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Modification of the Development of Female Reproductive Organs in the Guppy, *Poecilia reticulata*, Following an Androgen Treatment in Their Juvenile Period

Hiroya TAKAHASHI*

Abstract

Broods of guppies, *Poecilia reticulata*, were daily fed on diets containing methyltestosterone (MT, 15–300 μg/g) or 11-ketotestosterone (KT, 50–200 μg/g) for varying lengths of days starting on different days following birth, and subsequently raised on ordinary diet for more than a month after the end of the treatment (Table 1), in order to obtain some data concerning the outcome long after the cessation of the treatment.

MT caused the masculinization of the form of the urogenital sinus and the arrangement of associated ducts in females. The androgen-induced development of the gonopodial suspensorium in females was preceded by an induced formation of the associated ventromedian ridge, which often led to a persistent defect in the oviduct within the ridge.

An intersexualization of ovaries was the general result of the treatments with MT above 30 μg when initiated earlier than 20 days of age and lasting for more than 35 days, but a complete sex reversal was scarcely attained. The degree of intersexualization did not mutually correlate with the dose of MT and the duration of the treatment. The somatic cells in ovaries and oviduct anlages also responded to MT by differentiating into those of sperm ducts, which were always atypical and fragmentary. In some cases the affected females were entirely defective in oviducts caudal to the ovary. A marked decrease in number of ovarian germ cells, due to a progressive degeneration and suppressed production of oocytes, and a conspicuous thinning of the wall of the ovarian cavity were often followed by a sterilization of the gonads in adult females. In many cases, the oocytes surviving the treatment recovered to mature, but they subsequently suffered a dissolution and extrusion into the ovarian cavity persisting long after the cessation of the treatment.

KT acted as androgenic as MT in eliciting essentially similar changes in the affected females, but was much weaker in the action than MT when administered orally: 200 μg KT was nearly comparable to less than 30 μg MT in the effects. A stimulation of the testicular development was effectuated in males by KT as well as MT.

Among a variety of actions exerted by sex steroids in fishes[1], morphogenetic effects of androgens on secondary sex characters are most commonly known. In a viviparous cyprinodont, the guppy *Poecilia reticulata*, since the androgen-induced formation of gonopodia in juvenile females had been reported by Eversole[2], several external characters such as the body coloration and the shape of fins are

* *Laboratory of Fresh-Water Fish-Culture, Faculty of Fisheries, Hokkaido University*
(北海道大学水産学部淡水増殖学講座)

known to be dependent on the action of androgens. In addition, there are some evidences to show that the initial development of internal reproductive accessories of fishes is also influenced by androgen: the seminal vesicle of the goldfish is formed in juvenile females treated with androgen[3], and the sperm duct of the guppy is stimulated to differentiate and develop by exogenous androgen[4)5]. Since somatic elements of the gonadal system of teleosts have rather been exempted from detailed experimental observations, many more amounts of information should be accumulated in order to clarify a primary part taken by sex hormones in the morphogenesis of the teleostean reproductive system.

Artificial induction of sex reversal is another interesting subject of experimental application of steroid hormones throughout vertebrates, and several authors have succeeded in attaining a sex hormone-induced, functional sex reversal in a few species of teleosts.[6] In the guppy, Dzwillo[7] is the only one who has evidenced the occurrence of functional masculinization of genetic females treated with methyltestosterone before birth. Many other attempts to treat juvenile guppies with various androgens have failed to induce a functional sex reversal, only bringing about an intersexualization of varying degrees in the affected ovaries.[8)9)10]

Among those scarcely any observations have been extended to examine the androgen-treated fish continuously for long periods of days following treatment. Only the work of Clemens et al.[10] mentioned the deficiencies in spermiation and courtship behavior occurring in some treated guppies when mated with females, suggesting the malfunctioning of the pituitary caused by the androgen treatment in their juvenile period. These results are of particular interest because they are concerned with some undetermined effects of androgen which remain possibly long after the treatment in sexually modified fish.

During the course of serial studies on the effects of sex steroids on the developing gonads of the guppy, the writer observed some interesting functional modifications of ovaries and oviducts in adult females which had been affected by androgens in their juvenile period. These results of androgen treatment, along with comments on some androgen-dependent characteristics of the urogenital sinus and its related structures which seem to be new to literature, are mainly dealt with in this paper. In addition, androgenic effects of 11-ketotestosterone were tested first in the guppy and were compared with those of methyltestosterone.

The writer is deeply grateful to Prof. K. Yamamoto, Faculty of Fisheries, Hokkaido University, for his continued interest and criticism throughout the study.

Material and Methods

Broods of the guppy, *Poecilia reticulata*, employed in this study were obtained from isolated pregnant females of the fish of a yellow variety bred in the laboratory[5].

Twenty two separate experiments using the fish of 27 broods in total were performed annually through the years from 1971 to 1973. Newly delivered guppies of a single brood were divided into one or two experimental groups and a control group, and were kept in separate glass aquaria containing approximately 9 liters of constantly aerated water at 23–25°C, under the condition of natural light and darkness. Aquarium water was changed generally once a week.

A hormone treatment was carried out by feeding a hormone-containing diet daily to the guppies of the experimental groups. Crystalline methyltestosterone (MT) and 11-ketotestosterone (KT) were previously dissolved in 95% ethyl alcohol at the concentration of 100 μg/ml and served as stock solutions. A powdered commercial dry food was mixed thoroughly with an adequate amount of the stock solution to get a given dose of hormone per g diet, air dried, and then stored in a

Table 1. *Procedures of treatments of the guppy with methyltestosterone (MT) and 11-ketotestosterone (KT).*

Exp. No.	Treatment (μg per g diet)	No. of brood used	No. of fish treated	No. of fish died	Age of fish		
					at the start of treatment	at the end of treatment	at the last fixation
1	MT 15	1	18	1	2	36	91
2	MT 30	1	28	0	1	39	100
3		1	34	0	30	65	130
4	MT 50	1	23	0	1	20	70
5		4	78	4	1	35	131
6		1	21	0	1	40	87
7		1	32	3	2	62	126
8		1	10	0	2	93	223
9		1	24	0	1	119	149
10		1	85	0	10	49	330
11		1	14	0	21	62	93
12		1	22	0	30	64	100
13	MT 100	1	32	0	1	10	119
14		1	24	1	1	29	130
15		1	23	0	1	45	106
16		2	48	1	2	61	123
17		1	13	0	1	91	151
18		1	17	0	30	64	95
19	MT 300	2	49	2	1	36	91
20	KT 50	1	26	0	1	34	119
21	KT 100	1	30	2	1	34	96
22	KT 200	1	18	0	2	36	90
(Total)		(27)	(669)				
Control		15	212	2			

dark box. A diet for the control fish was similarly prepared by adding the same amount of solvent ethyl alcohol to the diet. Hormone doses used in this study were 15, 30, 50, 100 and 300 μg/g diet for MT and 50, 100 and 200 μg/g diet for KT. Daily administration of the hormone-containing and control diets was started one or two days following birth in most experiments, but from 10, 20 and 30 days of age in several ones. The amount of diet given was roughly estimated to be

Table 2. *Sex distribution, gonopodium formation, and changes of ovaries in the guppy following treatments with methyltestosterone and 11-ketotestosterone.*

Exp. No.[1]	Sex distribution[2]				Formation of gonopodium in females[3]	Change of ovary[4]		
	♀	♀	♂	(Total)		Dissolution and extrusion of mature oocyte	Tendency to sterilization	Defect in gonoduct system
1	9	0	8	(17)	I	+	−	−
2	2	14	12	(28)	C	+	−	±
3	19	0	15	(34)	C	−	−	−
4	3	5	5	(13)	I	+	−	+
5	11	22	29	(62)	C	++	±	+
6	2	5	14	(21)	C	++	−	+
7	6	8	10	(24)	C	+	++	++
8	2	3	5	(10)	C	+	++	++
9	0	12	7	(19)	C	++	+	+
10	39	1	37	(77)	C	++	+	−
11	2	2	6	(10)	C	+	−	−
12	13	0	9	(22)	C	±	−	−
13	20	0	12	(32)	S	±	−	−
14	10	0	13	(23)	C	++	−	±
15	5	5	9	(19)	C	++	+	++
16	10	7	30	(47)	C	±	+	++
17	3	3	7	(13)	C	−	+	++
18	10	0	7	(17)	C	−	−	−
19	17	7	23	(47)	C	++	+	++
20	13	0	13	(26)	I	−	−	−
21	16	2	10	(28)	I	++	−	±
22	6	0	6	(12)	I	++	−	±
Total	218	96	287	(601)				
Control	110	0	100	(210)	−	−	−	−

1) See Table 1.
2) Sex distribution was arranged among the sum of the fish aged more than 30 days.
3) Development of gonopodium in females of each group was examined at the end of treatment. C, complete; I, incomplete, S, slight.
4) Ratings of change: −, none; ±, slight, +, definite in some animals; ++, marked in most animals.

0.01–0.015 g per fish a day. The treatment lasted mostly for periods ranging from 30 to 40 days, though it was continued for more than 90 days for some groups. After the treatment had ceased, the fish of both experimental and control groups were sequentially maintained on an ordinary diet until they were sacrificed for the microscopic observations of their gonadal system. Detailed experimental procedures executed in each experiment are presented in Table 1.

Because of the final aim of this study to pursue the developmental changes of the gonadal system influenced previously by androgens, most of the treatdə fish were sacrificed, 6 to 10 at a time, at various times following the cessation of the treatment. They were fixed on about 35, 50, 70, 100 and 130 days of age in most cases, but in a few experiments the examination was done on the guppies killed when about 150, 180, 220, 240 and 330 days of age. Besides, some of the fish of several experimental groups were fixed at some times during the period of the treatment. The control fish of each experimental series were also sacrificed at the time of fixation of the treated ones. Some guppies of each brood used in this study were also preserved at the beginning of the treatment as an initial control. In addition, untreated guppies of many other broods were examined from the time of birth to the age of gonadal maturation to standardize observations of the development of their gonads and gonoducts. As the histological features of ovarian development were quite similar in all of the control groups, no distinction was made among those controls in this paper.

In order to leave the gonad and associated duct system intact, all of the treated and control fish were fixed *in toto* in Bouin's fluid or Bouin-Hollande solution, and were properly trimmed following fixation. Serial paraffin sections of the specimens were cut frontally or sagittaly at 8–10 μ in thickness, and stained with Delafield's hematoxylin and eosin or with Mallory's triple stain.

Results

External sex characters

The guppy employed in this study is characterized by bearing a pale yellow hue all over the body surface without accompanying notable black pigmentation of any pattern, on account of which it is named the "yellow variety". The yellowish hue is genetically recessive to the ordinary black pigmentation of the original stock of the fish. Mature males of this variety carry several orange-red spots or patches on the lateral sides of the caudal peduncle and on the dorsolateral sides of the trunk, but females never show such coloration even if they are under the influence of exogenous androgens. This means that the orange-red coloration may certainly be controlled by Y-linked genes in the guppy which is known to be male heterogametic.[11] Accordingly, the genetic males of the guppy of the present

variety are distinguishable from the genetic females by their orange-red pigmentation of a regular pattern appearing at early stages of their sexual development.

Under the rearing conditions of this study, the orange-red spots made their first appearance on both sides of the caudal peduncle of the control male guppies sometime after 40 days of age. An administration of MT evidently promoted the appearance of this male sex character in genetic males. In the cases of the treatment with 50 μg MT from the day following birth, the spots began to appear by 25–30 days of age in almost all the affected males. KT acted similarly on the male coloration, but the accelerating effect of KT seemed to be slighter than that of MT.

A transformation of the anal fins into gonopodia was not only accelerated in genetic males but also caused in genetic females, under the influence of both MT and KT. In general, an elongation of the anterior rays of the anal fin began to occur in control males 20 to 25 days following birth, and well-formed gonopodia came to appear in the males aged more than 35 days. By the treatment with MT at doses above 30 μg initiated the day following birth, an elongation of the anal fin rays was observed to emerge in the treated fish of both sexes of 5 to 10 days of age, and the formation of gonopodia proceeded at the same pace in both of the affected males and females to be completed within 20 days of hormone treatment. The resultant gonopodia of treated females were preserved constantly after the treatment had ceased.

MT at the dose of 15 μg was effective in evoking an elongation of the anal fin rays in females, but was insufficient to induce the complete formation of gonopodia in these females at least during the experimental period lasting for as long as 35 days of juvenile life (Table 2). The same was true in the cases of treatments with 50, 100 and 200 μg KT, though the development of gonopodia was clearly promoted in males influenced by KT as well as MT at the smallest dose. A notable elongation of the tips of the ventral fins was also noticed in the fish of either sex affected by the androgens.

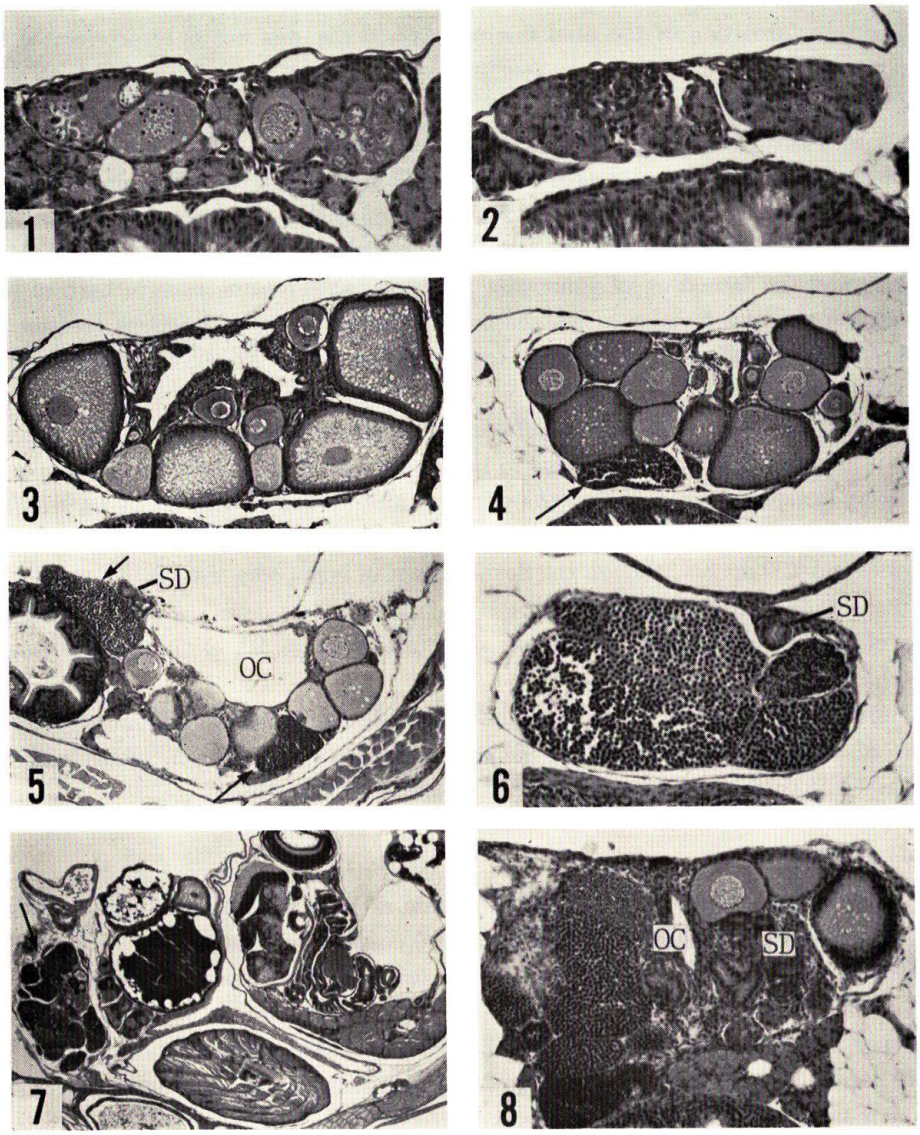
Morphological characteristics of the urogenital sinus and its related structures were also masculinized in genetic females treated with androgen, which will be described in some detail in the separate section of this paper.

Body growth

Adult female guppies are generally larger in body length than males. Body growth advances similarly in both sexes of control guppies during the first 20 postnatal days, but the females become to exceed the males in body length clearly about 35 days following birth. Feeding of MT at, and above, the dose of 30 μg starting the day after birth remarkably retarded the increase of body length in both sexes throughout the period of hormone treatment. The retardation appeared to be more pronounced when the administered dose of androgen was more elevated.

MT at 15 μg and KT at the three doses tested had no such obvious effect.

After the hormone administration was ended, the affected males continued to grow but quite slowly, whereas the affected females regained their growth at a greater rate than the males and eventually became as large as the control females of the same age, assuming a female appearance in body shape with the development of a so-called gravid spot and a masculinized anal fin. The restoration of body growth was noted also in the treated females in which ovaries were modified to varying extents by the occurrence of spermatogenetic tissues, although pronounced



intersexualization of the gonad tended to lessen the degree of the restoration of growth following the withdrawal of exogenous androgens.

Ovary

As has been ascertained by earlier authors[12)13)14)], in newly delivered guppies, ovaries are evidently discernible from testes mainly by the presence of synaptic oocytes and auxocytes and by the absence of hilar mass of stroma cells in the former (Figs. 1 and 2). During the normal course of ovarian morphogenesis, the formation of the ovarian cavity by means of a fusion of curving lateral edges of the single median ovary with the dorsal coelomic wall begins at the cephalic part of the ovary about a week after birth and proceeds caudalwards to be shaped through the entire length of the ovary 12 to 15 days of age. It was remarked that the formation of the ovarian cavity was not impeded by treatments with androgens at any level of the doses tested, even in the case when the treatment started the day following birth and lasted for more than 35 days.

However, the gonads of females subjected to androgen treatment exhibited some characteristic modifications such as the occurrence of intersexual traits, dissolution and extrusion of mature oocytes, and reduction in number of germ cells leading to sterilization of gonads. Although different combinations of doses of hormones and the time and duration of treatment elicited the modifications of a somewhat varying extent as revealed in Table 2, there was a generalized pattern of gonadal modifications in the results of these postnatal androgen treatments in the guppy.

The gonads of females treated with 50 μg MT for about 35 days following birth were evidently ovarian in their essential structures, with many auxocytes and definite ovarian cavities, at the end of treatment (Fig. 4, compare with Fig. 3). In these ovaries, however, auxocytes were generally less in number, were retarded in development, and those undergoing degeneration were noticed more frequently, when compared with the control ovaries. Ovarian cavities were much narrow in most of the affected ovaries (Fig. 4), but were expanded atypically in some of them

Figs. 1 and 2. Cross sections through the ovary (Fig. 1) and the testis (Fig. 2) of newly delivered guppies. ×220.

Fig. 3. Cross section through the ovary of a control guppy 35 days after birth. ×70.

Figs. 4–6. Cross sections through the intersexual gonads of female guppies at the end of 35-day treatment with 50 μg methyltestosterone from the day following birth. Arrows in the figures indicate spermatogenetic cysts in ovarian gonads. *OC*, ovarian cavity; *SD*, fragmentary sperm duct. Figs. 4 and 5, ×70; Fig. 6, ×140.

Fig. 7. Cross section through the gonad of a 90-day-old female guppy, 50 days after cessation of treatment with 30 μg methyltestosterone, revealing the existence of spermatogenetic cysts (arrow) in the functional ovary. ×20.

Fig. 8. Cross section through the intersexual gonad of a female guppy at the end of 35-day treatment with 100 μg 11-ketotestosterone. *OC*, ovarian cavity; *SD*, sperm duct. ×110.

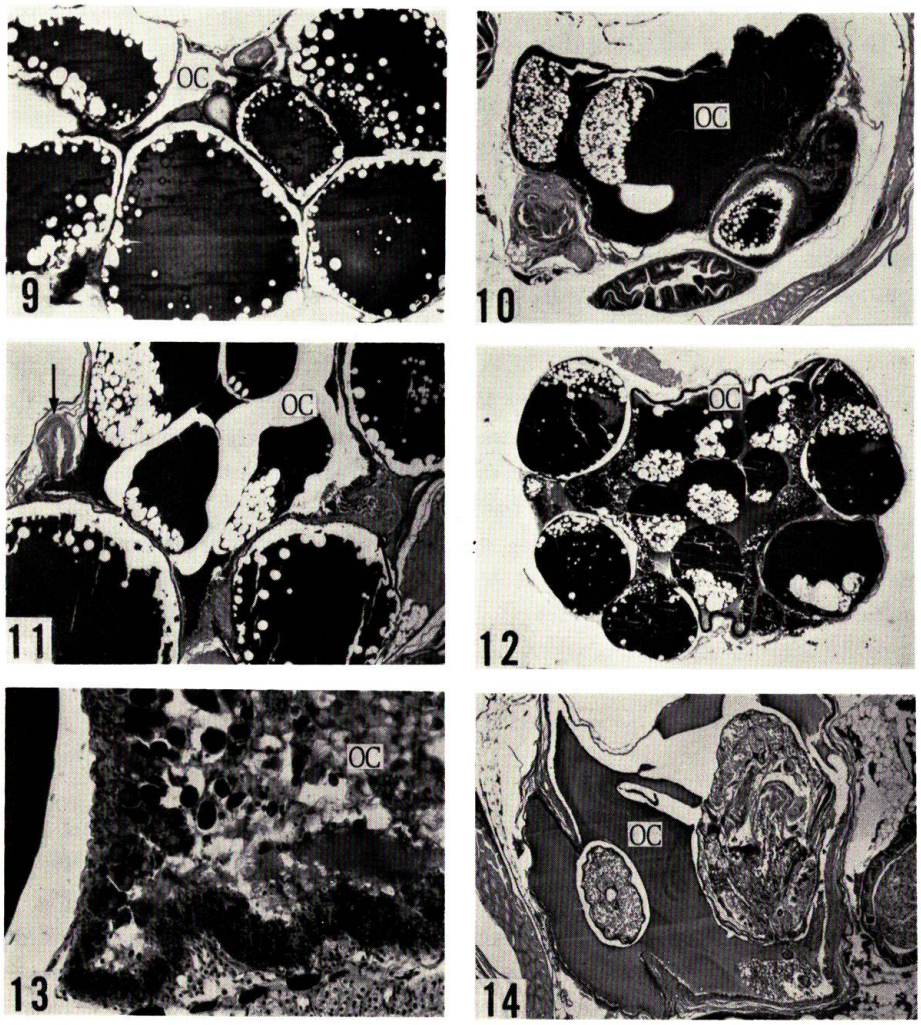


Fig. 9. Cross section through the ovary of a control, 70-day-old guppy. *OC*, ovarian cavity. ×20.

Figs. 10 and 11. Cross sections through the ovaries of 70-day-old female guppies treated with 50 μg methyltestosterone (Fig. 10) and 100 μg 11-ketotestosterone (Fig. 11) for the first 35 days, revealing dissoluted yolk and oocytes extruded from ovarian follicles (arrows in Fig. 11) in the ovarian cavity (*OC*). ×20.

Fig. 12. Cross section through the ovary of a 100-day-old female guppy, 50 days after end of treatment with 50 μg methyltestosterone, demonstrating the presence of several extruded oocytes in the ovarian cavity (*OC*). ×10.

Fig. 13. A part of the wall of ovarian cavity (*OC*) in the gonad of a 100-day-old guppy, 70 days after end of treatment with 50 μg methyltestosterone. Two separate regions with different phagocytotic cells are discernible in the epithelium. ×150.

Fig. 14. Cross section through the ovary of a 100-day-old guppy treated with 100 μg methyltestosterone for the first 45 days. Dead embryos are seen discharged into the ovarian cavity (*OC*). ×20.

(Fig. 5). Complete masculinization of ovaries was not caused by androgen treatments of almost all experimental series, except an experimental group treated with 100 μg MT for 90 days after birth (Exp. 17) in which the gonad of one out of two genetic females aged 151 days was constituted exclusively from atypically arranged spermatogenetic tissues. However, an intersexualization of the affected ovaries, which was characterized by the occurrence of varying amounts of spermatogenetic cell cysts among oocytes, was rather general to the treated females (Table 2).

The intersexualization was much various in aspect as well as in degree in different specimens of the same experimental group. Generally speaking, spermatogonial cells and the cysts of spermatogenetic cells, some of which were in the stage of early spermatids at the end of the 35-day treatment, were a few in number and distributed only dispersedly (Figs. 4 and 5). The development of intragonadal sperm ducts was hardly detectable, and the ducts, if existed, were formed atypically and arranged discontinuously along the testicular region (Fig. 5). In several cases, the spermatogenetic tissues with such abnormal ducts occupied the caudal region of the gonad (Fig. 6), but the ovarian tissues with a distinct ovarian cavity predominated in the median and cranial regions of the same gonad.

The degree of such masculinizing tendency revealed by ovarian germ cells did not mutually relate to the doses of MT used nor to the duration of the treatment. Even with 300 μg MT (Exp. 19), only 7 out of 24 females had ovarian intersexual gonads with merely a small amount of spermatogenetic tissues, and the treatment with 30 μg MT (Exp. 2) was also effective in eliciting a similar change in the affected ovaries. A prolonged treatment with MT for more than 60 days from the day soon after birth (Exps. 7–9) did neither cause a complete transformation of ovaries into testes nor promoted a degree of intersexualization of the ovaries. Following the withdrawal of androgen, the masculinized germ cells of the ovary advanced the spermatogenesis consecutively to form spermatophores, but in most cases they remained to exist still dispersedly, with fragmentary sperm ducts, along the periphery of the ovary (Fig. 7). Especially in a few aged fish treated with higher doses of MT for longer periods of days, most of the surviving germ cells in female gonads were spermatogenetic ones, existing in some separate regions along the wall of the ovarian cavity, but they were never accompanied with the development of the typical sperm duct system. In some cases, moreover, the sterilization of the ovaries due to an extensive degeneration of oocytes was a prominent feature rather than the masculinization, as explained later.

MT at the dose of 15 μg (Exp. 1) was ineffective in inducing the masculinization of female germ cells. The androgen at higher doses was also without effect when the treatment was initiated 30 days of age (Exps. 2, 12 and 18) or when it was confined to only 10 days following birth (Exp. 13). On the other hand, 100 μg KT showed a slight but definite effect of inverting ovarian germ cells into sper-

Table 3. *Changes of ovaries of the guppy treated with methyltestosterone (50 μg/g diet) for the first 35 days after birth.*

Exp. No.	Age of fish (days)	No. of ovaries examined	No. of ovaries with				
			developing embryo	intersexual trait	extrusion of mature oocyte	tendency to steriliza-tion	defect in oviduct system
5[1)]	35	12	0	10	0	0	7
	70	14	0	10	9	3	11
	100	5	0	2	5	0	2
	130	2	0	2	2	0	0
	(Total)	(33)	(0)	(24)	(16)	(3)	(20)
Cont.	35	5	0	0	0	0	0
	70	10	4	0	0	0	0
	100	3	3	0	0	0	0
	130	3	3	0	0	0	0
	(Total)	(21)	(10)	(0)	(0)	(0)	(0)

1) See Table 1.

matogenetic cells and differentiating atypical sperm ducts in the ovarian stroma (Exp. 21, Fig. 8). However, the effect of KT administered orally seemed to be much weaker than that of MT, as it was on the external sex characters (Table 2).

While the development of oocytes into vitellogenetic stages was completely arrested throughout the period of the androgen treatment, it was resumed after the cessation of treatment rather normally so that a few of the affected fish might become pregnant 90 to 100 days of age at least in the cases of the treatments with 15–30 μg MT and 50–100 μg KT (Fig. 7). It was remarked, however, that the females of most of the experimental groups, except those with the MT treatment initiated at the age of 30 days and those influenced by 50 μg KT, became to show a marked abdominal inflation about a month after the end of the androgen treatment. The abdominal inflation was due exclusively to a remarkable enlargement of the ovaries which had been exposed to the androgen treatment. In general, the ovarian cavities of these ovaries were extraordinarily expanded by packing themselves with a liquefied material which displayed the same histological stainability as the yolk of mature oocytes (Figs. 9 and 10).

Actually, an outflow of the liquefied yolk components of oocytes from their follicles into the ovarian cavity through opened delles was evidenced histologically in these ovaries (Fig. 11). Moreover, many roundish bodies, which were similar in size to mature oocytes, were frequently found buried in the liquefied yolk in the ovarian cavity (Fig. 12). It was once observed that, by an improper trimming of fixed specimens, several bodies presumed to be the extruded eggs were spilt out of the ruptured ovarian cavity. The epithelial wall lining the ovarian cavity with the degenerating residue was seen to consist of a layer of columnar cells in which two types of cells were discerned to occupy the separate regions of the wall (Fig.

13). The one was much irregular in aspects with large droplets of a dissolving yolk which was under the phagocytotic action, and the other was rather homogeneous in histological features with numerous fine granules in the cytoplasm. The latter seemed to be the epithelial cells of the ovarian cavity proper, serving as phagocytotic cells, whereas the former was certainly the cells of ovarian follicles which had extruded their contents and had extended to become a part of the wall of the ovarian cavity. Thus, it is certain that the changes of ovaries found in the females subjected to the androgen treatment were owing exactly to a dissolution of oocytes and an extrusion of them from their associated follicles into the ovarian cavity. The extrusion of oocytes seemed to be not always preceded by their degeneration in the follicles, for some eggs undergoing embryonal development could be occasionally observed in the ovary accompanying such abnormality and, in a few instances, dead embryos were found to be immersed in the liquefied yolk accumulated in the ovarian cavity (Fig. 14).

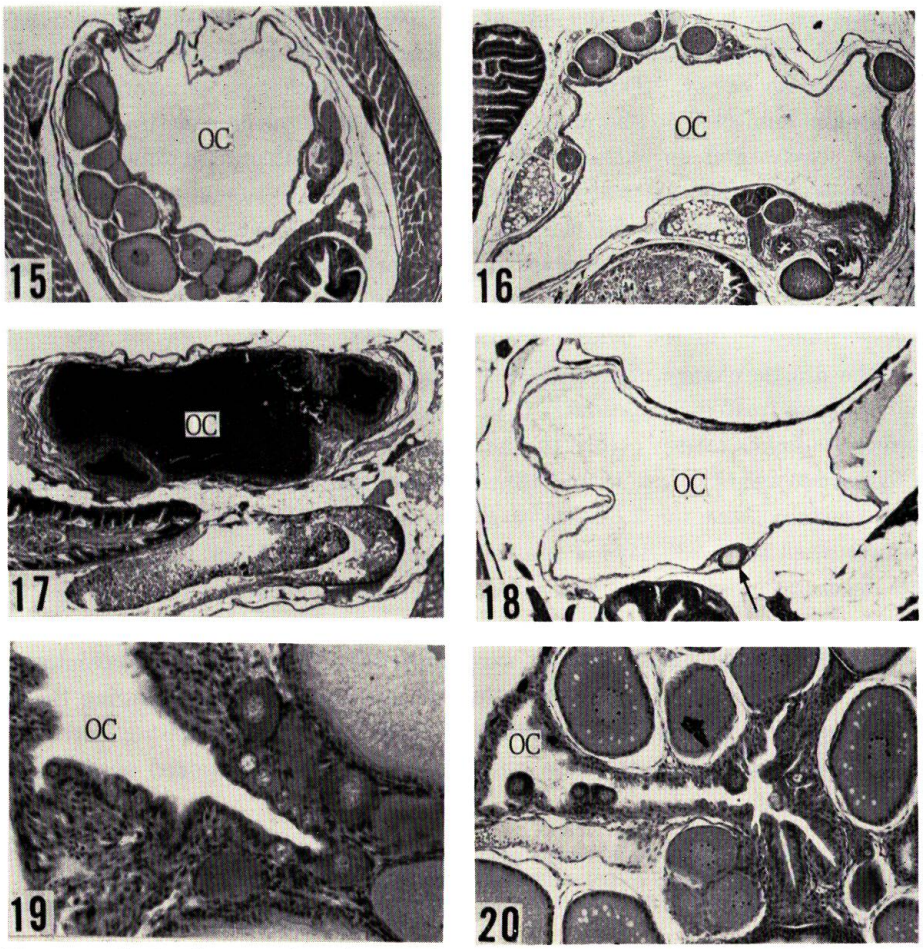
The drastic change of ovaries was rather a general phenomenon occurring in the androgen-treated females later than about a month after the discontinuance of hormone administration. Only in the cases of the treatment with MT from 30 to 64 days of age (Exps. 3, 12 and 18), ovaries of the affected females were exempted successfully from the influence of MT to become almost normal in histological features when 95 to 130 days of age. It was noted, in addition, that the characteristic degeneration of oocytes was as yet observable to occur extending over a long period of days (Table 3). In an experiment with 50 μg MT starting 10 days following birth and lasting for 40 successive days (Exp. 10), the dissolution of oocytes occurred continuously even after the lapse of 280 days following the end of the treatment (Table 4). Treatments with KT at 100 and 200 μg, but not at 50 μg, were also potent to cause the same changes as mentioned above in the affected ovaries.

Table 4. *Changes of ovaries of the guppy treated with methyltestosterone (50 μg/g diet) for 40 days from 10 days of age.*

Exp. No.	Age of fish (days)	No. of ovaries examined	No. of ovaries with				
			developing embryo	intersexual trait	extrusion of mature oocyte	tendency to sterilization	defect in oviduct system
10[1)]	50	7	0	0	0	0	0
	80	8	0	1	3	0	0
	100	10	0	0	9	4	0
	150	6	0	0	5	5	0
	180	3	0	0	3	3	0
	240	3	0	0	3	3	0
	330	3	0	0	3	3	0
	(Total)	(40)	(0)	(1)	(26)	(18)	(0)

1) See Table 1.

Another notable change found in the ovaries affected by MT was a reduction in number of the germ cells leading eventually to the appearance of nearly completely sterilized gonads in the affected females. This was especially conspicuous



Figs. 15 and 16. Cross sections through the ovaries of female guppies at the end of 35-day (Fig. 15) and 90-day (Fig. 16) treatments with 50 μg methyltestosterone. Expansion of ovarian cavities (*OC*) with thinned wall is remarkable. Fig. 15, $\times$30; Fig. 16, $\times$25.

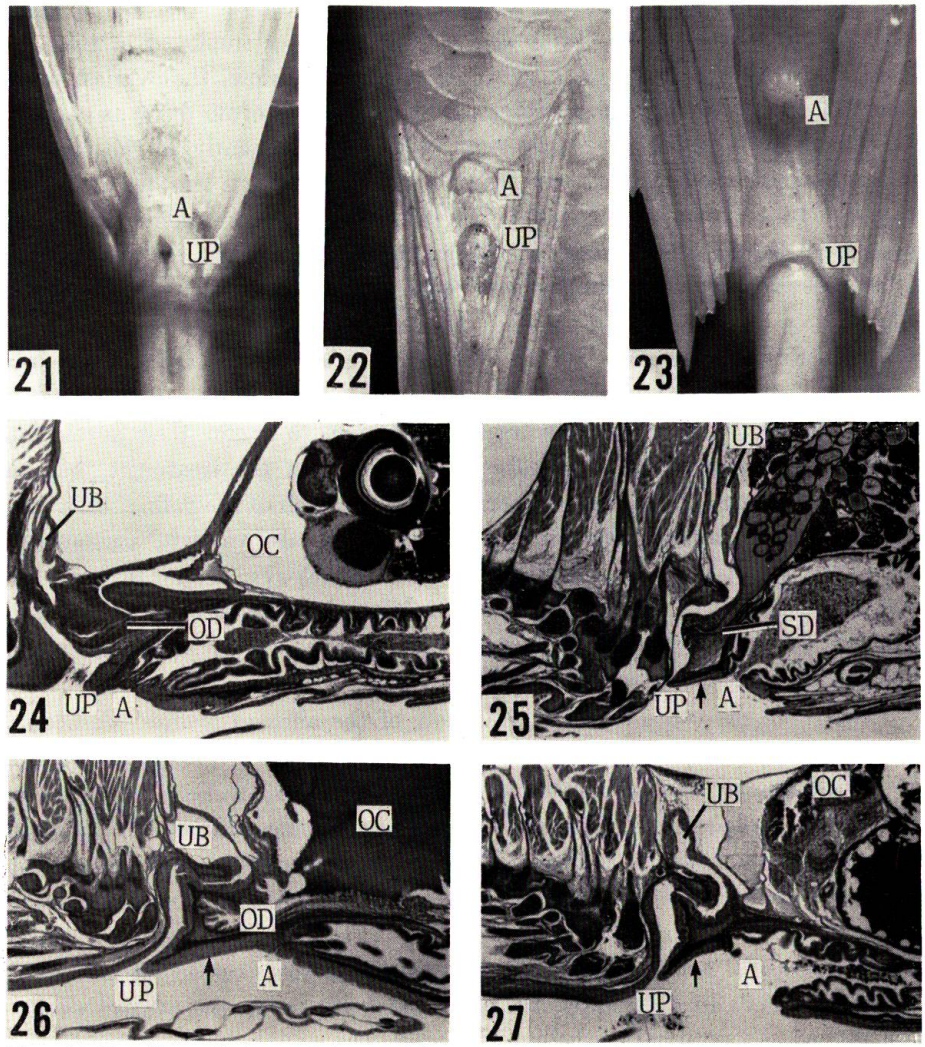
Fig. 17. Cross section through a sterilized gonad of a 150-day-old female guppy treated with 50 μg methyltestosterone for 40 days from 10 days of age. Dissolved yolk materials fill up the ovarian cavity (*OC*). $\times$25.

Fig. 18. Cross section through a sterilized gonad of a 90-day-old female guppy treated with 100 μg methyltestosterone for the first 60 days. Arrow indicates a fragmentary sperm duct with secretion in the lumen. *OC*, ovarian cavity. $\times$65.

Figs. 19 and 20. Parts of ovaries of a 35-day-old control (Fig. 19) and a 65-day-old female guppy treated with 50 μg methyltestosterone for 35 days from 30 days of age (Fig. 20). *OC*, ovarian cavity. Fig. 19, $\times$200; Fig. 20, $\times$100.

when the treatment was continued longer and when the dose level of the administered androgen was higher (Table 2). KT at the doses employed in this study did not reveal such a sterilizing effect. As mentioned before, the ovaries exposed to the MT treatment developed rather poorly at the age of 35 days as compared with the control ovaries, the female germ cells being frequently in the process of degeneration and the surviving ones being less in number and retarded in development (Fig. 15, compare with Fig. 3). The existence of intact oogonia was scarcely observable. During the periods of prolonged treatments, the oocytes exhibited neither a development into vitellogenetic stages nor an increase in number; on the contrary they became much diminished in number probably due to their progressive degeneration (Fig. 16). MT at 100 and 300 μg tended to cause the same changes of female germ cells earlier than those at lower doses. In some of the ovaries, the oocytes surviving the treatment seemed to regain their development to accomplish their vitellogenesis after the cessation of the treatment, but soon suffered some degenerative changes such as the dissolution of the yolk. As a result, only the ovarian cavity full of discarded yolk materials was left, accompanying none of the intact germ cells through its whole length (Fig. 17). In some other cases, in which most of the germ cells had possibly been affected severely, atypically expanded ovarian cavities with quite thinned walls merely remained without revealing any germ cell development (Fig. 18).

Concerning the cause of the sterilization of the affected ovaries, unsuccessful production of new crops of oocytes in the ovaries seemed to be also significant. The wall of the ovarian cavity of control ovaries was ordinarily lined by a uniform layer of cuboidal or columnar cells. In the developing ovaries, some roundish cells which were similar in appearance to gonial germ cells were found mounted among the epithelial cells of the ovarian cavity, becoming noticeable most frequently in the period from 20 to 35 days of age (Fig. 19). Cysts of a few synaptic oocytes and small, solitary auxocytes also existed in some cases. It is from this phenomenon that, in the guppy, the epithelial layer lining the ovarian cavity is often termed as "germinal epithelium". During the course of the androgen treatment, the germinal epithelium of the ovary retained in the suspended development became to lack its histological uniformity, the component cells displaying irregularity in their height. Concurrently the germ cells in the germinal epithelium became to protrude into the ovarian cavity (Fig. 20). Those bare of associated somatic cells were not rarely noticed in the ovarian cavity to be detached from the epithelium. In the ovaries revealing sterilizing tendencies, except those with the dissoluted yolk surrounded by a tall columnar, phagocytozing epithelium of the ovarian cavity, the wall of the ovarian cavity was usually very thin and seemed to be entirely deprived of the potency to produce new germinal elements (Fig. 16).



Figs. 21–23. Ventral views of the caudal region of the trunk of a control female (Fig. 21, b.l. 29.3 mm), a control male (Fig. 22, b.l. 23.5 mm), and a female guppy affected by 50 μg methyltestosterone for 50 days following birth (Fig. 23, b.l. 33.8 mm). *A*, anus; *UP*, urogenital pore. ×14.

Figs. 24–27. Sagittal sections through the caudal region of the trunk of a 70-day-old control female (Fig. 24), a 70-day-old control male (Fig. 25), a 100-day-old female treated with 50 μg methyltestosterone for 40 days from 10 days of age (Fig. 26), and a 70-day-old female treated with 50 μg methyltestosterone for the first 35 days (Fig. 27), demonstrating masculinized aspects of the urogenital sinus and its related structures in affected females. Arrows in Figs. 25–27 indicate the level of the ventromedian ridge of the male-typed duct system. *A*, anus; *OC*, ovarian cavity; *OD*, oviduct; *SD*, sperm duct; *UB*, urinary bladder; *UP*, urogenital pore. ×30.

Oviduct and urogenital sinus

In adult guppies, the urogenital pore of females is merely an ovoidal aperture, whereas that of males is overlaid by a transverse, crescent-shaped fold which is a caudal expansion of the anterolateral wall of the urogenital sinus (Figs. 21 and 22). In addition, the distance between the urogenital pore and the anus in the male is larger than in the female. This seems to correlate with the development of musculature of the gonopodial suspensorium in the male, and histological sections of the body show that some related differences exist between males and females in the shape of urogenital sinus and in the configuration of urethra and gonoduct adjoining the urogenital sinus (Figs. 24 and 25). In a sagittal plane, the female urogenital sinus is of an inversed triangle in shape, receiving a single oviduct at the dorsal side. A single urethra runs almost perpendicularly from the urinary bladder and opens into the sinus at its posterior corner. In contrast, the male urogenital sinus appears as a vertical, slit-like cavity which leans slightly to the anterior. Owing to a forward projection of the interhemals of the gonopodial suspensorial system, the urinary bladder comes to be located more craniad, and the urethra runs horizontally through a ventromedian connective tissue ridge lying between the anus and urogenital sinus, to open into the urogenital sinus at the dorsal edge of its anterior wall. A single sperm duct, or vas deferens, also penetrates the ventromedian ridge, passing between the urethra and the ventral body wall.

The ventromedian connective tissue ridge, which lacks completely in normal female, may serve as a support for the gonopodial suspensorial system. Its morphogenesis in the control males was observed to begin earlier than 20 days of age, resulting in the cranial positioning of the anus away from the urogenital pore. Treatments with MT and KT for more than 20 days starting from the day following birth, at all dose levels employed, could induce the development of the ventromedian ridge with the urethra and oviduct of the male-typed arrangement in the affected females (Fig. 26), bringing about the separated location of the anus and the urogenital pore and the development of the fold covering the sinus (Fig. 23). A masculinization of the shape of the urogenital sinus was also evident in the affected females, except those influenced by MT at 15 μg and KT at all the doses used in which the male-typed configuration of the urogenital sinus was always incomplete in the affected females. In the females treated with MT at 30, 50 and 100 μg for 35 days initiating from 30 days of age, the urogenital sinus tended to be retained, though more or less modified, in the female type, but the ventromedian ridge of the duct system was always constructed as in males, as well as in those treated with KT.

Furthermore, it was noticed that, in the experimental series in which the treatments were commenced soon after birth and continued for more than 20 days, many affected females completely failed to develop their gonoducts. The defect

in gonoduct was evident especially in the above-mentioned, male-typed ventromedian ridge, in which only the urethra was present (Fig. 27). This was recognizable to occur already 20 days of age when the ventromedian ridge had been induced to be shaped under the influence of exogenous androgen. Such a defect in the gonoduct was caused much prominently by prolonged treatments and by treatments with higher doses of MT. MT at 30 and 15 μg, and KT at all the dose levels as well, were ineffective in evoking such a change even if the treatment was initiated soon after birth, and MT at the doses higher than 50 μg was also impotent if the treatment was started later than 10 days of age (Table 2).

The defect in the gonoduct seems to be ascribable partly to a heteromorphic differentiation of the oviduct anlage into fragmentary sperm ducts and partly to an obstructed elongation of the oviduct anlage toward the urogenital sinus resulting from an improper formation of the male-typed ventromedian ridge evoked by the androgen treatment. In newly delivered females, bilateral genital ridges caudal to the ovary proper are fine and solid, and never reach the level of the anus. During the successive days they continue to extend caudalwards and, about a week after birth, come to conjoin to a single median mass of cells lying at the dorsal root of mesentery between the rectum and swim bladder. Later the bilateral ridges develop to form eventually a single lumen by a fusion of their lateral edges with the dorsal coelomic wall, and the single median mass of cells is sequentially penetrated to build the caudalmost part of the oviduct.

The androgen treatment during the neonatal period seemed to exert an inhibiting influence on the development of the oviduct anlage in the affected females. In some cases, the inhibition was so intense as to bring about a complete and permanent lack of gonoducts caudal to the gonad (Fig. 30); in others the androgen acted as inciting the somatic cells of the oviduct anlage to differentiate into those of sperm ducts (Fig. 29), though the resultant sperm ducts were not properly formed but appeared only fragmentary. When the effects of androgen had been milder, ill-developed and modified oviducts were formed in younger females in the postgonadal region except in the ventromedian ridge, and later they became to extend through the ventromedian ridge to come close to the urogenital sinus (Fig. 26), as was the case in the treated females of Experiment 9. In these cases, too, fragmentary sperm ducts were frequently formed in the subepithelial layer of oviduct lumen.

It should be added that, in a few instances, embryogenesis was progressing in the ovaries of the treated females aged more than a month after the cessation of the treatment while a deficient formation of the oviduct was quite obvious in these females. As mentioned before, spermatogenetic tissues occurring in the affected ovarian gonads were capable of developing mature sperms, which were often seen to be discharged into associated fragmentary sperm ducts. The sperm ducts were

on some occasions observed to open into the ovarian cavity. Accordingly, it seems most likely that the occurrence of embryogenesis in the affected ovaries of the "unfunctional" females was the result of self-fertilization. Identical cases of self-fertilization had been reported by Spurway[15] in virgin guppies with functional hermaphroditism, and by Dzwillo[16] in female guppies with intersexual gonads caused by a prenatal androgen treatment.

Testis and sperm duct

As reported in a previous paper[5], a stimulation of spermatogenesis and a promoted development of the sperm duct system were evident in all of the males treated with MT disregarding the dose of the hormone and the time and duration of the treatment. The same effects were demonstrated also by KT at every dose

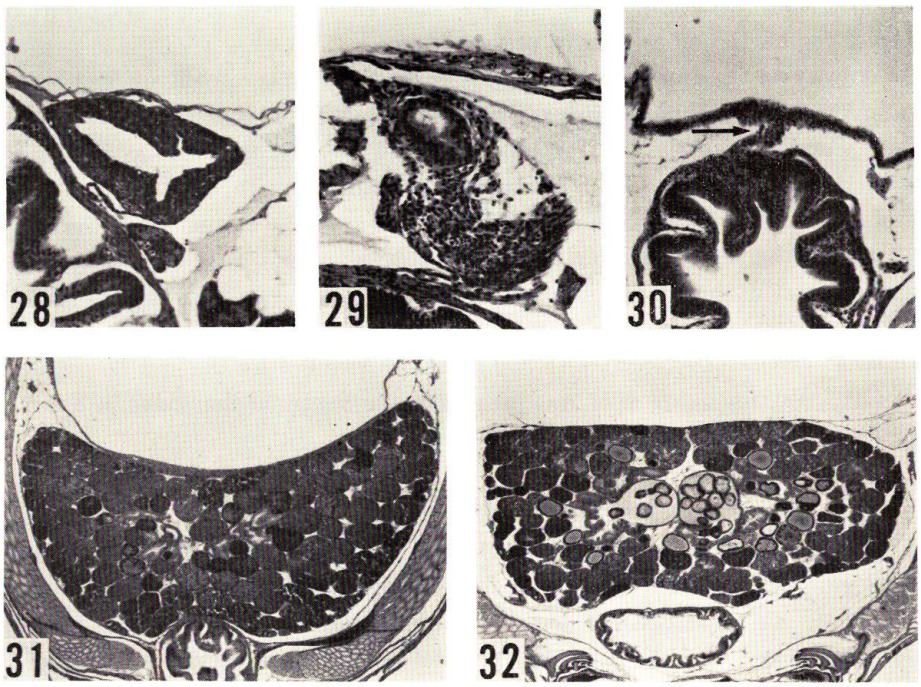


Fig. 28. Cross section through oviduct of a 17-day-old normal female guppy. ×150.
Fig. 29. Cross section through the region caudal to the gonad of a 70-day-old female guppy treated with 50 μg methyltestosterone for the first 35 days, revealing induced differentiation of an atypical sperm duct in the oviduct region. ×150.
Fig. 30. Cross section through the region caudal to the gonad of a 35-day-old female at end of treatment with 300 μg methyltestosterone, showing entire defect in gonoduct in its proper part indicated by arrow. ×110.
Figs. 31 and 32. Cross sections through testes of a 35-day-old control (Fig. 31) and a 35-day-old experimental (Fig. 32) guppy at end of treatment with 50 μg 11-ketotestosterone. ×30.

level employed: in the fish treated with 50 μg KT for 35 days following birth, the control testes were in a later phase of the spermatid stage (Fig. 31) whereas the affected ones were actively producing many spermatophores to be stored in well-developed sperm ducts (Fig. 32), at the end of the treatment. The degree of spermatogenetic stimulation seemed to have no mutual correlation with the dose of KT as well as of MT used.

In general, the testicular development in males influenced by MT and KT showed no notable differences from that in the control males after the end of the treatment, although spermatogenetic activities seemed to be slightly hindered especially by a prolonged treatment which might cause a thinning of the germ cell layer probably due to the suppression of a continual production of spermatocyte cysts. In a few affected testes, atypical spermatophores which displayed a disruption to liberate free sperms in the sperm ducts occasionally occurred intermingling among normally organized ones, but this was rather exceptional throughout the whole experimental series of the present study.

Contrary to the aforementioned cases of the deficiencies in the oviduct formation in the treated females, sperm ducts were well constructed in most affected males. Formation of the ventromedian ridge of the duct system was accelerated also in the affected males. In the cases of treatments with higher doses of MT and of those lasting for longer periods of days, however, the elongation of the sperm duct through the ventromedian ridge was liable to be retarded: in the control males the sperm duct came to open into the urogenital sinus by 40 days of age, while in some of the treated males the sperm duct lying in the ridge remained unopened until later. By the treatment with 300 μg MT carried out for the first 35 postnatal days (Exp. 19), no sperm duct was present in the ventromedian ridge in 4 out of 7 males examined 90 days after birth.

Discussion

Since the earliest work of Eversole[2)17)] on the effect of testosterone propionate on the guppy, many studies have rather demonstrated so far the formation of gonopodium, the appearance of some of the male pigmentation patterns, and the suppression of body growth in juvenile females in response to exogenous androgenic steroids.[8)9)18)19)] The results of the present study are fairly in accord with the former observations concerning the androgen-dependent external characters. It is remarked that the treated genetic females, if their ovaries have not been masculinized functionally, recover their body growth certainly after the cessation of the treatment, and that this may in turn afford a clue to assess the effectiveness of androgen on the gonad. The present results further provide evidence of the transformation of morphological characteristics of the urogenital sinus and its related structures from the female to the male type under the influence of exoge-

nous androgens. Particularly, the induction of the ventromedian connective tissue ridge, which is the structure associated with the gonopodial suspensorium peculiar to the male, in females treated with androgens seems to be much significant to deal with.

The formation of the ventromedian ridge seems to be quite sensitive to androgen: it is induced to be built in females by methyltestosterone (MT) and 11-ketotestosterone (KT) at their lowest doses within 20 days of postnatal treatment. The improper occurrence of this structure in females on the way to the full development of the oviduct system comes to obstruct the caudal extension of the oviduct anlage, bringing about a defect in the oviduct at least at its caudal part. If the hormone treatment is commenced later than 10 days following birth, the oviduct may differentiate properly in the affected females disregarding the induced formation of the ventromedian ridge. Actually, caudal genital ridges of the oviduct anlage in normal females complete their caudalward prolongation close to the urogenital sinus within 20 days following birth. A caudal elongation of sperm duct anlages in normal males occur earlier than that of oviduct anlages in normal females[5], so that the entire lack of sperm duct in the precociously formed ventromedian ridge was rather rarely observable in androgen-treated males only when MT was given at higher doses.

Similar cases of deficiency in oviduct formation have been noticed by Querner[19] in juvenile guppies raised in water solutions of androgens and by Dzwillo[16] in females treated with androgen during their prenatal period, though they have shown little interest in the cause of the abnormality. Besides the disturbance of the oviduct development described above, androgens, especially at higher dose levels, exercise a nearly complete suppression on the formation of the oviduct in the region caudal to the ovary; instead they induce the differentiation of the sperm duct in the same region on many occasions. However, the induced sperm ducts are always atypical in shape and appear only fragmentarily along the length of the genital ridge, never showing such a normal configuration as seen in males. This is also the case for the induced sperm ducts appearing in intersexual gonads. Thus, at least by the conditions of this study, it seems almost impossible to obtain a transformation of genetic females into "functional" males even though the masculinization of their germ cells may be effectuated extensively.

These findings indicate that androgens administered to newly born guppies are capable of not only stimulating the development of the sperm duct system in males[5] but also causing the heterotypic differentiation of oviduct anlages into sperm ducts in females. The occurrence of atypical and fragmentary sperm ducts may result from the scantiness of responding somatic cells, or from lowered competence of these cells to proliferate in response to androgenic stimulation, in ovaries at the time of onset of the treatment. In case of the complete and functional masculini-

zation of genetic female guppies treated with MT during their prenatal life, a hypertplastic development and precocious differentiation of the somatic cells in female gonads seem to be most significant in the process of sex reversal (Takahashi, unpublished).

The unsuccessful masculinization of ovarian somatic cells by the present androgen treatment seems to be disclosed also by an incomplete arrest of the ovarian cavity formation in the affected ovaries, though it is uncertain whether this is due to a lowered sensitivity to androgen of ovarian somatic cells or to a delayed appearance of the effect of androgen by means of oral administration. Since it has become clear that the gonad of teleost fishes is embryologically devoid of the medullary tissue (for review, see D'Ancona[20]), which is known to be the site of sexual induction toward the male as represented by sexual morphogenesis of amphibian gonads, less attention seems to have been devoted to the behaviour of somatic elements of teleostean gonads during sex differentiation and development. It seems necessary to reconsider the possible roles acted by these somatic elements in the organogenesis of the gonadal system in various teleosts.

It is often stated that an earlier and longer postnatal treatment with an adequate dose of androgens might be efficient in obtaining a complete sex reversal in the guppy. Several authors have remarked a frequent appearance of intersexual gonads in juvenile female guppies treated with androgens from the time immediately after birth, but have failed to prove a complete masculinization of females by means of the postnatal androgen treatment.[8][9] The same is true for the present results of treatments with MT in a wide range of its doses. The cause of the failure to induce a sex reversal may be attributable, at least in part, to the scantiness of sexually indifferent germ cells in the ovary at the time of the treatment. Sex differentiation of the gonad in the guppy occurs sometime before the day of birth, and newly delivered females have definite ovaries, though a wide spread in the developmental state of the ovaries is usually found in females of different broods and even in those of a single brood. A rather wide variety of the effect of androgen to bring about the partial and imperfect masculinization of germinal and somatic elements of the ovaries, represented by the results of the present study as well as those of the previous studies, seems to be a reflection of the difference in the developmental state of ovaries at the time of birth.

It seems clear from the results of the present study that, while the spermatogenetic cells induced to occur in ovarian gonads can develop to yield mature spermatozoa and can persist long in these gonads, they are neither increased in amount nor subjected to degeneration by a prolonged treatment and by a lapse of days after the cessation of the treatment. Based on his extensive work in the medaka, *Oryzias latipes*, Yamamoto[6] pointed out that, in order to achieve a complete and functional sex reversal in fishes, a hormone treatment should be done

beginning at the stage of indifferent gonad and continuing through the stage of sex differentiation. The importance of the time and duration of hormone treatment has been ascertained also in the feminization of a cichlid, *Tilapia mossambica*, induced by exogenous estrogen.[21] Dzwillo's success[7] in attaining a functional masculinization of genetic females of the guppy must be due to the adequacy of the time of the treatment done in the prenatal period. He immersed pregnant guppies in a water solution of MT (3 mg/l) for only 24 hours during the period from 8 to 12 days prior to the expected parturition.[7][16] Oral administration of MT (300–500 μg/g diet) to pregnant guppies lasting for some days before parturition is also effective in obtaining functionally masculinized, genetic female offspring.[22] Of course, in order to determine the conditions essential for securing functional masculinization in viviparous species such as the guppy, in particular, the competence to respond to exogenous androgen should be considered for both germinal and somatic elements of the gonad system, since a regular differentiation of the latter is indispensable for the functioning of reversed sex.

Clemens et al.[10] attempted to realize a sex reversal from females to males of the guppy by treating juveniles with MT (20–30 μg/g diet) for the first 60 days after birth. They found, after 146 days, the occurrence of males in a high ratio together with smaller numbers of females and intersexes, but failed to confirm the sex reversal since the fertile males of the treated group were only a few in number and always yielded young of both sexes when mated with normal females. They further noted deficiencies in spermiation and in courtship behaviour of some treated, infertile guppies, and suggested the malfunctioning of the pituitary in regard to the infertility of the treated guppies. Dzwillo[7] also noted the failure to mate his sex-reversed male guppy with normal females. Because of the lack of description about the results of histological examination of affected gonads in their report, it is only to be suspected that the occurrence of unfunctional intersexual gonads and a defect in the gonoduct may be also the cases in these infertile guppies. Anyhow, the work of Clemens et al.[10] seems to have an additional significance in the sense that it has first emphasized a persistent modification of endocrine organs other than gonads in fishes treated with androgen in their juvenile period.

It is interesting to note that, in the ovaries of the treated females, harmful influences of androgen are retained long after the completion of the treatment. Some of the ovaries came to be provided with considerably expanded ovarian cavities which were lined by extremely thinned epithelia, especially when the effect of androgen was intensified by a prolonged treatment or by elevated doses. Similar phenomena have been observed by Querner[19] and Mohsen[8] in androgen-treated, juvenile guppies. These ovarian cavities did not recover their normal structure after the cessation of the treatment: the thinning of the epithelium

seems to be an irreversible modification. The change of ovaries was generally followed by a pronounced decrease in number of the germ cells, which led to the sterilization of the gonad on many occasions. One of the causes to bring about such a diminution in number of the germ cells is certainly found in suppressed oogenesis and progressive degeneration of the cells in the course of the treatment. In addition, a production of new lines of germ cells appears to be checked completely even after the discontinuation of the treatment as a result of the thinning of the epithelium of the ovarian cavity; primitive germ cells preexisting in the "germinal" epithelium appear to be rejected into the ovarian cavity and lost eventually.

In control ovaries, a cuboidal or columnar epithelial layer bordering the ovarian cavity contains ovoidal cells of varying aspects, which are regarded as transitional germ cells giving rise to definitive germ cells.[13] Comparable cases have been mentioned also in other cyprinodonts such as *Xiphophorus*.[23][24] It is also known that, in the adult guppy, the primitive germ cells, or oogonia, multiply by mitoses to supply new oocytes.[25] Although it is uncertain whether any hormonal control may be involved in the function of the peculiar epithelium of the ovarian cavity, the long-lasting thinning of the epithelium seems to have a mutual relation with the sterilizing tendency of the ovary affected by androgen.

It is also unknown whether the suppression of the oocyte development during the androgen treatment is caused by a direct influence of androgen or by an inhibited secretion of pituitary gonadotropin. However, the fact that the oocytes surviving the treatment can rapidly accomplish the vitellogenesis to mature may admittedly mean the recovery of the pituitary gonadotropic potency following the withdrawal of androgen. Jalabert[26] described that hypophysectomy of the guppy brings about atretic changes of vitellogenetic oocytes, with no obvious influence on gestation, and that an administration of *Gambusia* pituitary extract to hypophysectomized guppies can evoke the vitellogenesis in remaining oocytes. Accordingly it may be affirmed that the observed dissolution and extrusion of mature oocytes in the affected ovaries is not ascribable to the lack of circulating gonadotropin. It is also distinct that the changes of mature oocytes are not associated with the aforementioned deficiencies in the oviduct system.

However, a wide opening of the delles of affected follicles, as a result of which the follicle cells become to face the ovarian cavity directly, seems to be characteristic of the present case of oocyte extrusion. The change of affected follicles seems to play a positive role in the oocyte extrusion. Such a change of follicles is not detectable in normal ovaries even when the delivery of young is occurring. The aforementioned functional modifications of the epithelium of the ovarian cavity may participate in causing the change of ovarian follicles. In ovaries affected by feeble doses of androgen, however, intact follicles of younger vitellogenetic oocytes are found abutting on the ovarian cavity while the extrusion of mature oocytes

takes place definitely.

It is remarked in the present study, moreover, that the changes of mature oocytes occur continuously for many months following the cessation of the androgen treatment, but that the treatment initiated on 30 days of age does not ensue the oocyte extrusion in the affected ovaries. An exact mechanism involved in the long-lasting modification of ovarian functions is quite uncertain at present. Spurway[15] observed that, in his stock population of the guppy, some females came to extrude unfertilized oocytes into the surrounding water, and suggested that the extrusion revealed a failure of the follicles to reabsorb unfertilized oocytes. This seems not to be the case in the present study because some of the oocytes evidently bore developing embryos. Some other inadequacy in ovarian functions must remain long after the androgen treatment.

It is premature, of course, to assert that the persistent deficiency in ovarian function may be attributable to a disturbed control of the pituitary, which has been improperly affected by exogenous androgen in the juvenile period, over adult ovaries. However, it is interesting to cite here the suggestion offered by Pandey[4], who implied that androgen may act to stimulate the development of the testis in juvenile guppies through the pituitary causing an early release of "gonadotropins of the male type". Provided that it may be so, the pituitary of juvenile females may respond in the same manner to the exogenous androgen, and might extend some persistent influence to adult ovaries.

KT, which is regarded as a potent androgenic steroid produced naturally in teleost gonads[27], has never been tested for its androgenic potencies in the guppy. It is capable of causing a complete sex reversal of the female medaka, *Oryzias latipes*, when administered orally.[28] In the guppy, KT has essentially the same androgenic action on the gonads of juvenile males and females: the hormone exerted masculinizing influences on female germ cells, and was also effective in inducing a precocious development of the testes. However, by means of oral administration, the effects of KT, except those on the testicular development, were much weaker than those of MT, though the former were as potent as the latter in eliciting the persistent modification of the ovaries of the affected females. It is presumed that 200 μg KT is nearly comparable to less than 30 μg MT in their effects on germ cells and secondary sex characters of the guppy. A comparison of the effects of the two hormones in the medaka produces a parallel result.[6][28] In contrast, it appeared, in other series of experiments on the guppy, that KT was much more potent in androgenic action than MT when the hormones were added to the rearing water, as it will be reported elsewhere.

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