The Effects of Some Sex Hormones on the Development of Bidder's Organ in *Bufo vulgaris formosus* Boulenger[1)]

By

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(With 1 Textfigure and 2 Plates)

Concerning the hormonal effects upon the sexual differentiation of Amphibians a good many works have been published since 1930. From these investigations, it has been ascertained that a certain amount of sex homones causes the modification of sexual differentiation of the gonads, at least in anuran larvae. In Bidder's organ of bufonids, however, the administration of androgenic substances has never been able to reverse its female differentiation, as reported by several authors such as Puckett (1939), Gallien (1947), Talluri and Podoa (1953), and Chang (1954). On the other hand, the development of the organs was arrested by treating the larvae with a high dosage of estrogen (Padoa, 1937; Padoa and Picchi, 1946). These results seem to support Witschi's opinion (1933) about the female differentiation of Bidder's organ. According to him, this organ retains the ovarian nature by reason of the lack of the medullary element, whereas some authors maintain the presence of the medullary element in the organ (Beccari, 1925; Vannini and Busetto, 1945; Talluri and Padoa, 1953).

In the present paper the writer reports some hormonal effects on the development of Bidder's organ in *Bufo vulgaris formosus* by administrating the several sex hormones.

Before proceeding further, the writer wishes to express his heartiest thanks to Professor Tohru Uchida, under whose direction this work has been carried out. The writer's gratitude is also due to Messrs. Tomoji Aoto and Kazuya Mikamo for their valuable advices.

Material and method

The tadpoles used in this experiment were of a brood laid in our laboratory by the toad, *Bufo vulgaris formosus*, which were collected in Saitama Prefecture. As is given in Table 1, 20 to 30 animals were reared in a liter of aquarium water, and these experimental groups of tadpoles were each treated with a sort of sex hormone respectively. The water was refreshed every other day and water temperature was controlled within a range of 18° to 20°C. The animals in all groups during the larval life were fed on spinach boiled

1) Contribution No.349 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

Table 1. Experimental method of groups

Experimental groups	Hormonal substances (Hormonal preparations)	Methods of administration (Total dose per an animal)	Number of animals	Age after fertilization (days)	
				Beginning of treatment	Stoppage of treatment
II. A.	testis extract (Spermatin, T.)[1]	rearing in 1 cc/l	26	2	52
II. B.	methyl testosterone (Enarmon Tablet, T.)[2]	feeding (0.9 mg, 21 times)	47	9	40
II. C.	testosterone propionate (Enarmon, T.)[3]	injection (0.2 mg, 5 times)	27	17	45
III. D.	estrone (Pelanin, M.)[4]	rearing in 1 mg/l	10	6	52
III. E.	estradiol (Ovahormon, T.)[5]	rearing in 1 mg/l	10	38	54
III. F.	estrone (Pelanin, M.)	rearing in 0.05 mg/l	25	10	42
IV.	testosterone propionate (Enarmon, T.) + anterior pituitary extract (Prae-Hormon, S.)[6]	injection (0.16m g, 4 times) + (0.006 mg, 4 times)	27	17	53

T : Teikoku Hormone Mfg. Co. M : Mochida Pharmaceutical Mfg. Co.
S : Shionogi & Co.

1) One cc of Spermatin, the whole extract of mammalian testes, corresponding to 1 g of fresh testes.
2) One tablet containing 1 mg of methyl testosterone. Two tablets finely smashed and mixed with food, were orally administered every other day.
3) Enarmon, oil solution, containing 2 mg/1 cc of testosterone propionate, injected 0.02 cc/week intraperitoneally under slight anesthesia.
4) Pelanin containing 0.05 mg/1 cc of estrone. Solution renewed every other day as well as other rearing experiment.
5) Ovahormon containing 0.02 mg/1 cc of estradiol.
6) Corresponding to 0.05 mg/1 cc of anterior pituitary extract, injected 0.02 cc of both Prae-Hormon and Enarmon intraperitoneally. The detailed data of this group shown in Table 3.

with flour, and on small insects afterwards. They were partly fixed just after the metamorphosis and partly on about the fifteenth day thereafter. The material was fixed with Bouin's solution, cut at 10 micra in thickness, and stained with Delafield's haematoxylin and eosin.

The maximum area of each Bidder's organ and the number of oocytes (in second growing stage) found in the area were adopted as the criteria describing the degree of the development of Bidder's organ.

Experimental results

The growth rate of the experimental animals was almost the same as that of the controls. It took generally 52 to 55 days from fertilization to completion of metamorphosis. The mortality is shown in Table 2.

Table 2. Mortality of each group

Group	I. (control)	II. A.	II. B.	II. C.	III. D.	III. E.	III. F.	IV.
Mortality	13/174	3/26	1/47	17/27	2/10	0/10	1/25	11/27
(%)	(7.5)	(11.3)	(2.1)	(62.9)	(20.0)	(0.0)	(4.0)	(40.7)

I. Development of Bidder's organ in control group

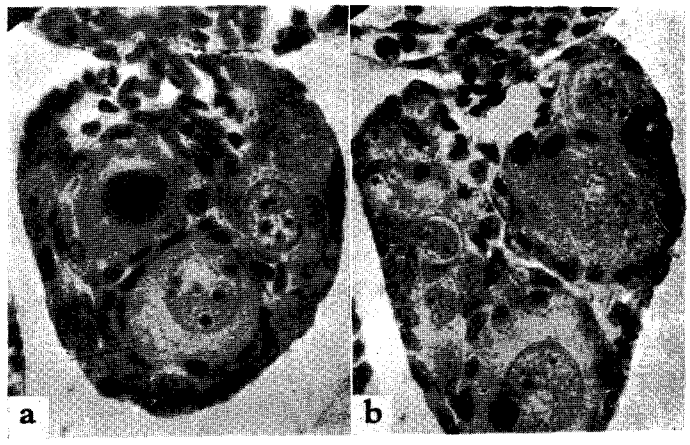
The gonads of the tadpoles were indifferent in feature at the end of metamorphosis. But on the fifteenth day after metamorphosis, 10 gonads out of 33 were observed to have just begun male differentiation. The female differentiation was not seen in any cases. Contrary to this, Bidder's organ was observed in all stages to consist of oocytes in growing period and young germ cells, with a few exceptional cases described later.

At the stage just before the metamorphosis, Bidder's organ had not much developed, with oocytes each having a round or bilobular nucleus. They were few in number and almost 20 to 28 micra in diameter. These oocytes gradually increased in number and enlarged in size, and on the fifteenth day after metamorphosis they measured 40 to 50 micra in diameter, sometimes containing several large ones, about 60 micra in diameter (Pl. VII, Figs. 1-3). These large oocytes were mostly irregular in shape probably because of enlargement, and their nuclear membrane was slightly undulated. In small oocytes the bilobular nucleus was not found. The oocytes were very frequently observed forming clusters in the peripheral region in early meiotic prophase, i. e. in first growing stage. Young germ cells with a polymorphic nucleus also existed in close contact with the periphery. The mitotic division of these cells was observed more frequently in the metamorphic stage than in other stages.

Out of 116 Bidder's organs examined, twenty-six, somewhat smaller in size than the rest, contained no growing oocyte. Some of them were solid in structure, composed of a few young germ cells and well-developed connective tissue, while some were packed with many young germ cells. In some organs, the germ cells were found only in the periphery, with a compact connective tissue in the center. Besides, there were found a few organs which were composed only of well-developed connective tissue and pigment granules (Pl. VII, Fig. 13).

In the hilum of Bidder's organ there could be often found a cord-like mass of cells connected with the mesonephros (Textfig. 1, a), or a round cavity (Textfig.

1, b). Generally the cord-like arrangement of cells was never seen in the inner part of the organ, and no cavity was formed by distinct row of cells.



Textfigure 1. Bidder's organs of animals at the end of metamorphosis in the control group. a : showing the cord-like cell mass in the hilum of the organ. ×430. b : showing the cavity in the same region. ×430.

II. Experiments with male sex hormones

A. *Effect of Spermatin added to aquarium water* : The influence of this treatment was clearly observed at the end of metamorphosis as follows. Bidder's organ enlarged strikingly, being about three times as large on the average as the controls. The oocytes became increased in number and mostly more enlarged in size than those of the controls, being 40 to 50 micra in diameter (Pl. VII, Fig. 4). Some of them were of large elongated form of 30 to 40 micra in minimum diameter and 60 to 100 micra in maximum. The nucleus of these oocytes was round and the nuclear membrane was finely wrinkled. The young germ cells or oogonia in the affected organs were less in number than in the controls, and the stroma was also narrower, the spaces between the oocytes being almost empty. The mitosis of young germ cells was not observed and the young oocytes in early meiotic prophase were scarcely found.

On the fifteenth day after metamorphosis, Bidder's organs came to resemble to those of control animals of the same age. In these organs which seemed to tend to decrease in size, oocytes of irregular contour, 40 to 50 micra in diameter, were also present, while large elongated oocytes described before could be sometimes observed (Pl. VII, Fig. 5). The young germ cells increased in number and were found to form clusters in early meiotic prophase in the peripheral region.

In some affected organs which were examined on the fifteenth day after metamorphosis, the following abnormal phases of the growing oocytes were

observed : the transfiguration of cytoplasm which caused separation of oocytes from their follicular layer (Pl. VII, Fig. 7) ; the appearance of vacuolization in the cytoplasm of younger oocytes ; irregular changes of the nuclear contents (Pl. VII, Fig. 6) ; the fragmentation of oocytes ; etc. These modifications seem to be due to the atrophy of oocytes.

The gonads still remained in an indifferent state and there could be seen no difference between the Spermatin-treated group and the control group.

B. *Effect of methyl testosterone mixed with food* : The oral administration of methyl testosterone caused retardation of the development of Bidder's organ. At the end of metamorphosis, the organs were of very diminished size, just half that of the controls. The oocytes were also reduced in number. The young germ cells were not so small in number, but the germinal epithelium was very thin owing to retarded clustering of cells, and the mitosis of these cells was scarcely observed. The young oocytes were also less in number. The growing oocytes, 20 to 30 micra in diameter, mostly contained a bilobular or horseshoe-shaped nucleus (Pl. VII, Fig. 8).

On the fifteenth day after metamorphosis, from histological studies, it can be surmised that the organs had gradually been released from the influence of administered androgen and retained the normal differentiation as seen in the controls. The organs were enlarged by increase of the oocytes. The growing oocytes are broadly divided into two groups ; one 35 to 50 micra in diameter and other 20 to 30 micra (Pl. VII, Fig. 10). The nucleus of the oocytes was more variable in shape than in the controls. Young oocytes in early meiotic prophase were observed frequently. The young germ cells or oogonia existed singly or forming clusters of cells in the periphery, but the mitotic division could not be observed.

There were some cases in which young germ cells of Bidder's organ appeared to be eliminated from the surface of the organ into the body cavity (Pl. VII, Fig. 9). Such elimination possibly causes the reduction of the germ cells and finally brings the organ to suppressed condition. No proliferation of the rete cells was observed in all affected Bidder's organs.

In nearly all the animals the gonads were in the process of male differentiation to various extents. In some cases, the elimination of the germ cells from the gonadal cortex seemed to occur. But neither the sterilization of the gonads nor the complete formation of seminal tubules was perceived.

C. *Effect of injection of testosterone propionate* : The effect of repeated injections of testosterone propionate caused a remarkable depression of Bidder's organ. At the end of metamorphosis, the organs were much smaller in size than those of the controls, and the oocytes were clearly reduced in number. Moreover, most of these oocytes, 20 to 24 micra in diameter, were arrested in development (Pl. VII, Fig. 11). The peripheral germ cells and clusters of young oocytes in first growing stage were present to a certain extent in all the organs,

but the mitotic division was rarely seen. A few Bidder's organs were nearly sterile, containing only a meagre number of young germ cells and oocytes.

At the post-metamorphic stage, Bidder's organs were reduced in size, composed of oocytes which had decreased in number. In a few cases, young germ cells were active and not decreased, but in most cases the organs revealed degenerating condition, containing two or three small oocytes with a round nucleus and ill-developed follicle cells, and nearly no young germ cell (Pl. VII, Fig. 12).

None of affected Bidder's organs had distinct rete cords, but there often existed compact cell masses, which seemed to be connected with mesonephric blastema, in the hilum of the organ. In the gonads, the masculinizing effect of testosterone propionate was noticed to various degrees. However, formation of distinct testicular structure such as seminal tubules was not observed. As the control of this study the same amount of sesame oil as that of Enarmon was injected to other tadpoles, but no special effect was obtained.

III. Experiments with female sex hormones

D. *Effect of a large amount of estrone added to aquarium water* : In these specimens, Bidder's organ did not alter in size. At the time of metamorphosis, the oocytes were dvided into two groups ; one those of 20 to 25 micra in diameter and the other those of 30 to 35 micra. The number of oocytes was nearly the same as those of the controls. The organs, however, were generally observed to be delayed in development by hormone administration, as shown in Fig. 17 (Pl.VIII). The phenomenon was noticed more clearly on the fifteenth day after metamorphosis ; all oocytes were still 20 to 30 micra in diameter, and seemed to be reduced in number (Pl. VIII, Fig. 18). Meanwhile, young germ cells were rather more rich in the organs of post-metamorphic stage than in those of metamorphic stage. The mitotic division of these cells was found in both stages, the young oocytes being in the stage of early meiotic prophase.

In the post-metamorphic stage the organs could not be observed to be reduced in size in spite of the tendency of the oocytes to decrease. This is due to the presence of numerous young germ cells, as was ascertained by histological observation.

The gonads mostly did not show sexual differentiation as well as in the controls. No adrenal hyperplasia, which was recently reported by some authors in ranid amphibians, was observed.

E. *Effect of a large amount of estradiol added to aquarium water* : The development of Bidder's organs in these experimental animals was nearly the same as that in the animals treated with a large amount of estrone (Pl. VIII, Fig. 19-20). In general, Bidder's organs increased in volume gradually, but the oocytes showed a tendency to decrease in number, while their growth was arrested distinctly. On the other hand, the young germ cells increased actively after the metamorphosis, though they were rather small in number at the

metamorphic stage. Although the reduction of oocytes was not so distinct as in the foregoing experiment, it was probably due to the active multiplication of young germ cells that the organ enlarged in size as in the controls.

The gonads were similar in state to those of the control group, so far observed. In the gonads of a few animals male differentiation occurred, but distinct female differentiation was never seen. Adrenal hyperplasia was not observed in this group.

F. *Effect of a small amount of estrone added to aquarium water* : At the end of metamorphosis, Bidder's organs were almost equal in size to those of the controls, possessing oocytes as many as in the controls. The oocytes were 20 to 30 micra in diameter and normal in development. However, their nuclei, as shown in Fig. 21 (Pl. VIII), were irregular in feature ; they appeared to be dividing amitotically. The phases were rarely observed in other groups, but they were conspicuous in this group. The young germ cells were generally numerous and clustered, but mitosis was rarely observed. In the post-metamorphic stage, the organs seemed to enlarge normally, containing oocytes in growth. The organs were generally similar to those of the controls (Pl. VIII, Fig. 22). The oocytes were 40 to 50 micra in diameter, having a nucleus which was nearly round, with nuclear membrane finely undulated. These oocytes were somewhat elongated in form, involving large ones which were present in less number than in the controls. The young germ cells and young oocytes were the same in condition as in the control animals of the same age.

From these observations, it has been ascertained that the effect of estrone upon Bidder's organ differs in accordance with the amount. The peculiar structure of the nucleus of the oocytes seems to suggest the influence of a small dosage of estrogen upon the growth of the oocytes.

No apparent effect was noticed on the sexual differentiation of the gonads, and most of them were in the indifferent condition.

IV. Experiment with male sex hormone injected in combination with anterior pituitary extract

Detailed data of this experiment are shown in Table 3. At the end of metamorphosis, the organs were observed to be half the size of those of the controls, with oocytes markedly reduced in number. It is noticeable in these organs that the multiplication of the young germ cells was obviously arrested. Though only a few germ cells were seen in these organs, they did not show mitotc division. The oocytes were 20 to 30 micra in diameter, with a round nucleus (Pl. VIII, Fig 16). The young oocytes were few in number and did not form clusters.

On the fifteenth day after metamorphosis, the oocytes were slightly increased in number and Bidder's organs were enlarged in size. But the oocytes were not so remarkably grown, being about thirty micra in diameter at this stage (Pl. VIII,

Table 3. Experiment with testosterone propionate injected in combination with anterior pituitary extract

Age after fertilization (days)	Date of injection	Injected substance	Injected dose per an animal (mg)	Number of animals injected	Remarks
17	March 24	t.	0.04	27	
22	29	p.	0.001	19	8 died
27	April 3	t.	0.04	16	3 died
32	8	p.	0.001	16	
38	14	t.	0.04	16	
43	19	p.	0.002	16	beginning of metamorphosis
47	23	t.	0.04	16	
53	28	p.	0.002	16	
54	29		total 160γ 6γ	(6 fixed)	completion of metamorphosis
69	May 15			(10 fixed)	

t : testosterone propionate p : anterior pituitary extract

Fig. 15). The young germ cells were increased in some organs, forming clusters of cells normally. The elimination of the germ cells was observed in affected Bidder's organs of both observed stages (Pl. VIII, Fig. 14).

The gonads of all experimental animals were more or less masculinized. These gonads were remarkably shrunken, and in some cases, the gonads became completely sterile, having only one or two germ cells in each section.

Discussion

Talluri and Podoa (1953) stated that the administration of testosterone to the tadpoles of *Bufo viridis* caused marked reduction in size of Bidder's organ. According to them, this reduction was due to the diminished number of oocytes as well as their minor growth. In the present study, the writer reached the same conclusion. In short, the development of Bidder's organ is inhibited by oral administration of methyl testosterone and also by repeated injections of testosterone propionate. This retardation of development is observed not only in the number and size of the oocytes, but also in several features of young germ cells. In the animals treated with androgens, at least at the time of stoppage of the treatment, young germ cells were not so many as in the controls; the clustering of cells was inhibited and mitosis was scarcely observed. Moreover, it is conceivable that these androgens caused elimination of germ cells, and then came to cause decrease of germ cells in Bidder's organ. In conclusion, the retardation of the development of Bidder's organ is due partly to depressed growth of oocytes and young germ cells, and partly to elimination of them.

As to the unexpected effect of Spermatin, it is difficult here to give a definite conclusion. The difference between the effect of synthetic androgens and that of Spermatin, however, seems to be attributable to the nature of the latter as a preparation of testis extract. The androgen content in Spermatin is rather smaller than the same dosage of the synthetic androgens used in this study. It is known that the androgen in smaller amount causes an acceleration of follicular maturation, while in larger amount it brings about ovarian atrophy. The accelerated maturation of ovarian follicles and the atrophy of interstitial cells affected by administration of androgen have been reported by many authors chiefly in mammals. The result of Spermatin-treatment in the present study may be the same as that described above.

With respect to the effect of a large amount of estrogen upon the gonads, Padoa (1937) reported first a paradoxical masculinization of genetical females in frog larvae. Recently, Witschi (1953) confirmed that 1 mg/l of estradiol caused partial or complete masculinization in several ranid amphibians. On the contrary, in toad larvae, a large amount of estrogen could not reverse the female differentiation of Bidder's organ, but could cause the diminution of its size (Padoa, 1937). Padoa and Picchi (1946) stated that this diminution of size of the organ had resulted from the reduced number and size of oocytes and was directly proportional to the concentration of the hormone administered. In the present study, 1 mg/l of estradiol and 1 mg/l of estrone caused neither the reversal of female differentiation nor the decrease in size of Bidder's organ. The noteworthy fact seen in these studies lies in the failure of oocytes to make remarkable growth even in the post-metamorphic stage, and in the continual multiplication of the young germ cells. Consequently, so far as the present study is concerned, the effect of a large amount of estrogen on the growth of oocytes may be said to be similar to the effect of synthetic androgen, but the former seems to be different in nature from the latter in respect to the effect on young germ cells. This difference may perhaps be due to the hypophyseal function. On the other hand, a small amount of estrogen had no distinct effect on the growth of Bidder's organ, so far observed.

As already described, there were found some Bidder's organs of unusual type, in which no oocyte was observed. The phenomenon was similar in both control and experimental groups. Witschi (1933), in his overripe experiment in *Bufo viridis*, reported that Bidder's organ is composed of degenerating oocytes or deficient in oocytes, and that the central space is filled with connective tissue and pigment cells. The unusual organs observed in the present study partly corresponded to those described by Witschi. Accordingly, the writer is inclined to the opinion that the experimental and control tadpoles had probably originated from overripe eggs.

It seems indubitable that Bidder's organ is similar to the functional ovary from the viewpoint of reaction to the sex hormones. Witschi (1933) asserted

that the female character of Bidder's organ is a consequence of the absence of medulla. After his explanation, it appears to be acceptable that the male hormone cannot reverse the ovarian nature of Bidder's organ. On the other hand, there are some authors who described the presence of medullary element in the organ (Beccari, 1925 ; Vannini and Busetto, 1945; Talluri and Padoa, 1953). In the writer's study, there could be found no evidence for the presence of distinct rete cord in Bidder's organ, but sometimes a structure resembling to the ovarian cavity and the rete cord was observable in both normal and affected Bidder's organs. Such a structure was chiefly found in the caudal portion of the organ and seemed to have never been effected by the administration of androgen. Further, in many cases, the cells, similar in structure to the mesonephric blastema cells, were crowded in the hilum of Bidder's organ. Takashima (1932) found out, in Bidder's organ of *Bufo formosus* larvae, that round cells, apparently distinguishable from the mesenchyme cells, formed the cell cord connecting the organ with the nephric body, and that these cells were also seen in the anlage of the pronephros. Further, he observed that a considerable number of these mesodermal cells developed into the Bidder's oocytes. These round cells may correspond to the cord-like cells described above. However, the writer is not able to determine whether or not these are identical in function with the gonadal medulla.

As seen from above, Bidder's organ is surely of the nature of a rudimentary ovary in structure, but the Bidder's oocytes seem to show some differences in nature from the ovarian oocytes. There were some cases where one or two small oviform cells appeared in the cortical layer of indifferent functional gonads. These oviform cells seemed morphologically to be Bidder's oocytes, and this fact may also suggest the specific nature of Bidder's germ cells.

Summary

(1) Tadpoles of *Bufo vulgaris formosus* were treated with androgenic and estrogenic hormones, and the affected condition of Bidder's organs was examined.

(2) The oral administration of methyl testosterone, as well as the injection of testosterone propionate, caused some suppression of the development of Bidder's organ. The injection of testosterone propionate with anterior pituitary extract brought about a marked diminution in size of the organ. However, they could neither reverse the female character of the organ nor cause its complete degeneration.

(3) This retardation of the development of the organ is shown not only in the decreased number and suppressed growth of the oocytes, but also in the prevention of multiplication of the young germ cells and their elimination from the organ.

(4) Spermatin, a preparation of whole extract of mammalian testis, brought about a remarkable growth of Bidder's organ. The oocytes were increased in

number and became larger. After the stoppage of treatment, some oocytes showed a certain degree of degeneration.

(5) By rearing the animals in a solution of a large amount of estrone as well as estradiol the growth of the oocytes was clearly inhibited. The multiplication of young germ cells seemed to be not prevented, in contrast with the effect of the synthetic androgen on these cells.

(6) Rearing the animals in an aquarium with a small amount of estrone showed no distinct effect on the organ. It seemed, however, that the activity of the oocytes was slightly modified by this treatment, the oocytes having nuclei which were irregular in feature.

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(Works marked with an asterisk were not accessible to the writer)

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Explanation of Plate VII

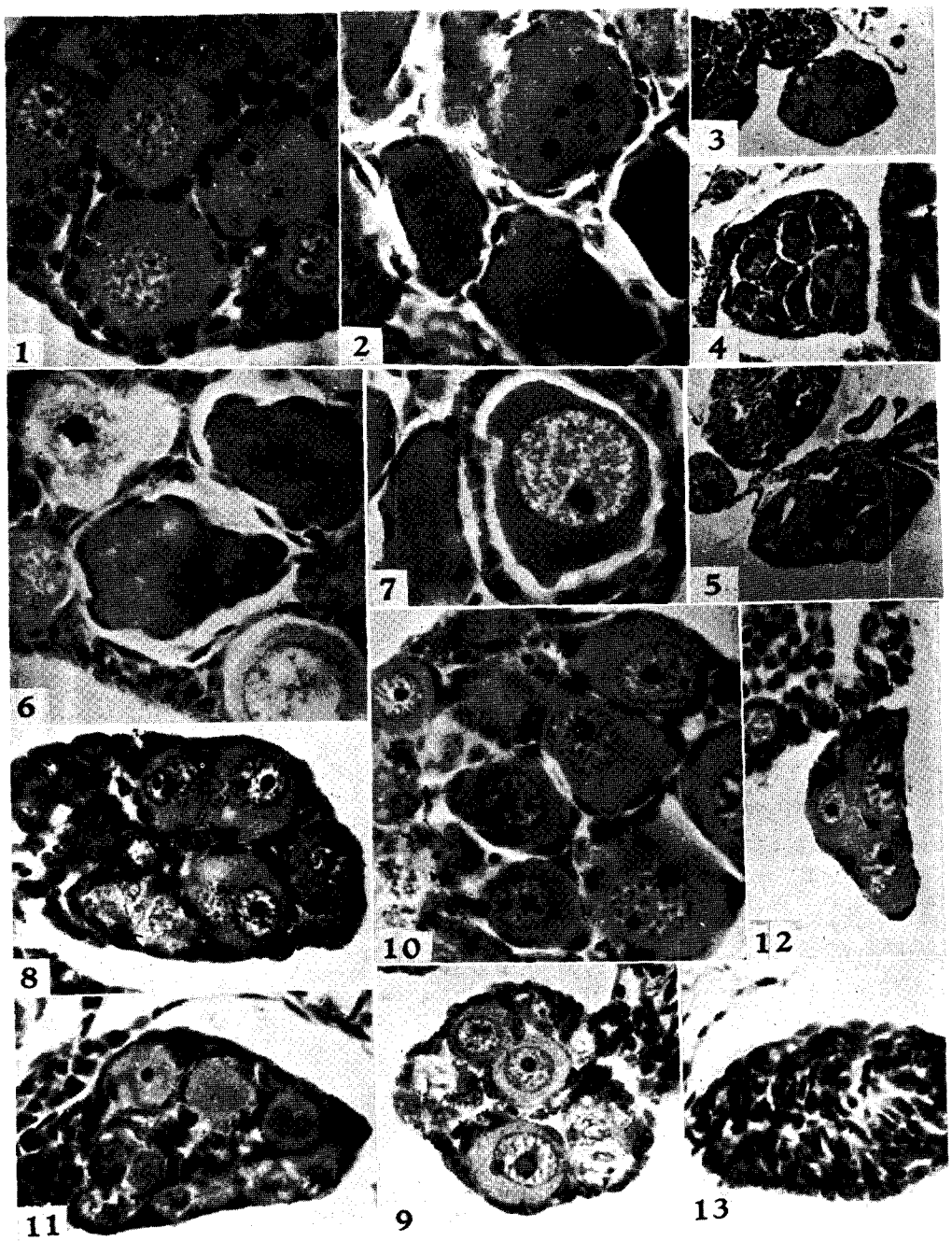
All figures are cross sections of Bidder's organs.

1–3. Control group (I.)
1, 3. Just after the metamorphosis. 1 : ×450. 3 : ×100
2. On about the fifteenth day after metamorphosis. ×450.
4–7. Spermatin group (II. A.)
4. Just after the metamorphosis. ×100.
5–7. On the fifteenth day after metamorphosis. In 6 and 7, some growing oocytes showing the atrophic feature. 5 : ×100. 6,7 : ×450.
8–10. Methyl testosterone group (II. B.)
8, 9. Just after the metamorphosis. ×450.
10. At about the fifteenth day after metamorphosis. ×450.
11, 12. Testosterone propionate group (II. C.)
11. Just after the metamorphosis. ×450.
12. At about the fifteenth day after metamorphosis. ×450.
13. Bidder's organ of unusual type in the control group. ×450.

Explanation of Plate VIII

All figures are cross sections of Bidder's organs.

14–16. Testosterone propionate plus anterior pituitary extract group (IV.)
14, 15. At about the fifteenth day after metamorphosis. ×450.
16. Just after the metamorphosis. ×450.
17, 18. estrone 1 mg/l group (III. D.)
17. Just after the metamorphosis. ×450.
18. At about the fifteenth day after metamorphosis. ×450.
19, 20. Estradiol 1 mg/l group (III. E.)
19. Just after the metamorphosis. ×450.
20. At about the fifteenth day after metamorphosis. ×450.
21, 22. Estrone 0.05 mg/l group (III. F.)
21. Just after the metamorphosis. ×450.
22. At about the fifteenth day after metamorphosis. ×450.



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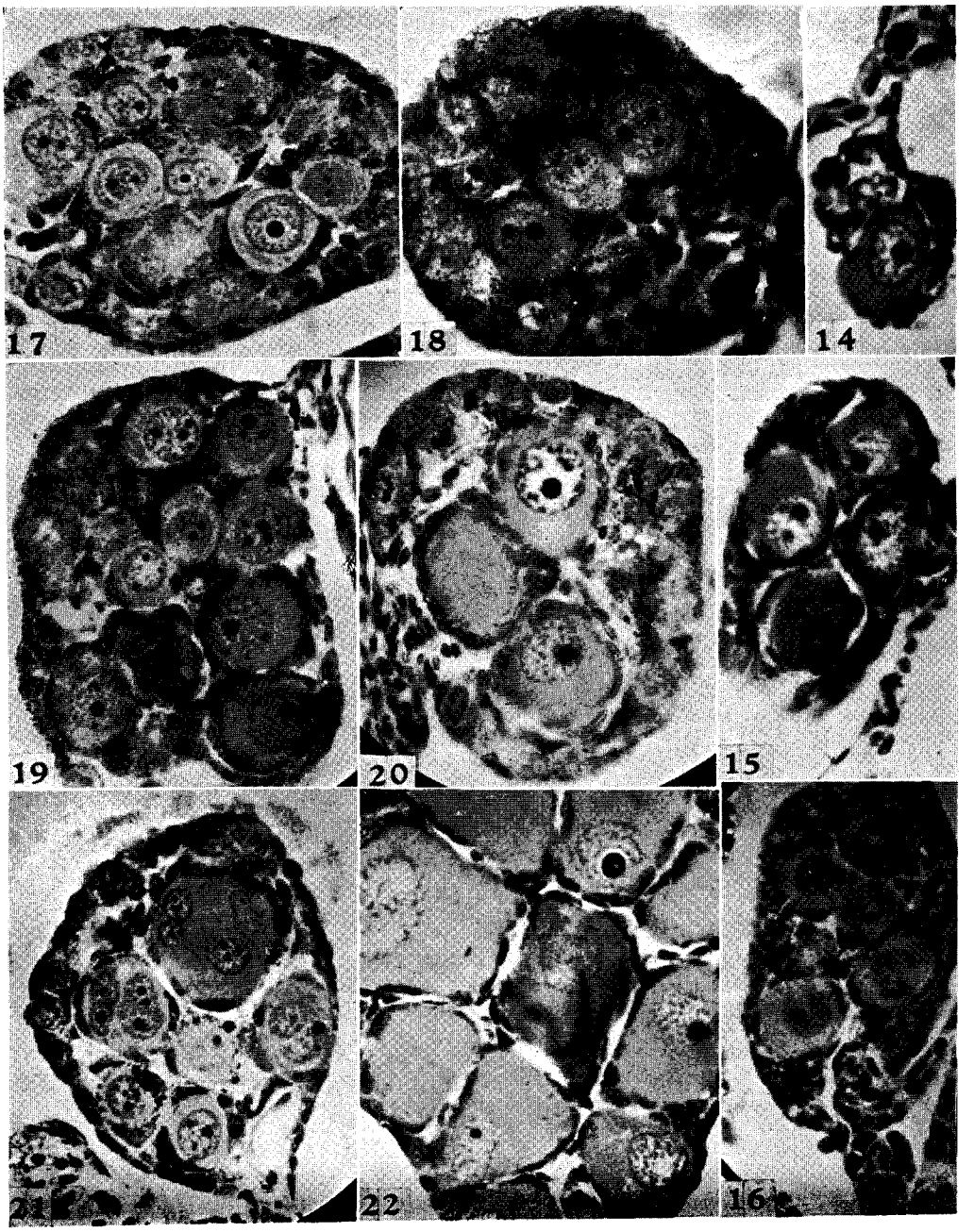

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(Zoological Institute, Hokkaido University)

(With 1 Textfigure and 2 Plates)

Concerning the hormonal effects upon the sexual differentiation of Amphibians a good many works have been published since 1930. From these investigations, it has been ascertained that a certain amount of sex homones causes the modification of sexual differentiation of the gonads, at least in anuran larvae. In Bidder's organ of bufonids, however, the administration of androgenic substances has never been able to reverse its female differentiation, as reported by several authors such as Puckett (1939), Gallien (1947), Talluri and Podoa (1953), and Chang (1954). On the other hand, the development of the organs was arrested by treating the larvae with a high dosage of estrogen (Padoa, 1937; Padoa and Picchi, 1946). These results seem to support Witschi's opinion (1933) about the female differentiation of Bidder's organ. According to him, this organ retains the ovarian nature by reason of the lack of the medullary element, whereas some authors maintain the presence of the medullary element in the organ (Beccari, 1925; Vannini and Busetto, 1945; Talluri and Padoa, 1953).

In the present paper the writer reports some hormonal effects on the development of Bidder's organ in *Bufo vulgaris formosus* by administrating the several sex hormones.

Before proceeding further, the writer wishes to express his heartiest thanks to Professor Tohru Uchida, under whose direction this work has been carried out. The writer's gratitude is also due to Messrs. Tomoji Aoto and Kazuya Mikamo for their valuable advices.

Material and method

The tadpoles used in this experiment were of a brood laid in our laboratory by the toad, *Bufo vulgaris formosus*, which were collected in Saitama Prefecture. As is given in Table 1, 20 to 30 animals were reared in a liter of aquarium water, and these experimental groups of tadpoles were each treated with a sort of sex hormone respectively. The water was refreshed every other day and water temperature was controlled within a range of 18° to 20°C. The animals in all groups during the larval life were fed on spinach boiled

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Table 1. Experimental method of groups

Experimental groups	Hormonal substances (Hormonal preparations)	Methods of administration (Total dose per an animal)	Number of animals	Age after fertilization (days)	
				Beginning of treatment	Stoppage of treatment
II. A.	testis extract (Spermatin, T.)[1]	rearing in 1 cc/l	26	2	52
II. B.	methyl testosterone (Enarmon Tablet, T.)[2]	feeding (0.9 mg, 21 times)	47	9	40
II. C.	testosterone propionate (Enarmon, T.)[3]	injection (0.2 mg, 5 times)	27	17	45
III. D.	estrone (Pelanin, M.)[4]	rearing in 1 mg/l	10	6	52
III. E.	estradiol (Ovahormon, T.)[5]	rearing in 1 mg/l	10	38	54
III. F.	estrone (Pelanin, M.)	rearing in 0.05 mg/l	25	10	42
IV.	testosterone propionate (Enarmon, T.) + anterior pituitary extract (Prae-Hormon, S.)[6]	injection (0.16m g, 4 times) + (0.006 mg, 4 times)	27	17	53

T : Teikoku Hormone Mfg. Co. M : Mochida Pharmaceutical Mfg. Co.
S : Shionogi & Co.

1) One cc of Spermatin, the whole extract of mammalian testes, corresponding to 1 g of fresh testes.
2) One tablet containing 1 mg of methyl testosterone. Two tablets finely smashed and mixed with food, were orally administered every other day.
3) Enarmon, oil solution, containing 2 mg/1 cc of testosterone propionate, injected 0.02 cc/week intraperitoneally under slight anesthesia.
4) Pelanin containing 0.05 mg/1 cc of estrone. Solution renewed every other day as well as other rearing experiment.
5) Ovahormon containing 0.02 mg/1 cc of estradiol.
6) Corresponding to 0.05 mg/1 cc of anterior pituitary extract, injected 0.02 cc of both Prae-Hormon and Enarmon intraperitoneally. The detailed data of this group shown in Table 3.

with flour, and on small insects afterwards. They were partly fixed just after the metamorphosis and partly on about the fifteenth day thereafter. The material was fixed with Bouin's solution, cut at 10 micra in thickness, and stained with Delafield's haematoxylin and eosin.

The maximum area of each Bidder's organ and the number of oocytes (in second growing stage) found in the area were adopted as the criteria describing the degree of the development of Bidder's organ.

Experimental results

The growth rate of the experimental animals was almost the same as that of the controls. It took generally 52 to 55 days from fertilization to completion of metamorphosis. The mortality is shown in Table 2.

Table 2. Mortality of each group

Group	I. (control)	II. A.	II. B.	II. C.	III. D.	III. E.	III. F.	IV.
Mortality	13/174	3/26	1/47	17/27	2/10	0/10	1/25	11/27
(%)	(7.5)	(11.3)	(2.1)	(62.9)	(20.0)	(0.0)	(4.0)	(40.7)

I. Development of Bidder's organ in control group

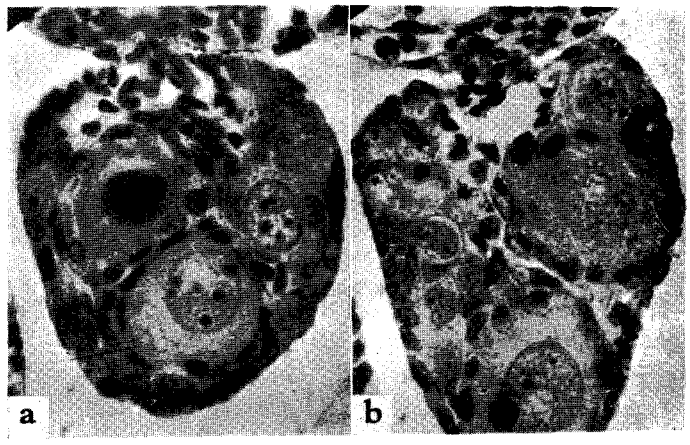
The gonads of the tadpoles were indifferent in feature at the end of metamorphosis. But on the fifteenth day after metamorphosis, 10 gonads out of 33 were observed to have just begun male differentiation. The female differentiation was not seen in any cases. Contrary to this, Bidder's organ was observed in all stages to consist of oocytes in growing period and young germ cells, with a few exceptional cases described later.

At the stage just before the metamorphosis, Bidder's organ had not much developed, with oocytes each having a round or bilobular nucleus. They were few in number and almost 20 to 28 micra in diameter. These oocytes gradually increased in number and enlarged in size, and on the fifteenth day after metamorphosis they measured 40 to 50 micra in diameter, sometimes containing several large ones, about 60 micra in diameter (Pl. VII, Figs. 1-3). These large oocytes were mostly irregular in shape probably because of enlargement, and their nuclear membrane was slightly undulated. In small oocytes the bilobular nucleus was not found. The oocytes were very frequently observed forming clusters in the peripheral region in early meiotic prophase, i. e. in first growing stage. Young germ cells with a polymorphic nucleus also existed in close contact with the periphery. The mitotic division of these cells was observed more frequently in the metamorphic stage than in other stages.

Out of 116 Bidder's organs examined, twenty-six, somewhat smaller in size than the rest, contained no growing oocyte. Some of them were solid in structure, composed of a few young germ cells and well-developed connective tissue, while some were packed with many young germ cells. In some organs, the germ cells were found only in the periphery, with a compact connective tissue in the center. Besides, there were found a few organs which were composed only of well-developed connective tissue and pigment granules (Pl. VII, Fig. 13).

In the hilum of Bidder's organ there could be often found a cord-like mass of cells connected with the mesonephros (Textfig. 1, a), or a round cavity (Textfig.

1, b). Generally the cord-like arrangement of cells was never seen in the inner part of the organ, and no cavity was formed by distinct row of cells.



Textfigure 1. Bidder's organs of animals at the end of metamorphosis in the control group. a : showing the cord-like cell mass in the hilum of the organ. ×430. b : showing the cavity in the same region. ×430.

II. Experiments with male sex hormones

A. *Effect of Spermatin added to aquarium water* : The influence of this treatment was clearly observed at the end of metamorphosis as follows. Bidder's organ enlarged strikingly, being about three times as large on the average as the controls. The oocytes became increased in number and mostly more enlarged in size than those of the controls, being 40 to 50 micra in diameter (Pl. VII, Fig. 4). Some of them were of large elongated form of 30 to 40 micra in minimum diameter and 60 to 100 micra in maximum. The nucleus of these oocytes was round and the nuclear membrane was finely wrinkled. The young germ cells or oogonia in the affected organs were less in number than in the controls, and the stroma was also narrower, the spaces between the oocytes being almost empty. The mitosis of young germ cells was not observed and the young oocytes in early meiotic prophase were scarcely found.

On the fifteenth day after metamorphosis, Bidder's organs came to resemble to those of control animals of the same age. In these organs which seemed to tend to decrease in size, oocytes of irregular contour, 40 to 50 micra in diameter, were also present, while large elongated oocytes described before could be sometimes observed (Pl. VII, Fig. 5). The young germ cells increased in number and were found to form clusters in early meiotic prophase in the peripheral region.

In some affected organs which were examined on the fifteenth day after metamorphosis, the following abnormal phases of the growing oocytes were

observed : the transfiguration of cytoplasm which caused separation of oocytes from their follicular layer (Pl. VII, Fig. 7) ; the appearance of vacuolization in the cytoplasm of younger oocytes ; irregular changes of the nuclear contents (Pl. VII, Fig. 6) ; the fragmentation of oocytes ; etc. These modifications seem to be due to the atrophy of oocytes.

The gonads still remained in an indifferent state and there could be seen no difference between the Spermatin-treated group and the control group.

B. *Effect of methyl testosterone mixed with food* : The oral administration of methyl testosterone caused retardation of the development of Bidder's organ. At the end of metamorphosis, the organs were of very diminished size, just half that of the controls. The oocytes were also reduced in number. The young germ cells were not so small in number, but the germinal epithelium was very thin owing to retarded clustering of cells, and the mitosis of these cells was scarcely observed. The young oocytes were also less in number. The growing oocytes, 20 to 30 micra in diameter, mostly contained a bilobular or horseshoe-shaped nucleus (Pl. VII, Fig. 8).

On the fifteenth day after metamorphosis, from histological studies, it can be surmised that the organs had gradually been released from the influence of administered androgen and retained the normal differentiation as seen in the controls. The organs were enlarged by increase of the oocytes. The growing oocytes are broadly divided into two groups ; one 35 to 50 micra in diameter and other 20 to 30 micra (Pl. VII, Fig. 10). The nucleus of the oocytes was more variable in shape than in the controls. Young oocytes in early meiotic prophase were observed frequently. The young germ cells or oogonia existed singly or forming clusters of cells in the periphery, but the mitotic division could not be observed.

There were some cases in which young germ cells of Bidder's organ appeared to be eliminated from the surface of the organ into the body cavity (Pl. VII, Fig. 9). Such elimination possibly causes the reduction of the germ cells and finally brings the organ to suppressed condition. No proliferation of the rete cells was observed in all affected Bidder's organs.

In nearly all the animals the gonads were in the process of male differentiation to various extents. In some cases, the elimination of the germ cells from the gonadal cortex seemed to occur. But neither the sterilization of the gonads nor the complete formation of seminal tubules was perceived.

C. *Effect of injection of testosterone propionate* : The effect of repeated injections of testosterone propionate caused a remarkable depression of Bidder's organ. At the end of metamorphosis, the organs were much smaller in size than those of the controls, and the oocytes were clearly reduced in number. Moreover, most of these oocytes, 20 to 24 micra in diameter, were arrested in development (Pl. VII, Fig. 11). The peripheral germ cells and clusters of young oocytes in first growing stage were present to a certain extent in all the organs,

but the mitotic division was rarely seen. A few Bidder's organs were nearly sterile, containing only a meagre number of young germ cells and oocytes.

At the post-metamorphic stage, Bidder's organs were reduced in size, composed of oocytes which had decreased in number. In a few cases, young germ cells were active and not decreased, but in most cases the organs revealed degenerating condition, containing two or three small oocytes with a round nucleus and ill-developed follicle cells, and nearly no young germ cell (Pl. VII, Fig. 12).

None of affected Bidder's organs had distinct rete cords, but there often existed compact cell masses, which seemed to be connected with mesonephric blastema, in the hilum of the organ. In the gonads, the masculinizing effect of testosterone propionate was noticed to various degrees. However, formation of distinct testicular structure such as seminal tubules was not observed. As the control of this study the same amount of sesame oil as that of Enarmon was injected to other tadpoles, but no special effect was obtained.

III. Experiments with female sex hormones

D. *Effect of a large amount of estrone added to aquarium water* : In these specimens, Bidder's organ did not alter in size. At the time of metamorphosis, the oocytes were dvided into two groups ; one those of 20 to 25 micra in diameter and the other those of 30 to 35 micra. The number of oocytes was nearly the same as those of the controls. The organs, however, were generally observed to be delayed in development by hormone administration, as shown in Fig. 17 (Pl.VIII). The phenomenon was noticed more clearly on the fifteenth day after metamorphosis ; all oocytes were still 20 to 30 micra in diameter, and seemed to be reduced in number (Pl. VIII, Fig. 18). Meanwhile, young germ cells were rather more rich in the organs of post-metamorphic stage than in those of metamorphic stage. The mitotic division of these cells was found in both stages, the young oocytes being in the stage of early meiotic prophase.

In the post-metamorphic stage the organs could not be observed to be reduced in size in spite of the tendency of the oocytes to decrease. This is due to the presence of numerous young germ cells, as was ascertained by histological observation.

The gonads mostly did not show sexual differentiation as well as in the controls. No adrenal hyperplasia, which was recently reported by some authors in ranid amphibians, was observed.

E. *Effect of a large amount of estradiol added to aquarium water* : The development of Bidder's organs in these experimental animals was nearly the same as that in the animals treated with a large amount of estrone (Pl. VIII, Fig. 19-20). In general, Bidder's organs increased in volume gradually, but the oocytes showed a tendency to decrease in number, while their growth was arrested distinctly. On the other hand, the young germ cells increased actively after the metamorphosis, though they were rather small in number at the

metamorphic stage. Although the reduction of oocytes was not so distinct as in the foregoing experiment, it was probably due to the active multiplication of young germ cells that the organ enlarged in size as in the controls.

The gonads were similar in state to those of the control group, so far observed. In the gonads of a few animals male differentiation occurred, but distinct female differentiation was never seen. Adrenal hyperplasia was not observed in this group.

F. *Effect of a small amount of estrone added to aquarium water* : At the end of metamorphosis, Bidder's organs were almost equal in size to those of the controls, possessing oocytes as many as in the controls. The oocytes were 20 to 30 micra in diameter and normal in development. However, their nuclei, as shown in Fig. 21 (Pl. VIII), were irregular in feature ; they appeared to be dividing amitotically. The phases were rarely observed in other groups, but they were conspicuous in this group. The young germ cells were generally numerous and clustered, but mitosis was rarely observed. In the post-metamorphic stage, the organs seemed to enlarge normally, containing oocytes in growth. The organs were generally similar to those of the controls (Pl. VIII, Fig. 22). The oocytes were 40 to 50 micra in diameter, having a nucleus which was nearly round, with nuclear membrane finely undulated. These oocytes were somewhat elongated in form, involving large ones which were present in less number than in the controls. The young germ cells and young oocytes were the same in condition as in the control animals of the same age.

From these observations, it has been ascertained that the effect of estrone upon Bidder's organ differs in accordance with the amount. The peculiar structure of the nucleus of the oocytes seems to suggest the influence of a small dosage of estrogen upon the growth of the oocytes.

No apparent effect was noticed on the sexual differentiation of the gonads, and most of them were in the indifferent condition.

IV. Experiment with male sex hormone injected in combination with anterior pituitary extract

Detailed data of this experiment are shown in Table 3. At the end of metamorphosis, the organs were observed to be half the size of those of the controls, with oocytes markedly reduced in number. It is noticeable in these organs that the multiplication of the young germ cells was obviously arrested. Though only a few germ cells were seen in these organs, they did not show mitotc division. The oocytes were 20 to 30 micra in diameter, with a round nucleus (Pl. VIII, Fig 16). The young oocytes were few in number and did not form clusters.

On the fifteenth day after metamorphosis, the oocytes were slightly increased in number and Bidder's organs were enlarged in size. But the oocytes were not so remarkably grown, being about thirty micra in diameter at this stage (Pl. VIII,

Table 3. Experiment with testosterone propionate injected in combination with anterior pituitary extract

Age after fertilization (days)	Date of injection	Injected substance	Injected dose per an animal (mg)	Number of animals injected	Remarks
17	March 24	t.	0.04	27	
22	29	p.	0.001	19	8 died
27	April 3	t.	0.04	16	3 died
32	8	p.	0.001	16	
38	14	t.	0.04	16	
43	19	p.	0.002	16	beginning of metamorphosis
47	23	t.	0.04	16	
53	28	p.	0.002	16	
54	29		total 160γ 6γ	(6 fixed)	completion of metamorphosis
69	May 15			(10 fixed)	

t : testosterone propionate p : anterior pituitary extract

Fig. 15). The young germ cells were increased in some organs, forming clusters of cells normally. The elimination of the germ cells was observed in affected Bidder's organs of both observed stages (Pl. VIII, Fig. 14).

The gonads of all experimental animals were more or less masculinized. These gonads were remarkably shrunken, and in some cases, the gonads became completely sterile, having only one or two germ cells in each section.

Discussion

Talluri and Podoa (1953) stated that the administration of testosterone to the tadpoles of *Bufo viridis* caused marked reduction in size of Bidder's organ. According to them, this reduction was due to the diminished number of oocytes as well as their minor growth. In the present study, the writer reached the same conclusion. In short, the development of Bidder's organ is inhibited by oral administration of methyl testosterone and also by repeated injections of testosterone propionate. This retardation of development is observed not only in the number and size of the oocytes, but also in several features of young germ cells. In the animals treated with androgens, at least at the time of stoppage of the treatment, young germ cells were not so many as in the controls; the clustering of cells was inhibited and mitosis was scarcely observed. Moreover, it is conceivable that these androgens caused elimination of germ cells, and then came to cause decrease of germ cells in Bidder's organ. In conclusion, the retardation of the development of Bidder's organ is due partly to depressed growth of oocytes and young germ cells, and partly to elimination of them.

As to the unexpected effect of Spermatin, it is difficult here to give a definite conclusion. The difference between the effect of synthetic androgens and that of Spermatin, however, seems to be attributable to the nature of the latter as a preparation of testis extract. The androgen content in Spermatin is rather smaller than the same dosage of the synthetic androgens used in this study. It is known that the androgen in smaller amount causes an acceleration of follicular maturation, while in larger amount it brings about ovarian atrophy. The accelerated maturation of ovarian follicles and the atrophy of interstitial cells affected by administration of androgen have been reported by many authors chiefly in mammals. The result of Spermatin-treatment in the present study may be the same as that described above.

With respect to the effect of a large amount of estrogen upon the gonads, Padoa (1937) reported first a paradoxical masculinization of genetical females in frog larvae. Recently, Witschi (1953) confirmed that 1 mg/l of estradiol caused partial or complete masculinization in several ranid amphibians. On the contrary, in toad larvae, a large amount of estrogen could not reverse the female differentiation of Bidder's organ, but could cause the diminution of its size (Padoa, 1937). Padoa and Picchi (1946) stated that this diminution of size of the organ had resulted from the reduced number and size of oocytes and was directly proportional to the concentration of the hormone administered. In the present study, 1 mg/l of estradiol and 1 mg/l of estrone caused neither the reversal of female differentiation nor the decrease in size of Bidder's organ. The noteworthy fact seen in these studies lies in the failure of oocytes to make remarkable growth even in the post-metamorphic stage, and in the continual multiplication of the young germ cells. Consequently, so far as the present study is concerned, the effect of a large amount of estrogen on the growth of oocytes may be said to be similar to the effect of synthetic androgen, but the former seems to be different in nature from the latter in respect to the effect on young germ cells. This difference may perhaps be due to the hypophyseal function. On the other hand, a small amount of estrogen had no distinct effect on the growth of Bidder's organ, so far observed.

As already described, there were found some Bidder's organs of unusual type, in which no oocyte was observed. The phenomenon was similar in both control and experimental groups. Witschi (1933), in his overripe experiment in *Bufo viridis*, reported that Bidder's organ is composed of degenerating oocytes or deficient in oocytes, and that the central space is filled with connective tissue and pigment cells. The unusual organs observed in the present study partly corresponded to those described by Witschi. Accordingly, the writer is inclined to the opinion that the experimental and control tadpoles had probably originated from overripe eggs.

It seems indubitable that Bidder's organ is similar to the functional ovary from the viewpoint of reaction to the sex hormones. Witschi (1933) asserted

that the female character of Bidder's organ is a consequence of the absence of medulla. After his explanation, it appears to be acceptable that the male hormone cannot reverse the ovarian nature of Bidder's organ. On the other hand, there are some authors who described the presence of medullary element in the organ (Beccari, 1925 ; Vannini and Busetto, 1945; Talluri and Padoa, 1953). In the writer's study, there could be found no evidence for the presence of distinct rete cord in Bidder's organ, but sometimes a structure resembling to the ovarian cavity and the rete cord was observable in both normal and affected Bidder's organs. Such a structure was chiefly found in the caudal portion of the organ and seemed to have never been effected by the administration of androgen. Further, in many cases, the cells, similar in structure to the mesonephric blastema cells, were crowded in the hilum of Bidder's organ. Takashima (1932) found out, in Bidder's organ of *Bufo formosus* larvae, that round cells, apparently distinguishable from the mesenchyme cells, formed the cell cord connecting the organ with the nephric body, and that these cells were also seen in the anlage of the pronephros. Further, he observed that a considerable number of these mesodermal cells developed into the Bidder's oocytes. These round cells may correspond to the cord-like cells described above. However, the writer is not able to determine whether or not these are identical in function with the gonadal medulla.

As seen from above, Bidder's organ is surely of the nature of a rudimentary ovary in structure, but the Bidder's oocytes seem to show some differences in nature from the ovarian oocytes. There were some cases where one or two small oviform cells appeared in the cortical layer of indifferent functional gonads. These oviform cells seemed morphologically to be Bidder's oocytes, and this fact may also suggest the specific nature of Bidder's germ cells.

Summary

(1) Tadpoles of *Bufo vulgaris formosus* were treated with androgenic and estrogenic hormones, and the affected condition of Bidder's organs was examined.

(2) The oral administration of methyl testosterone, as well as the injection of testosterone propionate, caused some suppression of the development of Bidder's organ. The injection of testosterone propionate with anterior pituitary extract brought about a marked diminution in size of the organ. However, they could neither reverse the female character of the organ nor cause its complete degeneration.

(3) This retardation of the development of the organ is shown not only in the decreased number and suppressed growth of the oocytes, but also in the prevention of multiplication of the young germ cells and their elimination from the organ.

(4) Spermatin, a preparation of whole extract of mammalian testis, brought about a remarkable growth of Bidder's organ. The oocytes were increased in

number and became larger. After the stoppage of treatment, some oocytes showed a certain degree of degeneration.

(5) By rearing the animals in a solution of a large amount of estrone as well as estradiol the growth of the oocytes was clearly inhibited. The multiplication of young germ cells seemed to be not prevented, in contrast with the effect of the synthetic androgen on these cells.

(6) Rearing the animals in an aquarium with a small amount of estrone showed no distinct effect on the organ. It seemed, however, that the activity of the oocytes was slightly modified by this treatment, the oocytes having nuclei which were irregular in feature.

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(Works marked with an asterisk were not accessible to the writer)

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Explanation of Plate VII

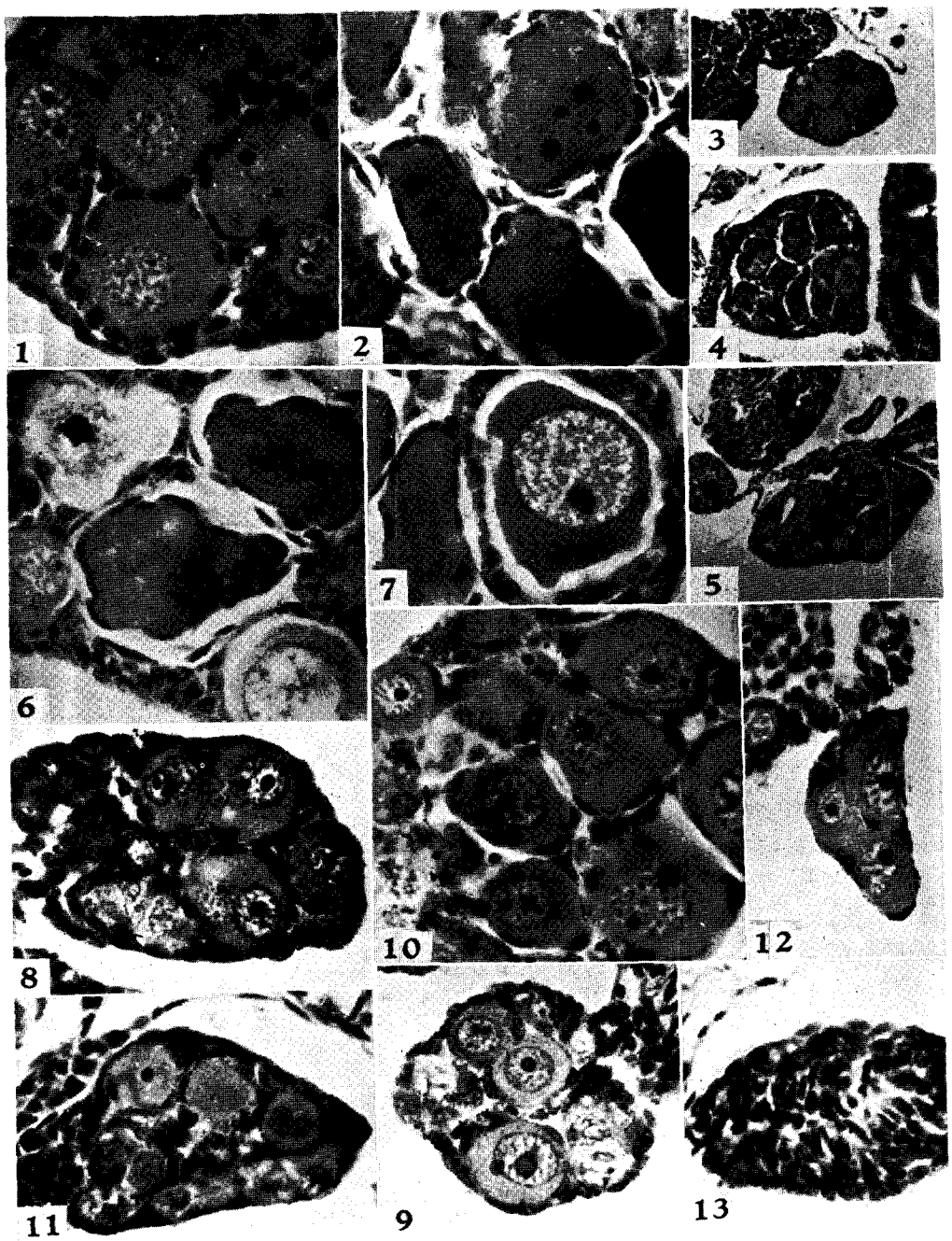
All figures are cross sections of Bidder's organs.

1–3. Control group (I.)
1, 3. Just after the metamorphosis. 1 : ×450. 3 : ×100
2. On about the fifteenth day after metamorphosis. ×450.
4–7. Spermatin group (II. A.)
4. Just after the metamorphosis. ×100.
5–7. On the fifteenth day after metamorphosis. In 6 and 7, some growing oocytes showing the atrophic feature. 5 : ×100. 6,7 : ×450.
8–10. Methyl testosterone group (II. B.)
8, 9. Just after the metamorphosis. ×450.
10. At about the fifteenth day after metamorphosis. ×450.
11, 12. Testosterone propionate group (II. C.)
11. Just after the metamorphosis. ×450.
12. At about the fifteenth day after metamorphosis. ×450.
13. Bidder's organ of unusual type in the control group. ×450.

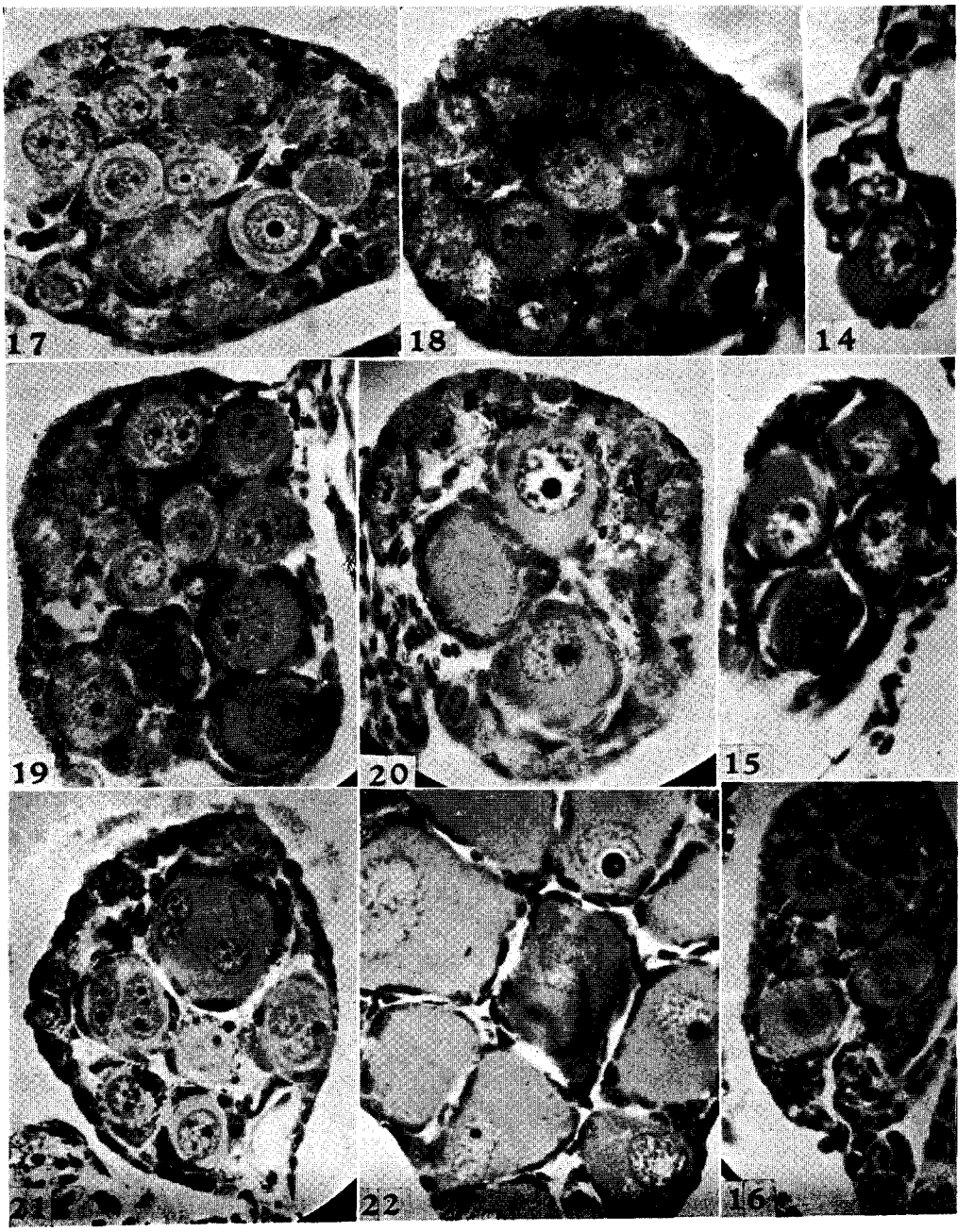
Explanation of Plate VIII

All figures are cross sections of Bidder's organs.

14–16. Testosterone propionate plus anterior pituitary extract group (IV.)
14, 15. At about the fifteenth day after metamorphosis. ×450.
16. Just after the metamorphosis. ×450.
17, 18. estrone 1 mg/l group (III. D.)
17. Just after the metamorphosis. ×450.
18. At about the fifteenth day after metamorphosis. ×450.
19, 20. Estradiol 1 mg/l group (III. E.)
19. Just after the metamorphosis. ×450.
20. At about the fifteenth day after metamorphosis. ×450.
21, 22. Estrone 0.05 mg/l group (III. F.)
21. Just after the metamorphosis. ×450.
22. At about the fifteenth day after metamorphosis. ×450.



H. Takahashi: Effects of Sex Hormones on Bidder's Organ



H. Takahashi: *Effects of Sex Hormones on Bidder's Organ*