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**Gonadal Sex Differentiation in *Tilapia mossambica*, with Special
Regard to the Time of Estrogen Treatment Effective in
Inducing Complete Feminization of Genetic Males**

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Abstract

In *Tilapia mossambica*, paired primordial gonads are formed 8-10 days after hatching, when germ cells begin their gradual multiplication and development into gonial ones. Sex differentiation of gonads either into ovaries or into testes becomes histologically discernible about 20 days of age: the development of some germ cells into meiotic prophase and the concomitant start of ovarian cavity formation characterize ovarian differentiation, while the occurrence of efferent duct anlagen in gonadal stroma confirms testicular differentiation. Henceforth, germ cells in the ovary advance their oogenetic processes smoothly, but those in the testis remain quiescent until about 50 days of age.

By oral administration of ethinylestradiol at a constant dose of 50 μg per g diet, a complete feminization of genetic males was obtained in an experiment in which the treatment was done during a period from 6 to 25 days of age covering the sexually indifferent stage through the stage of sex differentiation. The treatments lasting for 10 days in either of the two stages were without effect in causing any sexual modification, and an experiment carried out for 10 days extending through the two stages resulted in the occurrence of intersexual gonads in genetic males. It is presumed from these results that, in *T. mossambica*, the period sensitive to exogenous sex hormones for attaining complete sex reversal may lie between 10 and 25 days after hatching during which gonadal sex differentiation begins and proceeds.

Since Yamamoto¹⁾ achieved the functional feminization of *Oryzias latipes* by treatment with estrone, it has become generally thought that a complete and functional sex reversal can be induced rather easily in teleost fishes by the administration of heterologous sex hormones. From a viewpoint of fish propagation, artificial control of sex of fishes may be an important and interesting subject which awaits future development. There still remain, however, many problems to be solved in practicing the artificial direction of sex by exogenous sex hormones.

According to Yamamoto²⁾, the conditions to be fulfilled to attain a complete and functional sex reversal of fishes are that a heterologous sex hormone should be administered at adequate dosage levels to fish fry starting at the sexually indifferent stage and lasting through the stage of gonadal sex differentiation. The cases of functional sex reversal experimented so far in *Oryzias latipes*¹⁾³⁾, *Lebistes reticula-*

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*tus*⁴⁾⁵⁾, *Tilapia mossambica*⁶⁾ and *Carassius auratus*⁷⁾ support to some extent a general validity of his opinion. This in turn stresses that each item of the above conditions should be defined previously for the fish species that is to be practiced to realize functional reversal of one sex to another by treatment with sex hormones. Among the conditions, the time and the duration of hormonal treatment seem to be the most important factors which may be different in various species of fishes.

A functional masculinization of *Tilapia mossambica* treated with an androgen was obtained by Clemens and Inslee⁶⁾ and a feminization with estrogens by Hackman (cited from Reinboth⁸⁾), but the morphological process of gonadal sex differentiation in the fish has not been reported especially in connection with the time and duration of the hormonal treatment to induce a sex reversal. It is the aim of this study to determine the period during which the gonad is most responsive to exogenous sex hormones to accomplish its sexually reversed differentiation. For that purpose, the normal process of sex differentiation and development of the gonad was examined histologically, and its modification under the influence of an exogenous estrogen was studied by treating the fry with ethinylestradiol at various times of gonadal development and for varying lengths of days. The results of these observations will be dealt with in this paper.

Before going further, the writers wish to express their cordial gratitude to Professor Kiichiro Yamamoto, Hokkaido University, for his criticism and kind encouragement given throughout this study.

Material and Methods

Broods of *Tilapia mossambica* were obtained from adult females bred in laboratory aquaria. Five to 10 days after spawning, the fry were washed out from their mother's mouth and employed as a material. The age of the fish was counted after hatching, regarding the 5th day of oral incubation as 0 day since the hatching out of the fry was observed to occur on that day in most cases.

For the observation of the process of gonadal sex differentiation, several broods of fry and juvenile fish were reared under the natural condition of light in glass aquaria with well aerated water of $20\pm 2^{\circ}\text{C}$. They were fed daily on powdered or granulated commercial diet for carp culture. A histological examination of gonads was made at intervals of 3-5 days for the first 40 days after hatching and at intervals of about 10 days during the succeeding period of more than two months. Four other groups of fry, each consisting of experimental and control fish of the same spawn, were examined for the effect of daily oral administration of ethinylestradiol at a constant dose of $50\ \mu\text{g}$ per g diet on gonadal sex differentiation. The estrogen-containing and the control diet were prepared by the same manner as described in a previous report⁹⁾. The number of fish examined and the time and duration of the hormonal treatment in each group are shown in Table 1.

The experimental and control fish were raised in conditions identical to those described above. The rearing water was changed indefinitely during the experimental periods. Some of both the experimental and the control fish were autopsied during and at the end of the treatment, while others were cultured continuously after hormone withdrawal and killed at intervals of more than 20 days to examine the development of their gonads. At the time of autopsy, the fish were measured for the body length and body weight, their gonads attached *in situ* to the dorsal peritoneal wall were excised out and fixed in Bouin's fluid. Frontal paraffin sections of the gonads were cut serially at 6-8 μ in thickness and stained with Delafield's hematoxylin and eosin.

Results

Normal sex differentiation of the gonad

In newly hatched fry, the so-called primordial germ cells, which averaged about 40 in number in a fish, were found to be localized along the dorso-median region of the peritoneal wall at the dorsal root of undeveloped mesentery (Fig. 1). The germ cells were distinguishable easily from the somatic ones by their definite, roundish contour, a clear aspect of the cytoplasm, and larger nuclear and cellular sizes of 8-11 and 13-16 μ , respectively. Moreover, some of them contained a few yolk platelets in their cytoplasm (Fig. 1, inset). In only a few cases, presumable primordial germ cells were found to exist singly in some places away from the presumptive gonadal region (Fig. 2).

The germ cells still retained the cytological features of primordial germ cells 3 days after hatching. By that time they began to shift bilaterally from the dorsal root of mesentery to be located in the paired, presumptive gonadal regions and to protrude, with a small number of somatic cells enclosing them, into the peritoneal cavity (Fig. 3). This change was more remarkable in the anterior region than in the posterior one of the primordial gonad thus formed, and in the latter region the germ cells frequently occurred still closely adjacent to the dorsal root of mesentery. The germ cells showed cephalocaudally a roughly beaded alignment interspersed with obscure ridges of peritoneal cells which were sometimes undergoing mitotic divisions.

No prominent changes were observable in histological aspects and in the number of germ cells of the gonad 6 days after hatching. However, the paired arrangement of the gonads became to be established more distinctly than before. Active mitotic divisions of the germ cells which had become completely deprived of yolk platelets in the cytoplasm were seen in most of the gonads examined at 11 days of age. It was presumed, by inspecting the change in number of the germ cells in this and previous stages, active multiplication of germ cells had begun by 8-10 days of age. As a result, the germ cells became smaller than before, measur-

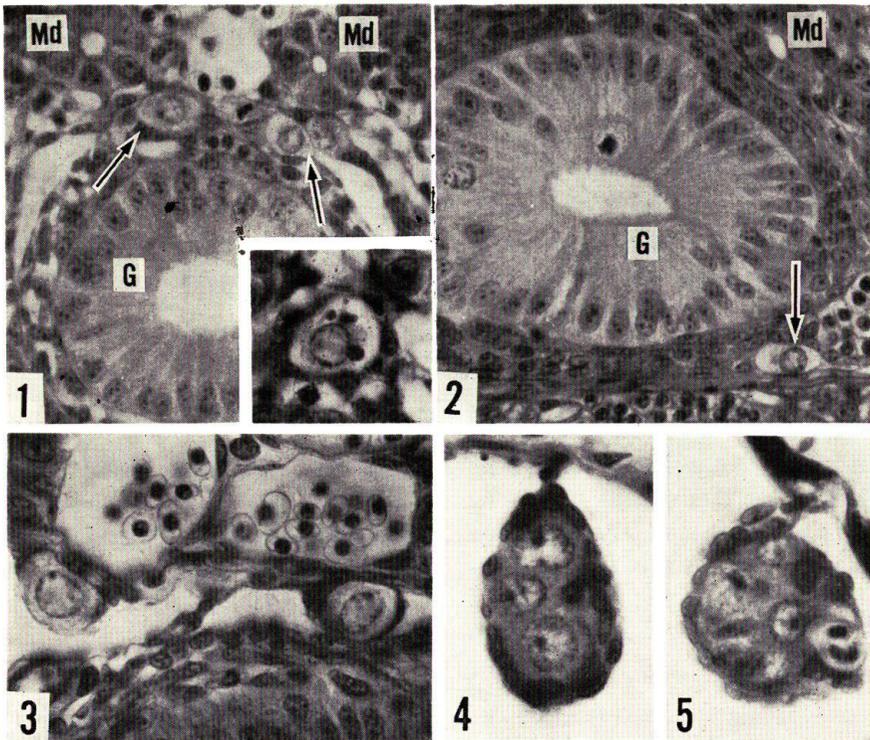


Fig. 1. Frontal section of a newly hatched fry. Arrows show primordial germ cells clustering at the dorsal root of mesentery. *G*, gut; *Md*, mesonephric duct. $\times 540$.
Inset: a primordial germ cell with yolk platelets in the cytoplasm. $\times 700$.
Fig. 2. Frontal section of a newly hatched fry, revealing a germ cell (arrow) lying in the mesodermal layer ventral to the gut (*G*). *Md*, mesonephric duct. $\times 540$.
Fig. 3. Frontal section of a fry 3 days after hatching. $\times 700$.
Figs. 4 and 5. Transverse sections through sexually indifferent gonads of specimens examined 10 days (Fig. 4) and 15 days (Fig. 5) after hatching. Fig. 4, $\times 1200$; Fig. 5, $\times 900$.

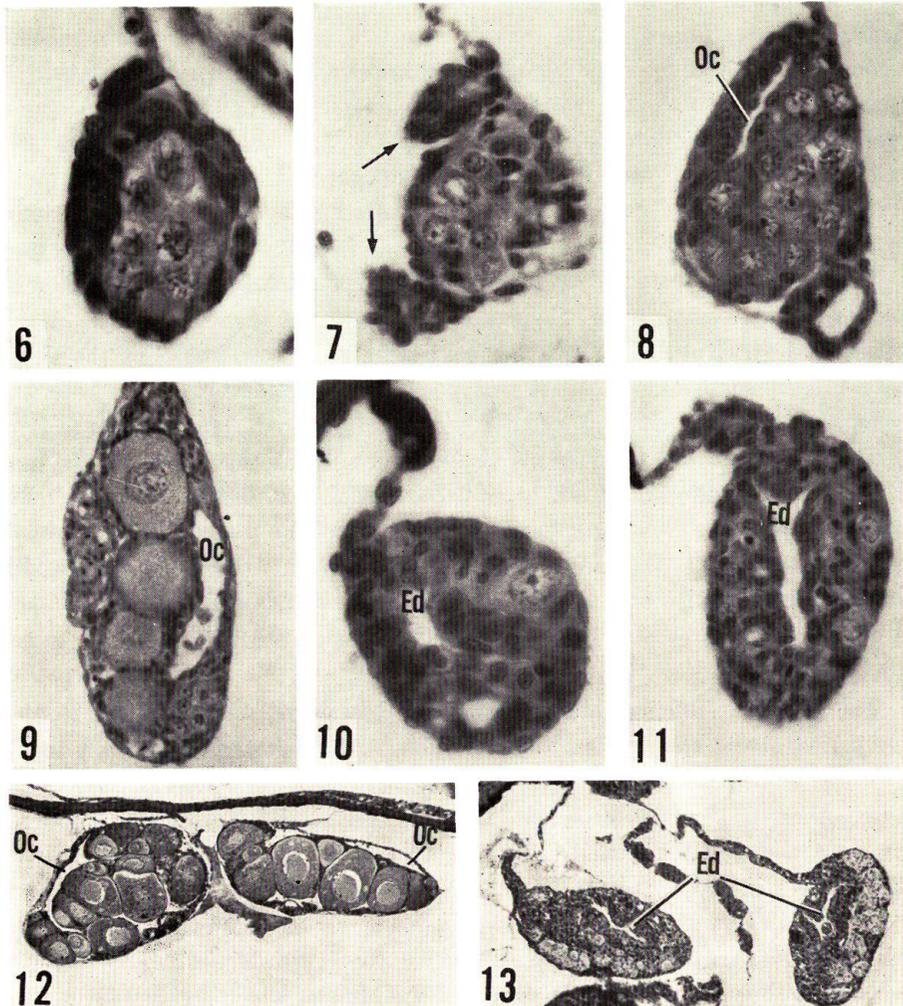
ing 11–12 μ in size, making clusters of several cells especially in the anterior region of the gonad (Fig. 4). In this stage each gonad was evidently seen to be suspended from the dorsal peritoneal wall into the coelomic cavity by a thin sheet of somatic cells, a mesogonium. For some days afterwards, the germ cells continued a gradual increase in number resulting in a gradual enlargement of the gonads. The somatic cells of the gonads, which constituted a stroma tissue embedding the germ cells, also showed a slight increase in amount. All the gonads observed 16 days after hatching were, however, as yet almost similar in their essential histology to one another and to those observed in the preceding stage, and were regarded as being sexually indifferent at least morphologically (Fig. 5).

The gonads of some of the fish examined 20 days after hatching possessed not

only a few single and clustered gonial cells but also many cysts of germ cells in meiotic prophase (Fig. 6). The somatic elements of these gonads also displayed concomitantly a characteristic change at this period: they began to extend two tissue sheets from both the distal and proximal edges of the gonad along the lateral side facing the lateral peritoneal wall (Fig. 7). The two elongating sheets of the gonadal stroma were developed each dorsad and ventrad to form grooves between them and the gonad proper, the ventrad elongation from the proximal edge being more conspicuous than the dorsad one from the distal edge of the gonad. They eventually came to be fused with each other at their free edges, thus constructing a flat cavity lying along the lateral side of the gonad (Fig. 8). The position and structure of this cavity coincided fairly with those of the ovarian cavity of defined ovaries, and it was possible to regard the gonads with these developments of germinal and somatic elements as ovaries. In general the formation of the ovarian cavity appeared to begin in the anterior region of the gonad and to proceed gradually in the caudal direction. A perfect construction of the ovarian cavity along the whole length of ovaries became noticeable more than 35 days after hatching. The paired ovaries were arranged converging progressively toward the caudal direction, and at their caudal ends nearby the urinary bladder the ovarian cavities became to be confluent with each other to open into a single oviduct.

The testicular differentiation of the gonad appeared to occur at about the same period as did the ovarian differentiation. At 20 days of age, while some of the fish examined were provided with the gonads of the above-mentioned ovarian nature, others had gonads in which the gonial germ cells did scarcely develop to form cysts and remained small in number. In these gonads, the germ cells were found to be sparsely distributed exclusively along the lateral periphery facing the lateral peritoneal wall. In most of these gonads, slit-like lumina were noticed to appear as splits in the stroma tissue packing the centro-lateral region facing the mesogonium (Fig. 10). These characters of the lumina, which contrasted definitely with those of the ovarian cavities (compare Fig. 12 with Fig. 13), were exactly coincident with those of the efferent duct in the developing testes. Thus, the testicular differentiation could be ascertained to be started by 20 days of age simultaneously with the ovarian differentiation. Just like in the case of the ovarian cavity, the efferent ducts came to join with each other at the caudal end of the paired testes and were connected with a single sperm duct which ran closely adjoining the urinary duct.

In the ovaries observed 26-31 days after hatching, there were some oocytes of the peri-nucleolus stage together with many oocytes of younger stages (Fig. 9). Henceforth ovaries were steadily enlarged in size in parallel with the growth of oocytes (Fig. 12), and began to show the initiation of vitellogenesis in oocytes 100-



Figs. 6-9. Transverse sections through ovaries of normal females 20 days (Figs. 6 and 7), 25 days (Fig. 8) and 35 days (Fig. 9) after hatching. The initiation of ovarian differentiation is noticeable in the occurrence of premeiotic germ cells (Fig. 6) and in the elongation of somatic ridges (Fig. 7, arrows) in the gonad at 20 days of age. *Oc*, ovarian cavity. Fig. 6, $\times 760$; Figs. 7 and 8, $\times 550$; Fig. 9, $\times 270$.

Figs. 10 and 11. Transverse sections through testes of normal males 20 days (Fig. 10) and 25 days (Fig. 11) after hatching. The development of efferent ducts (*Ed*) as slits in the stroma tissue begins at 20 days of age. Fig. 10, $\times 760$; Fig. 11, $\times 640$.

Figs. 12 and 13. Transverse sections through ovaries (Fig. 12) and testes (Fig. 13) of normal fish about 60 days after hatching. Note the difference in topographic position in the gonad between ovarian cavity (*Oc*) and efferent duct (*Ed*). $\times 80$.

150 days after hatching. On the other hand, the germ cells in the testis remained quiescent in their development until up to 50 days of age, though the stroma tissue with developing efferent ducts increased distinctly in amount (Fig. 11).

Active spermatogenesis including cystic conformation of spermatogonia seemed to take place about 50-70 days after hatching (Fig. 13).

Effects of ethinylestradiol on sex differentiation of the gonad

In a series of four experiments, the fry of *T. mossambica* were subjected to the treatment with ethinylestradiol (50 μ /g diet) starting at 6, 11 and 16 days of age and continuing for 10-20 days, with the design of determining the period of gonadal development critical for inducing complete feminization of genetic males by the estrogen. The resulted sex distribution in experimental and control groups is summarized in Table 1.

Table 1. *Effects of oral administration of ethinylestradiol (50 μ /g diet) on sex differentiation of *Tilapia mossambica*.*

Exp. No.	Group	Age of fish at start and end of treatment (days after hatching)	No. of fish examined*	Sex distribution			Mortality** (%)
				♀	♂	♂	
1	Exp.	6-15	16	4	0	12	11.5
	Cont.		16	8	0	8	0
2	Exp.	11-20	30	12	16	2	0
	Cont.		10	1	0	9	0***
3	Exp.	16-25	40	15	0	25	0
	Cont.		40	21	0	19	0
4	Exp.	6-25	46	46	0	0	13.0
	Cont.		55	21	0	34	0

* Sex distribution indicated in this table was determined by histological observations of gonads of the fish of more than 25 days of age in each group.

** Mortality in each group was estimated at the end of the hormone treatment in each of the experiments.

*** About 20 control fish died by accident after the hormone withdrawal and were excluded from the result.

In an experiment of treating the fish only during the sexually indifferent stage for 10 days earlier than 15 days of age (Experiment 1), no signs of sexual modification were detected in testes as well as ovaries examined 10 and 30 days after the completion of treatment. The gonads were similar in developmental degrees to those of the control fish of the same ages, and the occurrence of any intersexual traits was never noticed in either the germinal or the somatic elements of the affected testes. Similar results were obtained in Experiment 3 in which the estrogen administration was begun at 16 days of age and lasted for 10 days covering the period of gonadal sex differentiation. The ovaries of the treated fish were developed quite similarly to those of the controls. The testes of treated fish also showed a favorable development when examined 15-70 days of age, though

their germ cells seemed to be fewer in number as compared with those of the control testes. In the majority of the affected testes, however, a mass of stroma tissue displayed an elongation from the proximal border of the testis (Fig. 14). The elongation of the testicular stroma influenced by the estrogen was comparable to that which occurred in the initial process of ovarian cavity formation, though

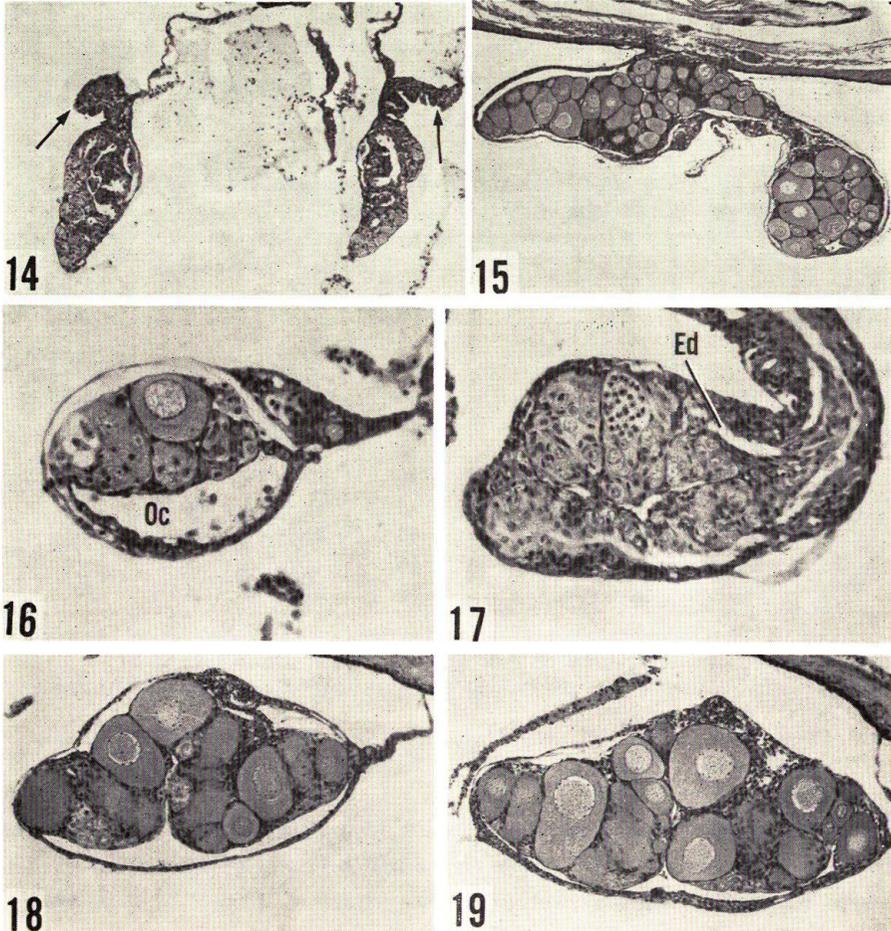


Fig. 14. Transverse section through testes of an estrogen-treated male in Experiment 3, 70 days after hormone withdrawal, revealing stromal elongation (arrows) from the proximal region of affected testes. $\times 100$.

Figs. 15-17. Transverse sections through gonads of an estrogen-treated female (Fig. 15) and an intersex (Figs. 16 and 17) in Experiment 2, 80 days after hormone withdrawal. Figs. 16 and 17 show an ovarian portion in the anterior region and a testicular portion in the posterior region of the same intersexual gonad, respectively. Fig. 15, $\times 45$; Fig. 16, $\times 270$; Fig. 17, $\times 250$.

Figs. 18 and 19. Transverse sections through ovaries of a control (Fig. 18) and an estrogen-treated female (Fig. 19) in Experiment 4, 60 days after hormone withdrawal. $\times 100$.

the former originated in no case from the distal border of the testis. It was considered that the estrogen treatment only during the period of histological sex differentiation of the gonad was capable of evoking a partial inversion of the somatic element, but not of the germinal one, of the gonads of genetic males toward female direction.

On the other hand, an incomplete feminization of genetic males was elicited in Experiment 2, in which the estrogen was administered for a period of 10 days starting at 11 days of age when the fish were in the sexually indifferent stage and lasting until 20 days of age when the sex differentiation of the gonads became noticeable histologically. Out of 30 fish examined 25 to 80 days after the hormone withdrawal, 12 were provided with defined ovaries and 2 with defined testes, though a median fusion of bilateral gonads was frequently observable in these gonads (Fig. 15). The remaining 16 possessed the gonads of prevailingly testicular nature in which intersexual features of various degrees were encountered. In most cases the intersexual gonads were ovarian in nature in the anterior region (Fig. 16) whereas they were evidently testicular in the posterior region (Fig. 17), the latter being predominant in the gonads in all cases. The occurrence of a few, small oocytes and the formation of ovarian cavity characterized the ovarian structure. In the testicular region of the gonads the beginning of spermatogenesis was observed 80 days after the completion of the estrogen treatment, as was the case of the control males of the same age.

A complete feminization of genetic males could be brought about only in Experiment 4, in which the estrogen administration covered the period of 20 days from 6 to 25 days of age, namely from a time of sexually indifferent stage to a time after the gonadal sex differentiation had definitely occurred. At the end of the treatment, all the treated fish had young ovaries which were similar in histology to those of the control females. All the treated fish examined at 30, 40 and 60 days of age were also provided with ovaries which were developed in almost a similar degree to those of the control females of the same ages (Figs. 18 and 19), though slight structural modifications such as an abnormal increase in amount of stroma tissue were noticed in some ovaries of the treated fish. The sex ratios totalling so far in Experiment 4 were 46 females and 0 males for the experimental group and 21 females and 34 males for the control one, as revealed in Table 1. Five other treated fish are now reared in the laboratory till sexual maturation, in order to examine, by mating them with normal males, if the expected sex reversal may be complete and functional.

Discussion

On the basis of plentiful experimental findings obtained by himself and his co-workers in *Oryzias latipes*, Yamamoto²⁾ pointed out that, in order to attain

a complete and functional sex reversal in fishes, some hormone treatment should be done beginning at the stage of indifferent gonad and continuing through the stage of sex differentiation. By histological observations on normal gonadal development in *Tilapia mossambica*, it was confirmed that the sex differentiation of the gonad in this species occurred during the period between 16 and 20 days of age when the body length was between 8 and 11 mm in an average. In the same species, Clemens and Inslee⁶⁾ mentioned that the gonadal sex differentiation took place between 35 and 48 days after hatching when the body length was between 15 and 30 mm. Although the difference between the observations made by the present writers and that by Clemens and Inslee is not explainable on account of the lack of detailed descriptions about the phenomenon in the latter, one of the causes is surmised to lie in morphological criteria adopted to decide the occurrence of sex differentiation of the gonad.

In various gonochoristic teleosts observed so far, morphological differentiation of the ovary emerges from the sexually indifferent state first, evidently preceding that of the testis. In a cichlid *Hemihaplochromis multicolor*, the ovarian gonads come to be provided with some auxocytes 17 days after spawning, while the testicular ones are not discernible to differentiate until more than 25 days of age¹⁰⁾. In another cichlid *Tilapia aurea*, the ovarian gonads begin to form the ovarian cavity about 30 days after hatching and begin to show auxocyte development 3 to 4 weeks later, whereas the testicular gonads remain quiescent in morphological differentiation during the period over 2 months¹¹⁾. In *T. mossambica* used in the present study, the gonadal sexes were indiscernible at least morphologically at 16 days of age, but became clearly distinguished by 20 days of age when some cysts of germ cells appeared to enter into meiotic prophase in some gonads. Simultaneously these gonads displayed an initiation of formation of the ovarian cavity, which further ensured the ovarian differentiation of these gonads. Thus the germ cell maturation in female gonads occurs in evident advance of that in male ones in this species as in other teleostean fishes.

In ovaries of some teleosts such as *Lebistes reticulatus*¹²⁾, and *Carassius auratus*⁹⁾, the ovarian cavity is not formed until germ cells have well progressed in oogenesis. In other fishes such as *Cottus bairdii*¹³⁾, however, the ovarian cavity initiates its formation concomitantly with the start of oogenesis, and in others like *Tilapia aurea*¹¹⁾ it makes its appearance during the period when the germ cells are still in a sexually indifferent phase. Especially in such cases as the latter two, a sex-specific behaviour of the gonadal stroma tissue seems to be successfully taken as a decisive criterion of gonadal sex differentiation. This was indicated also in the testicular differentiation in *T. mossambica*. Like in other cichlids cited before, the testicular gonads of the fish also remained quiescent in germ cell maturation for about 50 days after hatching. However, the formation of efferent duct anlagen

in gonadal stroma became apparent by 20 days of age, which made it possible to confirm the occurrence of testicular differentiation irrespective of the germ cell quiescence. The efferent duct anlagen were well discriminated from the ovarian cavity, since the former were the slits appearing in the stroma tissue of male gonads.

Thus it is concluded that, in *T. mossambica*, the gonads which are sexually indifferent at least in a histological sense may initiate their sex differentiation either into ovaries or into testes at some time between 16 and 20 days after hatching. A morphological differentiation of gonadal stroma into ovarian cavity or efferent duct appears to be started at the same age and usefully directs the gonadal sexes as was the case in *Cottus bairdii*¹³⁾.

So far as the present writers know, morphogenesis of the ovarian cavity in cichlid fishes has been described only for *T. aurea*¹¹⁾. In this cichlid the development of a stromal ridge from the proximal border of a presumptive ovary leads to the occurrence of two parallel ridges, and their eventual fusion at the distal edges completes the formation of the ovarian cavity. In *T. mossambica*, however, the ovarian cavity originates from two stromal ridges growing from both the proximal and distal borders of the ovary. A similar process of the gonad cavity formation was reported to be seen in the differentiation of ovaries of *Cottus*¹³⁾ and in that of the hermaphroditic gonads of some sparid fishes¹⁴⁾.

The results of the estrogen treatments in the present study seem to prove the propriety of the afore-mentioned Yamamoto's view²⁾ to be extended to *T. mossambica*. A complete sex reversal of genetic male gonads into ovaries was brought about only when ethinylestradiol was administered beginning considerably prior to the occurrence of testicular differentiation and continuing fully through the period of morphological sex differentiation of the gonad (Experiment 4). A shorter treatment with the estrogen during that period was effective merely in causing partial feminization of testicular gonads (Experiment 2). The treatment covering only the sexually indifferent stage was without effect (Experiment 1), and that being carried out only during the period of sex differentiation was at most capable of eliciting a feminizing modification of the testicular stroma (Experiment 3). Similar correlations between the time of androgen feeding and the masculinizing tendencies found in the affected gonads were evidenced also in a series of unpublished experiments in *T. mossambica* as made by the present writers.

These results may indicate that a harmonized development of germinal and somatic elements of the gonad toward a sexually reversed direction is expected when the treatment with a heterologous sex hormone covers the period between 10 and 25 days after hatching, centering at the time of morphological sex differentiation of gonads, in *T. mossambica*. As mentioned already, the appearance of efferent

duct anlagen at 20 days of age manifests the testicular differentiation whereas the germ cells in these gonads remain inactivated until about 50 days of age, showing a sharp contrast to those in the female gonads. It is probable that the germ cells in the male gonads may be *physiologically* differentiated into the male ones by the time of the stromal sex differentiation of the gonads, and that the differences in the effects of the present experiments may be ascribable to the change in the competence of the germ cells to respond to the treatment during the process of their *physiological* sex differentiation.

Ethinylestradiol has never been tested for its feminizing potency in cichlid fishes. The estrogen, especially given in high dosages, is liable to exert some toxic influences on various organs including gonads⁹⁾¹⁵⁾. Although the dose of 50 μg per g diet employed in the present study seems to be much higher than that of the same hormone required for inducing complete feminization of *Oryzias latipes*²⁾, it did scarcely exhibit any harmful effect on the general conditions and on the gonadal development in *T. mossambica*. The estrogen did neither inhibit nor accelerate the development of ovaries in genetic females. In the case of complete feminization the affected gonads of genetic males appeared to develop into ovaries through just the same process of differentiation as in those of genetic females, as were the cases of feminization by ethinylestradiol (125 $\mu\text{g}/\text{g}$) in *Lebistes reticulatus*¹²⁾ and by estrone (50 $\mu\text{g}/\text{g}$) in *Oryzias latipes*¹⁶⁾.

Accordingly it is highly possible to expect a complete and functional feminization of genetic males of *T. mossambica* by administering ethinylestradiol at a dose of 50 $\mu\text{g}/\text{g}$ so as to cover enough the sensitive period of gonadal development which is presumed to lie in the period from 10 to 25 days after hatching. Clemens and Inslee⁶⁾ succeeded in obtaining a functional masculinization of genetic females of the same species by treating them with methyltestosterone for 69 days from the time just after hatching. It remains to be elucidated if there are any correlations between the duration of effective treatment, or that of the sensitive period, and the dosage of hormones to be administered. In the case of the androgen-induced sex reversal in *Hemihaplochromis multicolor*, it was reported that the duration of the sensitive period could be delimited to about 48 hours (Hackmann, cited from Reinboth¹⁷⁾).

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