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Studies on Sex Differentiation in the Genus *Hynobius*

By

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(With 2 Text-figures)

INTRODUCTION

WITSCHI (1929) has reported in *Rana temporaria* that there are three different races, the differentiated, undifferentiated and semi-differentiated, in sex differentiation. The differentiated race is distributed throughout the northern cold districts, while the undifferentiated race is found in the temperate regions. The range of the semi-differentiated race lies between the other two races. In the urodelan species *Ambystoma maculatum*, WITSCHI (1933) has also reported the presence of two races. While the differentiated race is distributed throughout the cold regions, the other semi-differentiated race is found in the warmer districts. Thereafter, sex differentiation has been clarified in many anurans and urodeles by many authors. In Japanese urodeles, it has been reported that *Triturus pyrrhogaster* (ASAYAMA 1940), *Hynobius lichenatus* (HANAOKA 1942), and two local races of *H. nebulosus* (EBITANI 1952) are of the so-called differentiated type, and that *H. retardatus* (HANAOKA 1934) is of the semi-differentiated type.

In order to elucidate the mechanism of sex differentiation, many studies have been conducted up to the present on the effects of overripeness of eggs, high temperatures, implantation of a piece of gonads, parabiosis, administration of sex hormones, *etc.* It is especially noted that sex hormones are usually most effective in the differentiation of gonads. The masculinization of genetic females has been induced by administration of androgens in *Rana temporaria* (GALLIEN 1937), *R. pipiens* (WITSCHI and CROWN 1937, FOOTE 1938), *R. catesbeiana* (PUCKETT 1940), *R. sylvatica* (MINTZ and WITSCHI 1946, MINTZ 1948) and *R. japonica* (KAWAMURA and YOKOTA 1959). On the other side, a similar treatment induced feminization of genetic males in urodeles, *Ambystoma opacum* (FOOTE 1941), *A. mexicanum* (MINTZ 1947) and *Pleurodeles waltl* (GALLIEN 1950b, 1954). While administration of a low dose of estrogen induced feminization of males in *Discoglossus pictus* (GALLIEN 1950a) and *Xenopus laevis* (WITSCHI and ALLISON 1950, GALLIEN 1953, 1956), a high dose induced masculinization of females in *R. esculenta* (PADOA 1936). In urodeles, it is usual that feminization of males occurs by administration of estrogens, though masculinization of females does not occur with estrogen, as found in *Ambystoma punctatum* (BURNS 1938), *A. opacum* (FOOTE 1940) and *Pleurodeles waltl* (GALLIEN 1950b, 1954). In Japanese salamanders,

HANAOKA (1941a, b) reported that regression of germ cells was caused in both males and females of *Hynobius retardatus* by injection of testosterone propionate, and a slight feminization was induced by administration of follicular hormone. ASAYAMA and AMANUMA (1957) also observed an inhibitory effect of methyltestosterone on the development of the medullary part of the gonads in *Hynobius nebulosus* and confirmed feminization of testicular structures by administration of estrogens.

WITSCHI (1967) proposed hypothetic substances produced either by the medulla (medullarin and medullary antagonist) or by the cortex (corticin and cortical antagonist). These substances, controlled by sex genes situated in the sex chromosome, are the primary sex inductor. On the other hand, GALLIEN (1967) has proposed that the genetic constitution controls gonadal sex differentiation by means of steroid hormones and not by means of inductive substances like WITSCHI's corticin and medullarin. WACHTEL, KOO and BOYSE (1975a) reported that the H–Y antigen which causes the mammalian indifferent gonads to differentiate as testes was found in *Rana pipiens* and *Xenopus laevis*.

With the purpose of clarifying sex differentiation in the genus *Hynobius*, the present author carried out some studies by using five species, *Hynobius nebulosus*, *H. tokyoensis*, *H. nigrescens*, *H. lichenatus*, and *H. dunni*, each of which has a different distribution area in Honshu (the main island) and Kyushu of Japan. Of these species *H. nebulosus* and *H. tokyoensis* were each examined by using animals collected from three different locations which differ from one another in water temperature during the spawning season. Furthermore, he studied the effects of androgen and estrogen upon the differentiation of the gonads in *Hynobius nebulosus*, *H. tokyoensis* and *H. nigrescens*.

TABLE 1
Five *Hynobius* species used in the present studies

Species	Collecting station	Spawning season	Water temperature of the spawning place (°C)
H. nebulosus	Mt. Yoshozan, Okayama Prefecture	From middle of February to beginning of March	2~6
	Suburbs of Matsue City, Shimane Prefecture	From beginning of January to middle of February	4~8
	Daigo, Kyoto	From middle to end of March	8~12
H. tokyoensis	Iwafune, Ibaraki Prefecture	From beginning to middle of March	3~7
	Kinugasa and Kurihama, Kanagawa Prefecture	From beginning to middle of March	8~12
	Tahara, Aichi Prefecture	From end of February to biginning of March	9~10
H. nigrescens	Oze marsh, Gumma Prefecture	From beginning to middle of June	4~8
H. lichenatus	Asamushi, Aomori Prefecture	From end of March to middle of April	5~13
H. dunni	Suburbs of Ooita City, Ooita Prefecture	From middle of January to end of March	8~15

MATERIALS AND METHODS

Materials used in the present studies were eggs and embryos shown in Table 1. The larvae of the five *Hynobius* species were all reared in tap water at room temperature and fed on aquatic earthworms. In the experiment series, larvae of *H. nebulosus* from Matsue, *H. tokyoensis* from Kurihama and *H. nigrescens* from Oze marsh were reared up to the late larval stage in the same manner as stated above and then administered androgen or estrogen to the larvae. The androgen used in the present experiments was testosterone propionate, while the estrogen was synthetic hexoestrol. These two hormones were those offered by courtesy of Teikokuzoki Seiyaku Company. The method employed in administrating the hormones is described later for each experiment.

All the above larvae were fixed in NAVASHIN's solution, sectioned at 10 μ in thickness and stained with MAYER's acid hemalum for histological studies. The stages of development in this paper follow USUI and HAMASAKI's table in *Hynobius nigrescens* (1939).

OBSERVATION

I. Normal sex differentiation

1. *Hynobius nebulosus*

The process of sex differentiation in *Hynobius nebulosus* was observed in the Okayama, Matsue, and Kyoto races, all of which are different from one another

TABLE 2

Numerical data of sex differentiation in *Hynobius nebulosus*

Race	Stage at autopsy	Number of individuals	Days after hatching	Mean body length (mm)	Indifferent gonad	Ovary					Testis			
						I	II	III	IV	V	I	II	III	IV
Okayama	60th stage	33	29	14.4	33	0	0	0	0	0	0	0	0	0
	65th stage	33	48	17.9	17	8	0	0	0	0	8	0	0	0
	About two weeks before metamorphosis	40	65~70	20.8	0	6	16	4	0	0	7	6	1	0
	Immediately after metamorphosis	34	80~90	21.1	0	0	9	6	2	3	0	10	3	1
Matsue	60th stage	47	28	12.5	45	2	0	0	0	0	0	0	0	0
	65th stage	30	50	15.9	4	11	3	0	0	0	9	3	0	0
	About two weeks before metamorphosis	38	57~70	17.5	0	0	12	7	4	0	4	8	3	0
	Immediately after metamorphosis	52	72~85	17.6	0	0	2	12	10	5	0	13	5	5
Kyoto	60th stage	36	41	12.0	29	7	0	0	0	0	0	0	0	0
	65th stage	32	56	16.1	0	10	12	0	0	0	0	10	0	0
	About two weeks before metamorphosis	48	67~80	20.4	0	0	7	11	7	0	0	15	8	0
	Immediately after metamorphosis	46	82~95	20.8	0	0	0	8	7	7	0	14	4	6

I~V, Type of differentiation

in water temperature of their habitats at spawning. Observation of the Kyoto race was made first.

a. Indifferent gonads.

The indifferent gonads were flat and slender, being 1.5～1.6 mm long, 0.06～0.09 mm wide, and 0.05～0.06 mm thick. The larvae having gonads of this size was less than 12 mm in body length.

Each cross-section of the gonads had three to five germ cells, 25～30 μ in diameter, in the cortical layer. Their nuclei which were polymorphic and 15～20 μ in diameter had lightly stained chromatin granules. Many pigment granules were contained in the cytoplasm. In the medullary part, there were less than ten rete cells with deeply stained nuclei. The surface of the gonads was covered with peritoneal epithelium (Fig. 1a, b). The fat bodies were distinguishable from the gonad proper, although there was no accumulation of fat in the cytoplasm of component cells.

b. Ovaries

The ovaries were 1.5～1.7 mm long, 0.1～0.13 mm wide, and 0.05～0.09 mm thick. A sign of ovarian structure was recognized by the presence of proliferated germ cells in the cortical part and a change of arrangement of rete cells in the medullary part. Approximately ten germ cells which were 20～25 μ in diameter were observed in each cross-section. These germ cells had a large, spherical nucleus with deeply stained chromatin granules. There were more than ten rete cells which were arranged in the medullary part to form a narrow ovarian cavity. The surface of the gonads was covered with peritoneal epithelium (Fig. 1c). The fat bodies were already differentiated in all the larvae. Their component cells grew enormously large and contained an accumulation of fat. This type of gonad is called ovary of the first type in this paper.

The ovaries of more advanced larvae were 1.9～2.2 mm long, 0.15～0.18 mm wide, and 0.1～0.13 mm thick, and contained many oogonia which were 20～25 μ in diameter. They consisted of a thick cortical part containing many oogonia. The ovarian cavities were covered with a thin layer of rete cells. The nuclei of the oogonia were 15～20 μ in diameter and had deeply stained chromatin granules. Such ovaries will be called the second type (Fig. 1d).

In the ovaries which were 2～2.4 mm long, 0.2～0.25 mm wide, and 0.15～0.18 mm thick, a few auxocytes were observed. The auxocytes were 30～50 μ in diameter and had a deeply stained germinal vesicle. The cortical part was occupied by these auxocytes and young oocytes with large nuclei. The oocytes were 20～27 μ in diameter and their nuclei were 17～23 μ. Their nuclei were stained somewhat lighter than those of the oogonia. Such ovaries are called the third type in this apper (Fig. 1e).

The ovaries which were 2.2 mm long, 0.25～0.35 mm wide, and 0.18～0.23 mm thick contained some auxocytes which were 50～100 μ in diameter. In each cross-section, there were more than three such auxocytes with large germinal

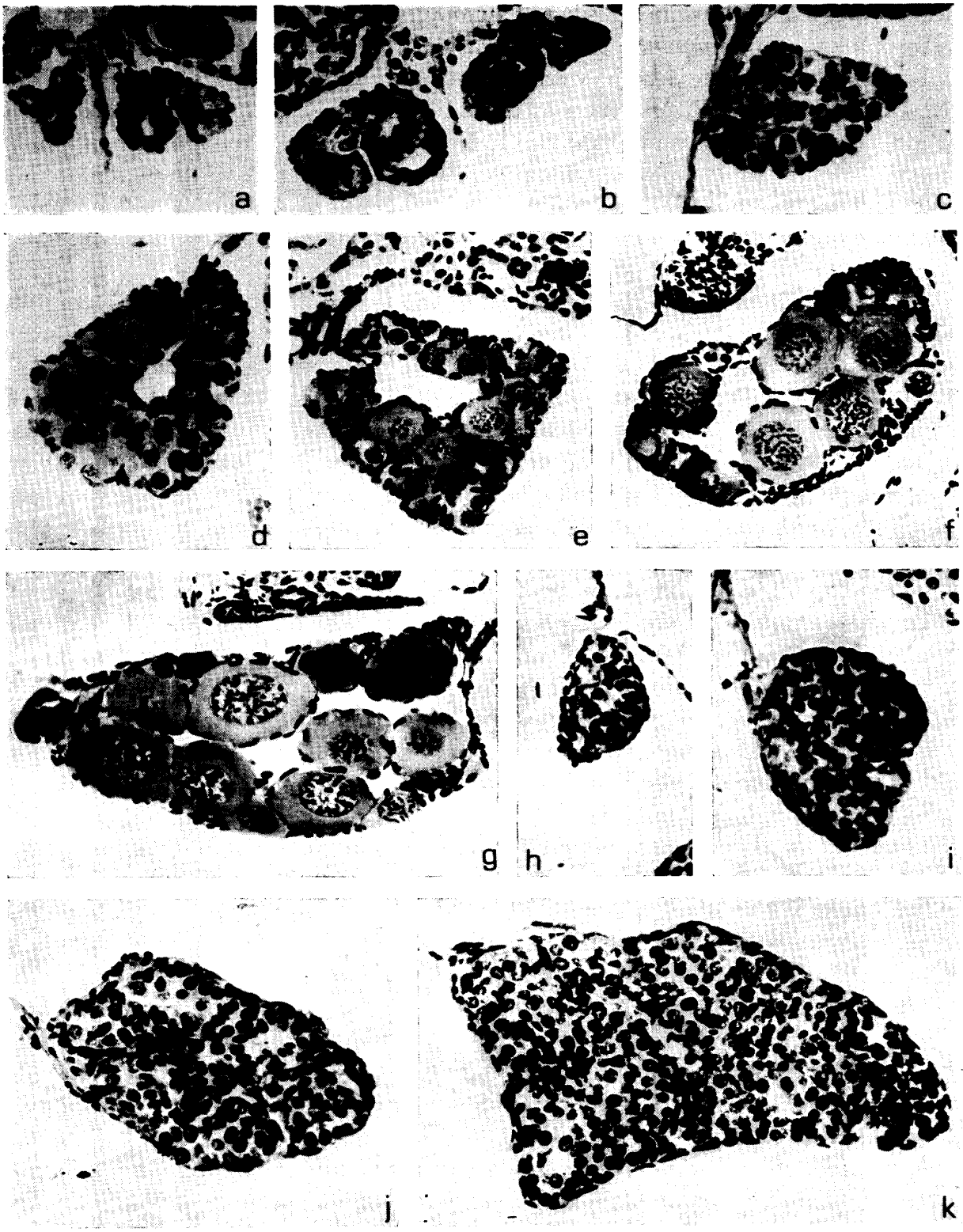


Fig. 1. Cross-sections of the gonads of normal *Hynobius nebulosus*. ×100

a, b. Sexually indifferent gonad.
c. Ovary of the first type.
d. Ovary of the second type.
e. Ovary of the third type.
f. Ovary of the fourth type.
g. Ovary of the fifth type.
h. Testis of the first type.
i. Testis of the second type.
j. Testis of the third type.
k. Testis of the fourth type.

vesicles and about the same number of auxocytes which were 30～50 μ in diameter, besides many young oocytes. These ovaries are called the fourth type (Fig. 1f).

Lastly, the ovaries which were 2.4～3 mm long, 0.4～0.6 mm wide, and 0.2～0.3 mm thick are described as those of the fifth type. These ovaries were found in some of metamorphosed individuals. They contained more than five well-developed auxocytes which were more than 100 μ in diameter in each cross-section (Fig. 1g).

c. Testes

The early differentiation stage of testes was found in larvae which were 15～17 mm in body length. The testes were 1.8～2 mm long, 0.13～0.17 mm wide, and 0.07～0.09 mm thick and covered with peritoneal epithelium (Fig. 1h). Such testes are called the first type in this paper. They had ten or less germ cells which were 17～20 μ in diameter in each cross-section. The germ cells had a large spherical nucleus with deeply stained chromatin granules. There were no pigment granules in their cytoplasm. These germ cells were 10～15 μ long and 5～7 μ wide and situated in the medullary part consisting of rete cells. They were gathered in a few masses.

The testes of the next stage in differentiation were found in larvae which were 16～18 mm in body length. These testes which were 1.8～2 mm long, 0.15～0.2 mm wide, and 0.1～0.12 mm thick contained more than twenty spermatogonia with deeply stained nuclei in each cross section. The difference from testes of the first type was in the number of spermatogonia and the existence of tubular structures. There were two or three seminiferous tubules constructed of rete cells and spermatogonia in each cross-section of the testis. Such testes are called the second type (Fig. 1i).

In more advanced testes which were 2～2.2 mm long, 0.2～0.24 mm wide, and 0.12～0.15 mm thick, there were three or four seminiferous tubules containing many spermatocytes with deeply stained nuclei in each cross-section. The spermatocytes were 15～18 μ in diameter and had a nucleus which was about 12 μ in diameter. Such testes are called the third type (Fig. 1j).

The testes at a still more advanced stage were found in some metamorphosing larvae. In these testes, 2.3～2.5 mm long, 0.28～0.32 mm wide, and 0.18～0.23 mm thick, there were many seminiferous tubules containing a number of spermatocytes in each cross-section. Such testes are called the fourth type (Fig. 1k).

As stated above, the ovaries and testes directly differentiated from indifferent gonads and no hermaphroditic stage was observed in the present species. The numerical data concerning sex differentiation are presented in Table 2.

There were no differences in sex differentiation among the Okayama, Matsue and Kyoto races. All of these races belong to the so-called differentiated type. It was found that the differentiation of gonads is the earliest in the Kyoto race which spawns in water of the highest temperature. This race is followed by the Matsue race and lastly by the Okayama race which spawns in water of the

lowest temperature. In these three races, the differentiation of testes seems to be a little later than that of the ovaries.

2. *Hynobius tokyoensis*

The sex differentiaitation of *Hynobius tokyoensis* was observed in three races collected from Ibaraki, Kanagawa and Aichi Prefecture. The process of sex differentiation of the gonads in these three races was very similar to that in the three races of *H. nebulosus*. The indifferent gonads directly developed into ovaries or testes and no hermaphroditic structures were observable in any stage. The differentiation of ovaries was a little earlier than that of testes. It is evident that the three races of *H. tokyoensis* all belong to the so-called differentiated type.

Among the three races of this species the following relationship was found in the rate of development of the gonads:

the Aichi race>the Kanagawa race>the Ibaraki race

Sex differentiation starts at the 60th stage in the Aichi race, while it starts a little later in the other two races. The numerical data concerning sex differentiation in this species are presented in Table 3.

TABLE 3

Numerical data of sex differentiation in *Hynobius tokyoensis*

Race	Stage at autopsy	Number of individuals	Days after hatching	Mean body length (mm)	Indifferent gonad	Ovary					Testis			
						I	II	III	IV	V	I	II	III	IV
Ibaraki	60th stage	13	34	14.3	13	0	0	0	0	0	0	0	0	0
	65th stage	28	48	17.0	7	15	0	0	0	0	6	0	0	0
	About two weeks before metamorphosis	20	60~75	19.7	0	1	9	2	0	0	3	4	1	0
	Immediately after metamorphosis	21	75~90	20.0	0	0	1	5	2	2	0	6	5	0
Kanagawa	60th stage	16	38	12.5	16	0	0	0	0	0	0	0	0	0
	65th stage	22	51	17.0	4	13	2	0	0	0	3	0	0	0
	About two weeks before metamorphosis	24	70~80	20.1	0	0	7	2	2	0	2	8	3	0
	Immediately after metamorphosis	13	85~95	20.5	0	0	1	4	4	2	0	1	1	0
Aichi	60th stage	24	43	12.9	20	4	0	0	0	0	0	0	0	0
	65th stage	30	53	16.8	1	9	1	0	0	0	2	17	0	0
	About two weeks before metamorphosis	25	70~85	19.6	0	0	4	3	5	0	0	7	6	0
	Immediately after metamorphisis	27	85~100	20.0	0	0	0	7	3	5	0	2	8	2

I~V, Type of differentiation

3. *Hynobius nigrescens*

The process of sex differentiation in *Hynobius nigrescens* was very similar to that in the above two species. Sex differentiation starts at the 60th stage of development. Differentiation of ovaries starts a little earlier than that of testes. No hermaphroditic structures are found in the gonads at any stage. Accordingly,

H. nigrescens belongs to the differentiated type. The numerical data concerning sex differentiation in this species are presented in Table 4.

4. *Hynobius lichenatus*

In this species, sex differentiation of the gonads starts at the 60th stage of development, although the ovaries differentiate somewhat earlier than the testes. No hermaphroditic structures were observed in the gonads at any stage. The process of differentiation of the gonads in this species was very similar to that in the above three species. Thus, it is evident that *H. lichenatus* is a true gonochorist. The numerical data concerning sex differentiation in *H. lichenatus* are shown in Table 4.

TABLE 4

Numerical data of sex differentiation in *Hynobius nigrescens*, *H. lichenatus* and *H. dunni*

Species	Stage at autopsy	Number of individuals	Days after hatching	Mean body length (mm)	Indifferent gonad	Ovary					Testis			
						I	II	III	IV	V	I	II	III	IV
H. nigrescens	55th stage	12	17	13.7	12	0	0	0	0	0	0	0	0	0
	60th stage	30	26	16.8	26	4	0	0	0	0	0	0	0	0
	65th stage	30	40	19.7	6	8	8	0	0	0	8	0	0	0
	About two weeks before metamorphosis	30	75~85	21.8	0	0	11	5	0	0	6	8	0	0
	Immediately after metamorphosis	32	90~100	22.3	0	0	5	6	3	0	3	15	0	0
H. lichenatus	55th stage	12	21	12.5	12	0	0	0	0	0	0	0	0	0
	60th stage	37	30	15.0	31	5	0	0	0	0	1	0	0	0
	65th stage	37	43	18.8	4	9	12	0	0	0	9	3	0	0
	About two weeks before metamorphosis	37	65~70	19.2	0	0	8	13	0	0	8	8	0	0
	Immediately after metamorphosis	37	80~85	21.5	0	0	11	3	5	0	1	11	5	1
H. dunni	55th stage	12	34	14.6	12	0	0	0	0	0	0	0	0	0
	60th stage	36	47	18.1	36	0	0	0	0	0	0	0	0	0
	65th stage	36	63	21.0	33	3	0	0	0	0	0	0	0	0
	About two weeks before metamorphosis	29	110~135	27.7	0	9	3	1	0	0	16	0	0	0
	Immediately after metamorphosis	38	135~150	28.0	0	0	0	0	23	0	0	4	9	2

I~V, Type of differentiation

5. *Hynobius dunni*

Sex differentiation of the gonads in this species was essentially very similar to that in the four species stated above. The ovaries began to differentiate at the 65th stage of development, while the testes began to differentiate a little later than the ovaries. No hermaphroditic structures were observed in the gonads at any stage. Thus, *H. dunni* evidently belongs to the differentiated type in sex differentiation. The numerical data concerning sex differentiation in this species are presented in Table 4.

II. Effects of androgen upon sex differentiation

1. *Hynobius nebulosus*

Each larva of the Matsue race was injected with testosterone propionate dissolved in 0.01 ml of olive oil at the 58th stage when the gonads were sexually indifferent. The larvae in the experiment series were divided into two groups according to the dose of hormone injected. The dose given to each larva was 100 μg in group I and 20 μg in group II. Histrological study of the gonads was made immediately after metamorphosis.

In the control series, there were 23 females and 16 males. Of the females, two had ovaries of the fifth type, 12 had ovaries of the fourth type and nine had ovaries of the third type. Of the males, one had testes of the fourth type, six had testes of the third type and nine had testes of the second type.

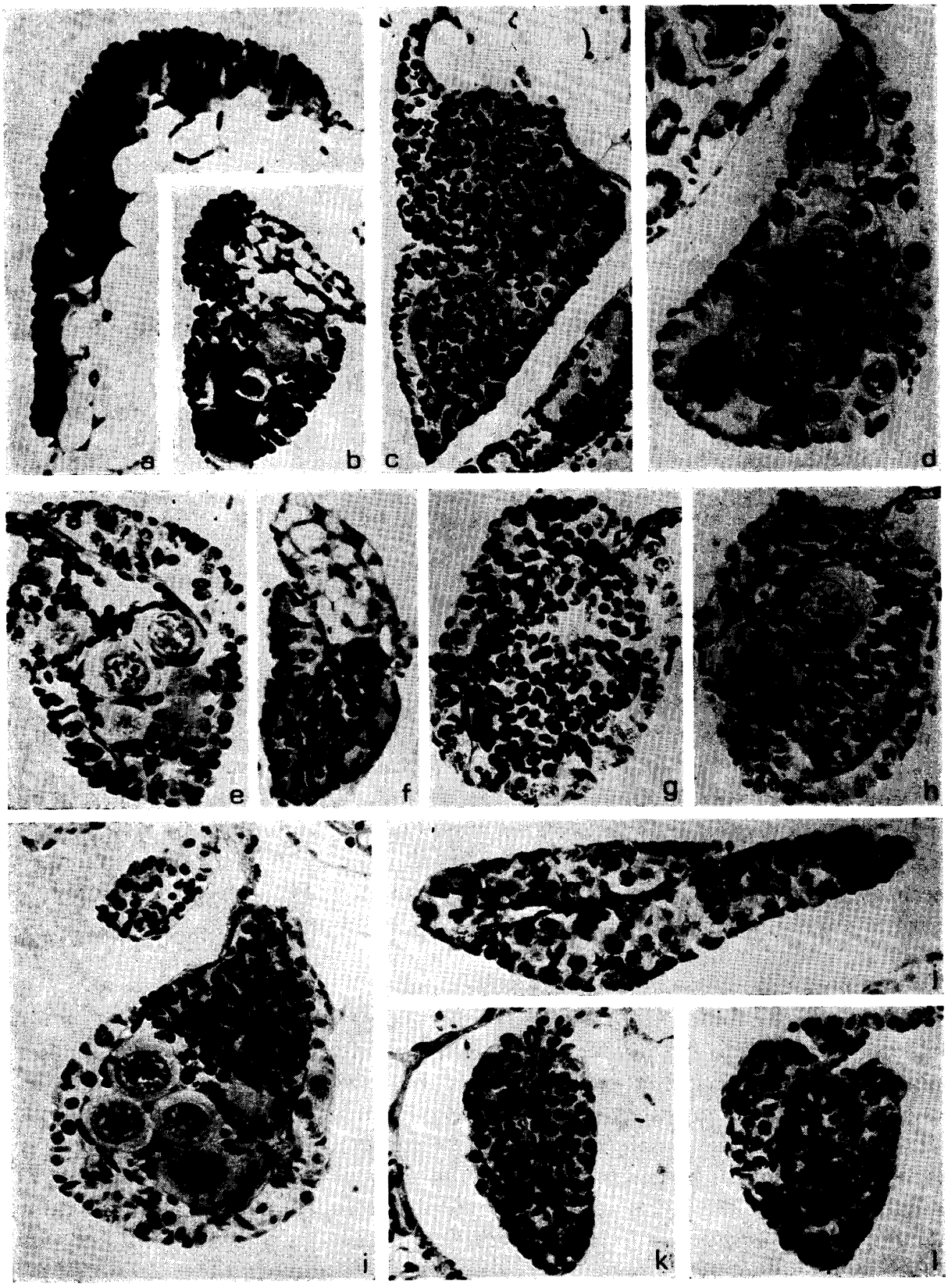
a. Group I

No males were found in 30 salamanders of group I, although eight had indifferent gonads. The ovaries of 18 females were almost normal in inner structure except that they were inferior to those of the control females in development. Of the 18 females, two had ovaries of the fourth type, eight had ovaries of the third type, five had ovaries of the second type and the remaining three had small ovaries which corresponded to the first type.

Four salamanders were not normal in structure of the ovaries. Two of them had ovaries which were incorporated with fat bodies and contained no ovarian cavities. A few auxocytes which were about 100 μ in diameter were found in each cross-section (Fig. 2a). The other two salamanders had gonads, in which the cortical part was separated from the medullary part by a layer of rete cells at their end portions. In these end portions, auxocytes and oogonia were found in both the cortical and medullary part (Fig. 2b). Thus, these end portions were hermaphroditic in structure, while the other portions of the gonads were of almost normal ovarian structure.

b. Group II

No males were found in 27 salamanders of group II as in group I. Of these 27 salamanders, six had indifferent gonads and 15 had normal ovaries. Five of these salamanders having normal ovaries were of the third type. It seems evident that androgen exerts a repressive effect on the development of ovaries. The remaining salamanders had abnormal gonads. Three of them had ovaries which were incorporated with fat bodies as found in the two salamanders of group I. The gonads of two of the other three were well-developed ovaries in appearance, although their inside was divided into several sections by membranous structures. These ovaries contained many young oocytes and oogonia (Fig. 2c). Besides, there were a few auxocytes which were 100 μ in diameter in the ovaries of one of the two salamanders. The gonads of the remaining salamanders had auxo-


a
b
c
d
e
f
g
h
i
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k
l

TABLE 5

Sex of *Hynobius nebulosus* which were injected with testosterone propionate at the 58th stage

Experiment	Dosage (μg)	Number of individuals										
		Lost	Indifferent	♀							⚥	♂	Total
				I	II	III	IV	V	F	D			
Group I	100	5	8	3	5	8	2	0	2	0	2	0	35
Group II	20	8	6	5	5	5	0	0	3	2	1	0	35
Control	0	1	0	0	0	9	12	2	0	0	0	16	40

I~V, Type of differentiation

F, Ovary which was incorporated with fat body

D, Ovary which was divided into several sections in inner structure

cytes and oogonia in both cortical and medullary parts as found in two individuals of group I. Thus, this salamander is a hermaphrodite in structure of gonads. The sex distribution in the salamanders of these two groups is shown in Table 5.

From the above findings the administration of testosterone propionate in *Hynobius nebulosus* seems to suppress the development of the ovaries of genetic females to a small degree, while it almost completely inhibits the differentiation of testes and induces ovarian or ovotesticular structures in the gonads of genetic males.

2. *Hynobius tokyoensis*

Larvae of the Kanagawa race were injected with testosterone propionate, dissolved in 0.01 ml of olive oil, at the 58th stage, when the gonads were indifferent. The larvae were divided into three groups and each group was again subdivided into two series, according to differences in hormone administration. Such differences among experimental groups and series are shown in Table 6. Histological study of the gonads of hormone-treated individuals was made immediately after metamorphosis.

a. Group I

No males were found in this group. In series A, eight of 21 females had ovaries which were similar to those of the fifth type in the control series. Eight other females had ovaries of the fourth type. The remaining five salamanders

Fig. 2. Cross-sections of the gonads in experiment series. ×95

a. Ovary of *H. nebulosus* injected with 100 μg of testosterone propionate.

b. Hermaphroditic end portion of the ovary of *H. nebulosus* injected with 100 μg of testosterone propionate.

c. Ovary of *H. nebulosus* injected with 20 μg of testosterone propionate.

d. Ovary of *H. tokyoensis* injected with 200 μg of testosterone propionate.

e. Ovotestis of *H. tokyoensis* injected with 10 μg of testosterone propionate.

f. Ovotestis of *H. nebulosus* reared in hexoestrol solution of 1000 μg/l.

g, h. Ovotestis of *H. tokyoensis* reared in hexoestrol solution of 1000 μg/l.

i. Ovotestis of *H. tokyoensis* reared in hexoestrol solution of 400 μg/l.

j, k. Ovary of *H. nigrescens* reared in hexoestrol solution of 400 μg/l.

l. Ovotestis of *H. nigrescens* reared in hexoestrol solution of 400 μg/l.

TABLE 6

Difference in hormone administration among experimental groups and series

Experiment		Dosage of the first injection (μg)	Dosage of the second injection* (μg)
Group I	Series A	200	0
	Series B	100	100
Group II	Series A	50	0
	Series B	25	25
Group III	Series A	10	0
	Series B	5	5

* The second injection was made on the 15th day after the first injection.

had smaller ovaries, although three of them were almost normal in inner structure. The ovaries of these three ovaries were covered with a layer of epithelial cells and had narrow ovarian cavities surrounded with rete cells. In each cross section, there were a few small auxocytes and young oocytes in the cortical part, although there were some areas which contained no germ cells. The remaining two individuals had ovaries, which contained auxocytes of various sizes and young oocytes, while there were no ovarian cavities (Fig. 2d).

Nine of 23 salamanders in series B had well-developed ovaries of the fifth type, and four others had a few auxocytes which were less than 100 μ in diameter. Although there were no ovarian cavities in the ovaries of these four salamanders, auxocytes and young oocytes were arranged in a compact mass and especially some of the auxocytes were surrounded with rete cells. Thus, the gonads of these four salamanders are ovotestes in structure. The gonads of the remaining 10 individuals were ovaries of the second type.

b. Group II

All the salamanders in series A and B of this group were females. The ovaries of the individuals in series A were underdeveloped as a whole, as compared with those of the control series. Thirteen of 26 salamanders had ovaries of the third type. In nine others, rete cells of the ovaries were gathered in the central part and there were no auxocytes. These ovaries were superior in size to those of the second type, although they were inferior in differentiation to those of the third type. Three other individuals had ovaries of the second type, while the remainder was a single female having ovaries of the fourth type.

In series B, seven of 23 females had normal ovaries of the fourth type. Eight other females had fewer auxocytes although the latter were rather large. These ovaries were slightly inferior in size to those of the above females. Six other females had ovaries with distinct or narrow ovarian cavities and a thick cortical part. In each cross-section of the ovaries, there were ten or more oocytes and a few auxocytes which were about 50 μ in diameter. These ovaries were of the third type in inner structure. In the remaining two females, the ovaries were underdeveloped; each cross-section of the ovaries contained less than 20 oogonia in the cortical part. The ovarian cavities were very narrow, although rete cells

were arranged into an epithelial layer of the cavity.

c. Group III

In this group, no males were found. The ovaries of females were similar in inner structure to those found in groups I and II. Eight of 23 salamanders of series A had ovaries of the fifth type and five others had those of the fourth type. Seven other salamanders had ovaries of the third type, while the remaining three had solid gonads constructed of a mixture of auxocytes, young oocytes and rete cells. Each cross-section of these solid gonads revealed that a few auxocytes were surrounded with rete cells and separated from the other germ cells. Thus, these gonads were ovotestes, in which germ cells were found in both the cortical and medullary parts. However, these gonads contained small ovarian cavities (Fig. 2e).

In series B, three of 20 salamanders had normal ovaries of the fifth type. These ovaries contained several well-developed auxocytes as found in the females of the control series. Three other salamanders had ovaries of the fourth type. The ovaries of six others were similar to those of the third type. In the remaining eight salamanders, the ovaries were of the second type. The ovaries of the 20 females in series B were quite normal in inner structure, although some of them were inferior in differentiation to those of the control salamanders.

The control salamanders consisted of 32 females and 29 males. Of these females, eight had ovaries of the fifth type, 14 had ovaries of the fourth type and

TABLE 7

Sex of *Hynobius tokyoensis* which were injected with testosterone propionate at the 58th stage

Experiment	Dosage (μg)	No. of injection	Number of individuals										
			Lost	Indifferent	♀							⚥	♂	Total
					I	II	III	IV	V	O	U			
Group I														
Series A	200	once	9	0	0	0	0	8	8	2	3	0	0	30
Series B	100	twice*	7	0	0	10	0	0	9	0	0	4	0	30
Group II														
Series A	50	once	4	0	0	12+	13	1	0	0	0	0	0	30
Series B	25	twice*	7	0	0	2	14++	7	0	0	0	0	0	30
Group III														
Series A	10	once	7	0	0	0	7	5	8	0	0	3	0	30
Series B	5	twice*	10	0	0	8	6	3	3	0	0	0	0	30
Control	0	none	0	0	0	0	10	14	8	0	0	0	29	61

*, The second injection was made on the 15th day after the first injection.

I~V, Type of differentiation

U, Ovary which has some areas having no germ cells in the cortical part

O, Ovary which has no ovarian cavity

+, Nine females whose ovaries were more developed than those of the second type but less than those of the third type were added.

++, Eight females whose ovaries were slightly inferior in size to those of the fourth type were added.

the remaining ten had ovaries of the third type. Of the males 18 had testes of the third type and the other 11 had testes of the second type. The sex of salamanders in these three groups and the controls is shown in Table 7.

From the above findings, it seems evident that administration of testosterone propionate does not suppress the development of the ovaries of genetic females, while it almost completely inhibits the differentiation of testes and produces ovarian structure in the gonads of genetic males.

3. *Hynobius nigrescens*

Thirty larvae of *Hynobius nigrescens* collected from Oze marsh were used in this experiment. At the 58th stage, each larva was injected with 10 μg of testosterone propionate dissolved in 0.01 ml of olive oil. The larvae at this stage had indifferent gonads.

While seven larvae died within a few days after injection, the others were reared until the completion of metamorphosis at room temperature. Their gonads were histologically examined immediately after metamorphosis.

Among 40 control salamanders, there were 22 females and 18 males. All the females had ovaries with distinct ovarian cavities. The ovaries of four females were of the fifth type and filled with well-developed auxocytes which were more than 100 μ in diameter, while those of three females were of the fourth type and had several auxocytes which were 50~80 μ in diameter in each cross-section of the ovaries. The ovaries of the other 15 females were similar to those of the third type in size, but no auxocytes were contained. Of the 18 males, eight had testes of the fourth type and the other ten males had testes of the third type.

In contrast with the control salamanders, all the individuals in the experiment series had more or less underdeveloped ovaries or abnormal gonads. Of 23 salamanders, 18 had almost normal ovaries and five had abnormal gonads. Seven of the former 18 females had ovaries of the second type, while the other 11 had ovaries which were more advanced in differentiation; eight had ovaries of the third type and three had ovaries of the fourth type. The gonads of the remaining five salamanders were ovotestes. The medullary part contained some germ cells, while the cortical part had also many young oocytes. In the medullary part there were no ovarian cavities. The germ cells in the medullary part were very similar to those in the cortical part. These germ cells were 18~23 μ in diameter and had a large, deeply stained nucleus. These abnormal gonads were very

TABLE 8

Sex of *Hynobius nigrescens* which were injected with testosterone propionate at the 58th stage

	Dosage (μg)	Number of individuals								
		Lost	Indifferent	♀ I	♀ II	♀ III	♀ IV	♀ V	⚥	♂	Total
Experiment	10	7	0	0	7	8	3	0	5	0	30
Control	0	0	0	0	0	15	3	4	0	18	40

I~V, Type of differentation

similar to those found in the experiment series of *Hynobius nebulosus* (II, 1, a). The sex of salamanders in the experiment series is presented in Table 8.

These findings suggest that administration of 10 μg of testosterone propionate almost completely inhibits testicualr differentiation and induces ovarian structures in the gonads of genetic males. At the same time the development of ovaries in genetic females seems to be more or less suppressed by hormone injection.

III. Effects of estrogen upon sex differentiation

1. *Hynobius nebulosus*

Larvae of the Matsue race were divided into two groups and reared in water solution of synthetic estrogen, hexoestrol. The concentration was 1 mg per liter in the first group and 400 μg per liter in the second group. The experiment was begun at the 58th stage, when the larvae were sexually indifferent, and continued until the completion of metamorphosis. A group of 35 larvae was kept in 1.5 liter of water solution which was renewed on alternate days. Histological observation of gonads was made immediately after metamorphosis. The control series was the same as that of experiments of androgen-treatment described above (II, 1).

a. Group I

Of 29 salamanders in this group, 25 were females, two were hermaphrodites and two had indifferent gonads. Of 25 females, 21 had generally underdeveloped ovaries; three were of the fourth type, eight were of the third type, four were of the second type and one was of the first type in size and structure of ovaries. The remaining five had ovaries which were similar to those of the second type in size, but a few auxocytes being less than 100 μ in diameter were usually found in each cross-section.

The other four females had ovaries which were incorporated with fat bodies. Two of them had one or two auxocytes being about 100 μ in diameter in each cross-section of the ovaries. The two hermaphrodites had gonads which were abnormal in inner structure, like those found in some of the androgen-treated salamanders. There were germ cells in both cortical and medullary parts. In one of these salamanders, the gonads were incorporated with fat bodies (Fig. 2f).

b. Group II

All 28 salamanders were females. The ovaries of these females were generally inferior in development to those of group I. Seven of them had ovaries of the first type, four had ovaries of the second type, 11 had ovaries of the third type and one had well-developed ovaries of the fourth type. The remaining five had ovaries which were incorporated with fat bodies. Two of the latter had a few auxocytes which were 50$\sim$100 μ in diameter in each cross-secion of their ovaries. The sex of salamanders in the two experimental groups is shown in Table 9.

These findings seem to indicate that hexoestrol works as a suppressor against

TABLE 9

Sex of *Hynobius nebulosus* which were reared in water solution of hexoestrol from the 58th stage

Experiment	Concentration (μg/l)	Number of individuals									
		Lost	Indifferent	♀						⚥	♂	Total
				I	II	III	IV	V	F			
Group I	1000	6	2	1	9*	8	3	0	4	2	0	35
Group II	400	7	0	7	4	11	1	0	5	0	0	35
Control	0	1	0	0	0	9	12	2	0	0	16	40

I~V, Type of differentiation

F, Ovary which was incorporated with fat body

*, Five females whose ovaries were more developed than those of the second type but less than those of the third type were added.

the differentiation of testicular structures and at the same time exercises a feminizing effect upon genetic males. Moreover, this hormone seems to suppress the development of ovaries to some extent.

2. *Hynobius tokyoensis*

Larvae of the Kanagawa race were reared in water solution of hexoestrol from the 58th stage up to metamorphosis. The larvae were divided into three groups which were reared in three different solution containing 1 mg, 400 μg and 100 μg of hexoestrol per liter, respectively. Twenty-five larvae were kept in 1.5 liter of water solution which was renewed on alternate days. The control larvae were kept in tap water. Histological study was made immediately after metamorphosis which took place about three and a half months after the hormone-treatment.

The control series was the same as that of the androgen-treatment stated above (II, 2).

a. Group I

No males were found among 34 salamanders of this group. Six of them had ovaries of the first type. In the other females, the ovaries were underdeveloped as compared with those of the control females. Eight of them had ovaries in which the cortical part was thick and contained numerous oogonia. In the medullary part, there were very narrow, slit-like ovarian cavities surrounded with flattened rete cells.

Sixteen other salamanders had gonads of a hermaphroditic structure (Fig. 2g). While the cortical part of these gonads contained normal oocytes and oogonia, the medullary part was also filled with a mass of oogonia. The medullary part was clearly distinguished from the surrounding cortical part by the existence of a layer of rete cells. The gonads with such a structure were previously observed in some of the androgen-treated salamanders. In 14 of these salamanders, a few auxocytes were contained in the medullary part in each cross-section of the gonads. These auxocytes were often about 80 μ in diameter (Fig. 2h).

The remaining four salamanders were females which had normal ovaries corresponding to the fifth type.

b. Group II

No males were found among 43 salamanders of group II. There were 40 females and three hermaphrodites. Nineteen of the females had normally developed ovaries with well-developed auxocytes such as found in the control females. Twelve females had ovaries of the third type. Of the other nine females, six had ovaries of the second type. The other three females had ovaries, in which ovarian cavities were barely observable and the cortical part contained a few small auxocytes besides numerous oocytes and oogonia.

In the hermaphroditic gonads of the remaining three salamanders, the medullary part contained a number of oogonia and several auxocytes which were isolated from the cortical part by the existence of an epithelial arrangement of rete cells. In the cortical part there were a small number of oocytes (Fig. 2i).

c. Group III

A total of 35 salamanders in this group consisted of 30 females and five hermaphrodites. Six of the females had ovaries of the fifth type. The ovaries of 15 other females belonged to the fourth type. Nine females had underdeveloped ovaries of the third type.

The remaining five salamanders had gonads in which germ cells were contained in both cortical and medullary parts, as found in some of the androgen-treated salamanders. The sex of salamanders in the three experimental groups is presented in Table 10.

TABLE 10

Sex of *Hynobius tokyoensis* which were reared in water solution of hexoestrol from the 58th stage

Experiment	Concentration (μg/l)	Number of individuals									
		Lost	Indifferent	♀						⚥	♂	Total
				I	II	III	IV	V	S			
Group I	1000	16	0	6	0	0	0	4	8	16	0	50
Group II	400	7	0	0	6	15*	19	0	0	3	0	50
Group III	100	15	0	0	0	9	15	6	0	5	0	50
Control	0	0	0	0	0	10	14	8	0	0	29	61

I~V, Type of differentiation

S, Ovary having slit-like ovarian cavity and thick cortical part in which numerous oogonia were found

*, Three females whose ovaries were more developed than those of the third type but less than those of the fourth type were added.

These findings seem to show that hexoestrol exerts a remarkable effect of inhibition on the differentiation of testicular structures and induces ovarian structures in the gonads of genetic males. Moreover, it seems that a large dosage

of hexoestrol acts as a suppressor to the development of ovaries in the genetic females.

3. *Hynobius nigrescens*

The larvae of *Hynobius nigrescens* which were collected from the same place as the material used in the experiment of androgen-treatment were reared in water solution of hexoestrol. In 1.5 liter of water solution containing 400 μg hexoestrol per liter, 25 larvae were kept. When the experiment started, the larvae were at the 58th stage, when the gonads were indifferent. The solution was renewed on alternate days. Of 50 larvae treated with estrogen 12 larvae died within a few days after the beginning of treatment, while the remaining 38 reached the completion of metamorphosis. Histological study was made immediately after metamorphosis.

The control salamanders were the same as those of androgen-treated ones described above (II, 3)

In the experiment series, it was found that there were no males. Although seven of the total of 38 salamanders had indifferent gonads, 28 others were females with normal or abnormal ovaries, and the remaining three had ovotestes. Two of the females had ovaries which had developed further than those of the fifth type in the controls. One female had ovaries of the fourth type. Eight females had ovaries of the third type. Twelve females had ovaries which had a thick cortical part and narrow ovarian cavities. There were no auxocytes. The cortical part of these ovaries were filled with an epithelial layer of rete cells (Fig. 2j). In five females, the ovarian cavities were very narrow slits which were barely observable (Fig. 2k).

The three hermaphrodites had gonads of characteristic structure (Fig. 21). A number of oogonia were observed as a mass in the solid medullary part, while there were many oocytes and oogonia in the cortical part. These two parts were distinctly separated by a layer of rete cells. The gonads of the three hermaphrodites were similar in structure to those of some of the androgen-treated salamanders stated above. The sex of salamanders in this experiment is shown in in Table 11.

The above findings seem to indicate that hexoestrol exerts a distinct inhibitory

TABLE 11

Sex of *Hynobius nigrescens* which were reared in water solution of hexoestrol from the 58th stage

	Concentration (μg/l)	Number of individuals									
		Lost	Indifferent	♀							⚥	♂	Total
				I	II	III	IV	V	T	N			
Experiment	400	12	7	0	0	8	1	2	12	5	3	0	50
Control	0	0	0	0	0	15	3	4	0	0	0	18	40

I~V, Type of differentiation

T, Ovary which had a thick cortical part and a narrow ovarian cavity

N, Ovary which had a very narrow ovarian cavity

effect on the differentiation of testicular structures in the gonads of genetic males. The ovaries of genetic females do not seem to be affected with this hormone.

DISCUSSION

1. Normal sex differentiation in *Hynobius*

Five *Hynobius* species, *H. nebulosus*, *H. tokyoensis*, *H. nigrescens*, *H. lichenatus* and *H. dunni* are all true gonochorists which reveal no hermaphroditic tendency. This finding agrees with that obtained in *H. lichenatus* by HANAOKA (1942) and in *H. nebulosus* by EBITANI (1952). Although the three races of *Hynobius nebulosus* or *H. tokyoensis* are different from one another in water temperature of their spawning place, there are no differences among them in the type of sex differentiation, in contrast to those of *Rana temporaria* or *Ambystoma maculatum* observed by WITSCHI (1930, 1933).

In the process of gonad formation, the differentiation of ovaries always proceeds to that of testes in all five species. This phenomenon seems to support WITSCHI's (1929) suggestion that the various sex types represent steps in orthogenic evolution from hermaphroditism to gonochorism.

Sex differentiation generally occurs earlier in the species and local races inhabiting temperate districts than those inhabiting cold districts. The temperature in the breeding seasons of five species shows that it is the highest in *Hynobius dunni*, the second in *H. tokyoensis* and *H. nebulosus* and the lowest in *H. lichenatus* and *H. nigrescens*. On the other hand, the number of larvae whose gonads differentiated into ovaries at the 60th stage was nearly the same in all of the four species except *H. dunni*. At the 65th stage, the ratio of larvae with differentiated gonads to those with indifferent gonads was the highest in *Hynobius nebulosus* and *H. tokyoensis* and followed in sequence by *H. lichenatus*, *H. nigrescens*, and *H. dunni*. It is evident from these findings that sex differentiation takes place earliest in *H. tokyoensis* and *H. nebulosus*, secondly in *H. lichenatus* and *H. nigrescens*, and lastly in *H. dunni*. A similar relationship is also observed among the local races of *Hynobius nebulosus* as well as *H. tokyoensis*. In the temperature of the spawning place, the Kyoto race is the highest and the Okayama race is the lowest in *H. nebulosus*. The temperature of the spawning place gradually decreases in the order, the Aichi, Kanagawa and Ibaraki race in *Hynobius tokyoensis*. Sex differentiation of *Hynobius nebulosus* occurs earliest in the Kyoto race and latest in the Okayama race, while that of *H. tokyoensis* occurs earliest in the Aichi race and latest in the Ibaraki race.

When larvae of the five species were compared with one another at the same developmental stage, *Hynobius dunni* is the largest, and *H. nigrescens*, *H. lichenatus*, *H. nebulosus* and *H. tokyoensis* are gradually smaller.

The period from hatching to the 65th stage is the longest in *Hynobius dunni* and gradually shorter in order of *H. nebulosus*, *H. tokyoensis*, *H. lichenatus* and *H. nigrescens*. The period from hatching to metamorphosis also decreases in nearly

the same order as the above. Sex differentiation occurs earlier in the species which takes more time for development except *Hynobius dunni*. A similar relationship is also found among the local races of *Hynobius nebulosus* as well as *H. tokyoensis*.

The fact that *Hynobius dunni* is an exception in most of the above correlation may indicate that this species is remotely related to the other four species in phylogeny.

2. Effects of androgen upon sex differentiation

Several investigators, particularly GALLIEN (1937) in *Rana temporaria*, MINTZ and WITSCHI (1946) and MINTZ (1948) in *R. sylvatica*, and KAWAMURA and YOKOTA (1959) in *R. japonica*, have reported that androgen induces complete masculinization of genetic females. However, the responses shown by other anuran and urodeles to androgen are in remarkable contrast to that of *Rana*. Feminization of genetic males by administration of androgen has been reported in *Bufo americanus* by CHANG (1955), in *Ambystoma opacum* by FOOTE (1941), in *A. mexicanum* by MINTZ (1947), in *A. tigrinum* and *A. opacum* by BRUNER (1952), in *Pleurodeles waltl* by GALLIEN (1950b, 1954), and in *Hynobius nebulosus* by ASAYAMA and AMANUMA (1957).

In the present study, sex reversal of genetic females to males did not occur by injection with testosterone propionate, whereas sex reversal of genetic males to females seemed to occur in all the three species *Hynobius nebulosus*, *H. tokyoensis* and *H. nigrescens*. In each species, there were a few hermaphrodites besides females. Especially in *Hynobius nebulosus*, a fairly number of salamanders with indifferent gonads were observed, while there were no salamanders with indifferent gonads in the other two species. In *Hynobius nebulosus* and *H. tokyoensis*, there were no remarkable differences in the response to androgen administration among salamanders given different doses.

The present author assumes that the females with normal ovaries which were nearly the same in differentiation as those of the control series are genetic females. On the other hand, females with underdeveloped ovaries and salamanders with indifferent gonads and ovotestes are considered to be genetic males. The ovaries of genetic males in *Hynobius tokyoensis* were superior in development to those of *H. nebulosus* and *H. nigrescens*. The rate of hermaphrodites was remarkably lower in *H. tokyoensis* than those in the other two species. These findings seem to indicate that the gonads of genetic males of *H. tokyoensis* have the strongest tendency to develop into ovaries through the effect of androgen among the three species. In contrast, the gonads of genetic males of *H. nebulosus* seem to resist the feminizing effect of androgen.

The effects of androgen observed by the present author in *Hynobius nebulosus* are somewhat similar to those observed by ASAYAMA and AMANUMA (1957) in the same species. ASAYAMA and AMANUMA raised larvae in aqueous solution of methyl-testosterone, and confirmed that this treatment provoked preponderance of females. There were a few males and no hermaphrodites among the salamanders obtained by them, in contrast with the results obtained by the present author.

However, such a difference seems to have been caused by a difference in the method of hormone-treatment.

3. Effects of estrogen upon sex differentiation

It has been reported in anurans that estrogen in lower concentration produced feminization of genetic males in *Rana esculenta* (PADOA 1942) and *Xenopus laevis* (WITSCHI and ALLISON 1950; GALLIEN 1953, 1956), while the same hormone in higher concentration caused masculinization of genetic females in *Rana esculenta* (PADOA 1936, 1942).

In urodeles, on the contrary, feminization of genetic males caused by administration of estrogen has been reported in *Ambystoma punctatum* (BURNS 1938), *A. opacum* (FOOTE 1940), *Hynobius retardatus* (HANAOKA 1941b) and *Pleurodeles waltl* (GALLIEN 1950b, 1954). An inhibitory effect of estrogen to the development of the medullary part has been observed in each of these urodeles.

GALLIEN (1954) made an analysis of the mechanism by which feminization occurred in *Pleurodeles waltl*. According to him, estrogen exerts an inhibitory effect on the differentiation of the meduallary part in the gonads of genetic males and induces a compensatory development of the cortical part.

In the present study, it was evident that all the genetic males were reversed to females by the effect of estrogen, although a few of them remained as hermaphrodites or had indifferent gonads. However, the ovaries of genetic females of *Hynobius nebulosus* which had been reared in estrogen solution were all retarded in differentiation when compared with those of the control females. The ovaries of genetic females of *Hynobius tokyoensis* which had been reared in estrogen solution of a lower concentration were definitely normal. Those of the same species reared in a higher concentration were all underdeveloped. The ovaries of genetic females in *H. nigrescens* which had been reared in estrogen solution seemed to show the same degree of differentiation as those of the control females. The ovaries of genetic males of the three species seemed to be underdeveloped when compared with those of genetic females. There were remarkably numerous hermaphrodites in *H. tokyoensis*, but there were only a few hermaphrodites in the other two species. These hermaphrodites seem to be genetic males which were partly feminized by estrogen. The abundance of hermaphrodites in *Hynobius tokyoensis* treated with estrogen correlated with the paucity of hermaphrodites in the same species treated with androgen. These findings seem to show that the male-determining genetic tendency in males of *Hynobius tokyoensis* is more powerful than those of males in the other two species. This powerful tendency in male *Hynobius tokyoensis* agrees with the fact that the testes of normal males in this species are more differentiated than those in the other two species.

4. General effects of sex hormones upon sex differentiation

According to WITSCHI (1967), genes controlling sex differentiation are inactive at first. At stage 25 (15 days) sex specific messenger RNA, sex specific poly-

peptides and enzymes are present in gonadal primordia and sex differentiation begins at stage 26 (21 days). In the genetic males, the gonadal medulla produces both medullarin and medullary antagonist (anticortex) so that the testes are differentiated. In the genetic females, the gonadal cortex produces both corticin and cortical antagonist (antimedulla). At stage 33 (2 months) steroid sex hormones are produced. This opinion is based on the data in *Xenopus laevis*. In contrast, GALLIEN (1967) proposed the metabolic pathway related to sex differentiation as follows. Sex gene activity begins at the early larval stage and produces mediators which control the direction of differentiation in the gonads. These mediators are linked to the biosynthesis of the steroid hormones in the course of sex differentiation.

According to WACHTEL (1977), the primary determination of mammalian sex depends on the presence of the H–Y antigen. The indifferent embryonic gonad is induced to differentiate as a testis in the presence of H–Y antigen, and as an ovary in the absence of H–Y antigen. WACHTEL, KOO and BOYSE (1975a) and WACHTEL, OHNO, KOO and BOYSE (1975b), gave evidence for the occurrence of murine H–Y antigen in *Rana pipiens* and *Xenopus laevis*. The H–Y antigen may direct the indifferent gonad towards typificantion of the heterogametic sex of each species, like testes in XY males and ovaries in ZW females.

The above described data in the present study and the considerations cited led the present author to consider as follows.

He considers on the basis of the results of the experiments performed by him that androgen or estrogen administered at the early larval stage does not seem directly to determine the future course of differentiation of the indifferent gonads, but the hormones block some metabolic pathway controlled by sex genes. It is probable that the medullary part of the gonad is suppressed and compensatory development of the cortical part is induced in urodeles, in contrast to *Rana*, in which the cortical part is proven to be suppressed and compensatory development of the medullary part occurs by hormone administration. In *Rana*, sex reversal always occurs from females to males which are the heterogametic sex. The assumption that females are heterogametic in urodeles has been made by many cytologists from observation of lampbrush chromosomes in oocytes. Heteromorphism of a lampbrush bivalent was reported by MANCINO and NARDI (1971) in *Triturus marmoratus*, by HAUSCHKA and BRUNST (1964) in *Siredon mexicanum*, by LACROIX (1968) in *Pleurodeles waltl* and *P. poireti*, by LEÓN and KEZER (1974) in *Siren intermedia nettingi* and by SCHMID, OLERT and KLETT (1979) in *Triturus alpestris*, *T. vulgaris* and *T. helveticus*. Although it has not yet been elucidated, which sex is the heterogametic in *Hynobius*, it is very probable that females are ZW in sex chromosome constitution, as all the urodeles studies hitherto are female-heterogametic.

It is conceivable that the genetic function in the larvae of the homogametic sex may be disturbed by male and female hormones, and compensatory development of the gonads of the heterogametic sex occurs in *Hynobius*, just as observed in *Rana*.

SUMMARY

1. Normal sex differentiation was examined in *Hynobius nebulosus*, *H. tokyoensis*, *H. nigrescens*, *H. lichenatus* and *H. dunni* which had been reared in the laboratory. In these five species, no juvenile hermaphrodites were observed at any stage of sex differentiation. Thus, all the species belong to the so-called differentiated type.

Sex differentiation occurs earliest in *Hynobius tokyoensis* among the five species and is followed in order by *H. nebulosus*, *H. lichenatus*, *H. nigrescens* and *H. dunni*. This order of sex differentiation is the order arranged from higher to lower water temperature at which the spawning of each species occurs with the exception of *H. dunni*.

2. In each of *Hynobius nebulosus* and *H. tokyoensis*, the cold adapted race was generally later in sex differentiation than the warm adapted race.

3. Effects of sex hormones upon sex differentiation were examined in *Hynobius nebulosus*, *H. tokyoensis* and *H. nigrescens*.

In all of the three species, various doses of androgen had a feminizing effect upon the gonads of genetic males, although there were a few hermaphrodites. The ovaries of sex-reversed genetic males were remarkably inferior in development to those of the control females.

The sex-reversed males of *Hynobius tokyoensis* were superior in development of the ovaries and fewer in the rate of hermaphrodites than those of the other two species. The ovaries of genetic females in *Hynobius tokyoensis* seemed almost completely normal after androgen treatment, while those of *H. nebulosus* and *H. nigrescens* seemed somewhat underdeveloped.

4. Estrogen feminized the gonads of genetic males in all the three species, although there were some hermaphrodites.

The ovaries of sex-reversed males in *Hynobius tokyoensis* were the best in development, while those in *H. nebulosus* were the most inferior among the three species. However, all these ovaries were distinctly inferior in development to those of the control females. Hermaphrodites were especially numerous in *Hynobius tokyoensis*. The ovaries of genetic females of the three species seemed generally normal after estrogen treatment.

5. In *Hynobius*, like other urodeles, sex hormones administered in the early larval stage seem usually to induce the indifferent gonads to differentiate into gonads of the heterogametic sex.

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