa cells under the pituitary control to play indispensable roles in supporting some reproductive phenomena (cf. Barr¹⁾), though much extensive work is needed to substantiate the possible significance of the enzyme activity.

So far as the present writer knows, only a few works are concerned with the seasonal alterations of the activity of steroidogenic enzymes in fish ovaries, but no one has hitherto mentioned a different localization of the enzyme in the different periods of reproductive cycle. In the medaka during the resting period of annual reproductive cycle, when the ovary had depleted the oocytes in active vitellogenesis and no more showed the 3 β -HSD activity in the follicles, a histochemical response of the enzyme reappeared in some of the cells which were localized in interfollicular areas neighbouring the oocytes mostly in the yolk vesicle stage. The presence of 3 β -HSD in ovarian interstitial cells was described also by Wiebe⁵⁾ in viviparous *Cymatogaster aggregata*, who described that the enzyme occurred both in follicles and interstitial areas of ovaries of pregnant and non-pregnant fish. In oviparous *Oryzias latipes*, however, the enzyme response was detectable in interfollicular cells during the resting period in contrast to that seen in granulosa cells of the follicle during the spawning period. In this respect, it is interesting to note the findings presented by Yamamoto and Onozato⁴⁾ in *Brachydanio rerio*. According to them, some cells with ultrastructural features of steroidogenesis exist in the area among small follicles of immature ovaries, and they seem to become located in the theca layer of large oocytes in maturing and mature ovaries. By the present observations, however, it remained quite uncertain whether or not it is also true for the present case of *Oryzias latipes*.

It has been remarked, moreover, that the enzyme response found in the interfollicular cells was more distinct and intense than that seen in the granulosa cells. The former was not constantly revealed but only infrequently in a few specimens examined during the resting period. The occurrence of the enzyme-positive interfollicular cells was more frequently distributed in the specimens of November and December than in those of the other months. During those two months, ovaries of the medaka begin to restore their development as represented

by complete absorption of atretic oocytes and an increase in number of young oocytes, as shown already by Yamamoto and Yoshioka¹¹⁾, but gonadotropic influence of the pituitary gland is still completely suppressed as suggested by Kasuga and Takahashi¹³⁾. The possible relationship between these phenomena and the physiological significance of the dual occurrence of 3β -HSD-positive cells in the ovary of the medaka will be clarified by further studies including an ultrastructural comparison of cells of the two different sites of the ovary.

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