Instructions for use

Studies on the Sexuality of Amphibia. II. Sexual Induction in a Sexually Semidifferentiated Salamander[1]

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With Plates II-IV and three text-figures

1. Introduction and review

Since the appearance in 1925 of Burns' parabiosis experiment of *Amblystoma punctatum = A. maculatum*, suggested by the case of the free-martin, investigations on the free-martin effect have been repeatedly performed in Amphibians by Burns, Humphrey and Witschi. Thanks to these investigators the sexual induction has been made clear in the case of Amphibians in comparison with other Vertebrate groups. The experiments devised for Amphibians so as to bring about the reaction between two opposite sex glands are the following. 1) Two amphibian embryos prior to sexual differentiation are united in parabiotic twins or chains. 2) The prospective gonad-forming area is grafted in either ectopic or orthotopic positions on to host embryos where sexual differentiation has not yet occurred. 3) Fragments of sexually differentiated or undifferentiated gonads are grafted into host embryos of indifferent stages. 4) Amphibian larvae before sexual differentiation are reared in sex-hormonal solutions. The material used in these experiments includes the following genera, *Amblystoma*, *Triturus* (both belonging to the Urodela), *Rana*, *Hyla* and *Bufo* (all belonging to the Anura).

The sexual induction in the Amphibian has been elucidated in several points mainly by means of the two former methods. The first procedure has been most frequently used by the American

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investigators such as Burns (1925, 1930, 1931, 1935), Witschi and his colaborators (1927, 1929, 1930, 1931, 1936, 1937) and Humphrey (1936) in homogenous or heterogenous parabiosis in the following species; *Amblystoma maculatum, A. tigrinum, A. jeffersonianum, A. mexicanum, Triturus torosus, Rana sylvatica, R. aurosa, Hyla regilla, Bufo americanus.* The second method has been employed successfully only by Humphrey (1929, 1931, 1933, 1935) in the three afore-mentioned species of *Amblystoma* and *Rana sylvatica.* In these two experiments the sexual induction is at least at the beginning due to the results of the interactions of primitive larval gonads which do not yet produce so-called sexual hormones. From the results obtained by himself and by Burns and Humphrey, Witschi is of the opinion that the larval sexual inductors, cortexin and medullarin, act in heterosexual parabiotic twins only as inhibitive substances but not as stimulatives, and furthermore pointed out that these sexual inductors resemble Spemann's "organizers" of amphibian gastrula, because, as no blood circulation has yet been established, the inductive substances disperse as in the case of the organizers. He concluded in 1937 (p. 314) that, "It was also found that there exist species differences with respect to the ease with which these substances are transmitted. The inhibitors act only within the gonad in which they are produced in toads (type 1); they spread over a limited distance in frogs (type 2), while in salamanders and newts, they are able to reach all parts of the twin system (type 3)."

The third and fourth experiments are slightly different from the former two, because in these experiments the sexual induction is mainly the result of the effect of sexual hormones from the adult gonads upon the primitive indifferent gonads of larvae. In 1937 Witschi transplanted adult testicular pieces of *Rana temporaria* in the tadpoles of the same species and found no effect of these grafts upon the larval gonads. In the same year Burns reported that mutual interactions occurred in heterosexual cases between grafted indifferent larval gonads of *Amblystoma maculatum* and *A. tigrinum,* and larval gonads of the host larvae. Burns' material, however, seems to have been rather limited and moreover in these Urodelans the normal (unoperated) larvae frequently indicate bisexual conditions (Humphrey, 1929 and Witschi basing on Gilbert's work, 1937); therefore, this experiment must be carefully checked in consideration of the bisexual tendency. The fourth experiment has scarcely been tried in the case of Amphibians;

recently Padoa (1936) briefly reported the effect of the follicular hormone upon larval gonads of *Rana esculenta*.

In this paper has been described a testicular grafting experiment on *Hynobius* larvae prior to sex-differentition, which has been carried out through the financial aid of the Imperial Academy. The writer's cordial thanks must be extended to Mr. K. Hanaoka for making preparations and also to Mr. H. Yamaguchi for making photo-micrographs.

2. Semidifferentiated Urodelans

The existence of sexually undifferentiated and semidifferentiated strains in ·Amphibians has been well established by E. Witschi's work (1914). Since then there have been described a few semidifferentiated species either in the Anura or in the Urodela. Concerning the bisexual tendency of the male larval gonads of Urodelans, Humphrey (1929) reported that the larval testis, uninfluenced by sexual inductors, in *Amblystoma maculatum* and *A. tigrinum* possesses morphologically the capacity for development into a typical ovarian cortex. When such development occurs, the gonad frequently becomes bisexual in character, with the cortical part similar to the ovarian cortex in the delimited distal part. This fact seems to be more firmly established by the un-published work of Gilbert. Describing Gilbert's findings Witschi (1937, p. 321) mentions, "Secondly, it has been shown by the studies of Gilbert that in any group of *A. maculatum* there appear some males with extremely pronounced hermaphrodite features. The regularity of their occurrence, even under various experimental conditions, makes it quite certain that they are genetically determined and constitutionally different from the average males of the group."

In 1933 Witschi and Humphrey independently pointed out that the ovaries of the Arkansas strain of *A. maculatum* develop normally, while the gonads of the male exhibit a pronounced bisexual condition. In this strain a large number of germ cells remain in the peripheral germinal epithelium and differentiate into ovogonia. The medullary cords at the outset of testicular differentiation often possess a central cavity, rudiment of an ovarial sac. At the time of metamorphosis the testicular component is usually well-organized, while the cortex becomes relatively less important. Though the case is not so marked as in the undifferentiated races of frogs, these embryological facts

prove clearly that the *Amblystoma* forms a race of semidifferentiated type. In 1934 Hanaoka reported that the salamander, *Hynobius retardatus* in the environs of Sapporo is an undifferentiated one, characterized by protogynous tendencies in the male. In this species the appearance of gonads occurs in larvae of about 20 mm and gonads soon come to retain the indifferent condition, having the germinal epithelium on the periphery and the rete cord in the central position. When the larvae reach approximately a length of 40 mm, all the gonads come to be feminized in form, featured by the formation of a distinct ovarian cavity and germinal cells arranged in the cortical portion. In these stages males cannot be distinguished from females. Just before the metamorphosis which occurs in larvae about 50 mm in length; the gonads of the genotypic male begin to assume male characteristics; the ovarian cavity being gradually occluded by rete cells and consequently having been wholly obliterated, while the rete cord in the medulla coming to contain germ cells and interstitial cells. This salamander also seems to be one of the semidifferentiated types, slightly more remarkable than in the Arkansas strain of *Amblystoma maculatum*.

3. The experiment

Taking into consideration of the semidifferentiated condition of the larvae of *Hynobius retardatus*, the writer undertook an experiment with the intention of testing the sexual induction in the Urodelans by grafting adult testes on larvae in which the primordial gonads are scarcely developed or in an indifferent condition. When the sexual induction is positive, the two following results may be expected: 1) Influenced by grafted pieces of testis, the rudimentary gonads of both the male and female develop as in the male gonads of the differentiated salamander, possessing a solid medulla without the ovarian cavity, or 2) larvae of zygotic females develop as in the normal female, while larvae of zygotic males differentiate as in the males of differentiated salamanders.

The testes of the donors were in the stage immediately after the breeding season and were found to contain spermatogonia which were increasing and grouped in cysts.

The larvae used in these experiments differed in growth and were operated on in the following stages. Most of them were in the indifferent stage, but in those belonging to groups L, M and N the

gonads presumably had just made their appearance. In group K the gonads were supposed already to have had the ovarian cavity.

A. Body 30 mm long, from the snout to the anus 17 mm long, fore legs 2.5 mm, hind legs rudimentary, balancers still present.

B. Body 31 mm from the sount to the anus 17 mm, fore legs 3.5 mm, with three rudimentary digits, hind legs rudimentary, balancers already atrophied.

C. Body 31 mm long, from the snout to the anus 16 mm long, fore legs 2.5 mm, with three rudimentary digits, hind legs rudimentary, balancers already atrophied.

D. Body 32 mm long, from the snout to the anus 16 mm long, fore legs 2.5 mm, with three rudimentary digits, hind legs rudimentary, balancers atrophied.

E. Body 37 mm long, from the snout to the anus 21.5 mm long, fore legs 4.5 mm, with four rudimentary digits, hind legs 1.5 mm long. No balancers.

G. H. Body 27 mm long, from the snout to the anus 14 mm long, fore legs 2 mm, with three rudimentary digits, hind legs rudimentary, balancers degenerated.

I. J. Body 33 mm long, from the snout to the anus 16 mm long, fore legs 2 mm, with three rudimentary digits, hind legs rudimentary.

K: Body 38.5–45 mm, from the snout to the anus 21.5–25 mm long, fore legs 5–6 mm, with four digits, hind legs 4–4.5 mm, with three rudimentary digits.

L, Body 21 mm long, from the snout to the anus 11.5 mm long, fore legs rudimentary, no hind legs, balancers well-developed.

M. Body 21 mm long, from the snout to the anus 16 mm long, fore legs rudimentary, no hind legs, balancers well-developed.

N. Body 23 mm long, from the snout to the anus 13 mm long, fore legs 1 mm, hind legs represented by only minute rudimentary buds, balancers well-developed.

P. Body 30–35 mm long, from the snout to the anus 16–20 mm, fore legs 4.5 mm, with four digits, hind legs 2–3 mm with three rudimentary digits.

For the comparison of these animals, the following table of controls has been kindly made at the writer's request by Mr. K. Hanaoka on the basis of his preparations (see also Text-fig. 1, p.). In the development of the gonads of this species the indifferent stage lasts for a fairly long time and then the stage with the ovarian cavity and cortical germ cells sets in. This stage has been named for convenience in this paper the " ovarian stage." Following the " ovarian stage " the sex differentiation begins to take place.

It must be noted that in the best-developed animals differentiation of male characteristics takes place 80 days after fertilization, but when the larvae are more or less starved, the gonads remain generally in the indifferent stage and their germ cells gradually decrease in number. Excellent illustrations may be had of hermaphrodites changing into males after sex differention has taken place relatively late in time.
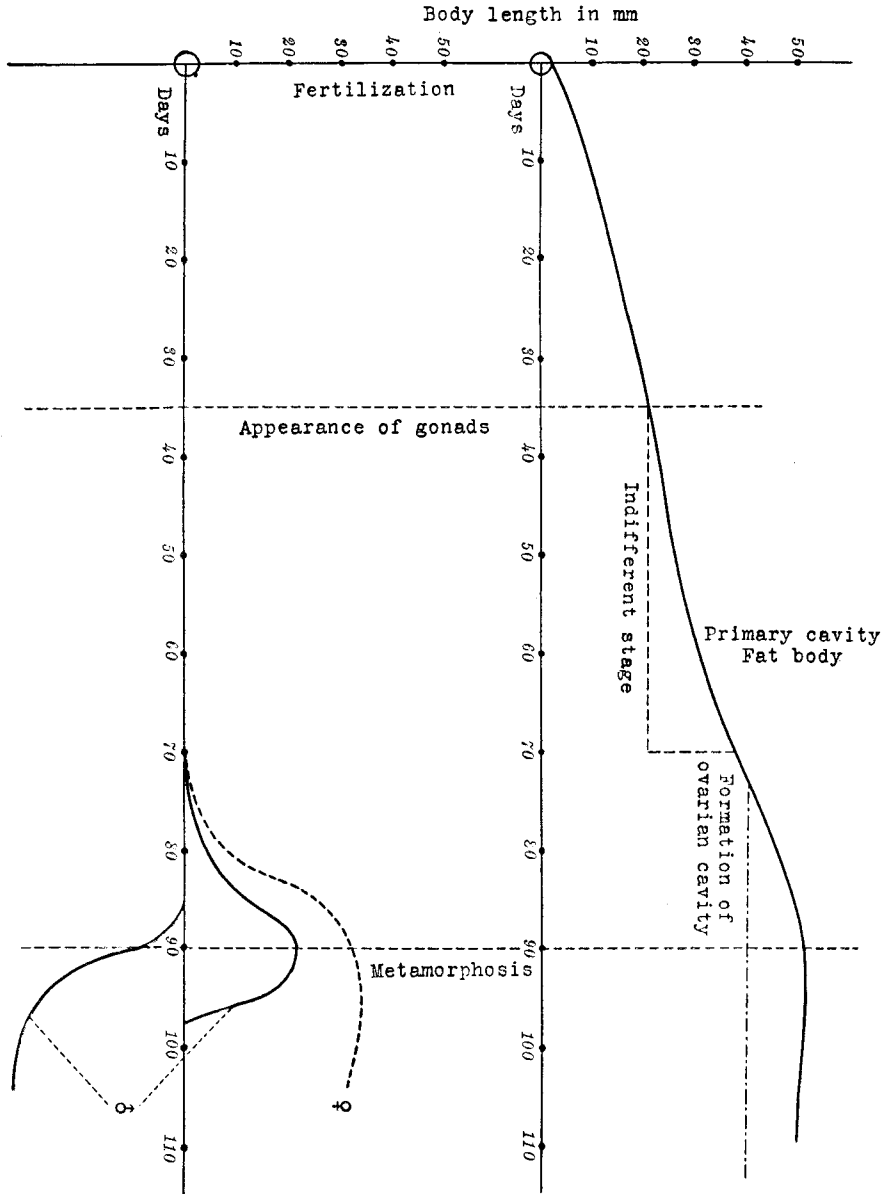
Fig. 1. a. Graph expressing the growth rate of *Hynobius retardatus*. b. Graph representing inductive substances released by male and female gonads of this semidifferentiated salamander in relation to the rate of growth. The indifferent stage lasts about 35 days after the appearance of gonads which occurs about 35 days after fertilization. At the age of approximately eighty-five days only the female substance is released and thence the occurrence of the ovarian stage. Simultaneously with the metamorphosis which takes place in the age of about 90 days, the male differentiation occurs.

The larvae were narcotized by 0.2 % chroletone solution before the operation. After two or three days they were kept separate because of a marked cannibalistic tendency and were reared with ease with liver or flesh. In due time they were fixed in Bouin's solution, sectioned and doubly stained in Delafield haematoxylin and eosin in the normal way.

TABLE I.

Days after fertilization	Length in mm	Gonads	External features
37.	21.	1) Appearance of gonads.	Fore legs rudimentary, no hind legs.
50–60.	28–30.	1) Occurrence of primary gonad cavity 2) Development of fat-bodies.	Fore legs 3 mm, hind legs 1 mm.
65–70.	35–40.	1) Secondary ovarian cavity packed with rete cells (indifferent stage)	Fore legs 5 mm, hind legs 4 mm.
Approximately 80.	45–50.	1) Secondary ovarian cavity being opened.	Fore legs 8 mm, hind legs 8 mm.
↓	↓	Enlargement of ovarian cavity.	Metamorphosis occurring in a few individuals.
Approximately 90.	50–58.	↓	Beginning of degeneration of gills.
Approximately 100.	47–55 (Metamorphosis).	1) Typical female gonads or 2) Differentiation of male characters.	Gills already disappeared.

4. The behavior of the testis grafts

Testicular pieces grafted into the recipients were generally found attached to the coelomic body wall, the wall of the gut and the mesentery. Some cases were observed where implantation took place on the liver, the fat bodies and gonads. A few grafts were lying freely between or on the dorsal side of the alimentary tract. As shown in Fig. 2 and Table II, most of these testicular grafts contained active

germ cells, though often more than half was vesiculated. They were generally found to have more or less enlarged and contained germ cells (Gc) forming cysts and actively proliferating interstitial cells. Some of these grafts had greatly degenerated and vesiculated, only containing interstitial cells (I) which were sometimes active and sometimes inactive. In some grafts mainly composed of interstitial cells

Fig. 2. Regenerated testicular graft in the larva H 3, showing
the formation of seminiferous tubules. ×105.

are found a few germ cells which are dispersedly distributed (Gs). There were found some hosts in which no testicular piece was found in sections. Concerning these cases one can suppose either that the graft had been utterly absorbed by the host tissue or that it had been lost at the time of dissection or fixation.

5. The gonads of hosts

The indifferent gonads of the young larvae of this salamander have the capacity for development to both sexes ; when the cortical part develops and the ovarian cavity is opened in the medulla, they become female gonads, but when the cortical part becomes thin and the medulla is filled with sex-cords, they become male gonads. Though the indifferent gonads of the larvae of this salamander at first all come to indicate the female condition as shown on page 40, the gonads of operated larvae mostly were found to have been modified by the testicular grafts. Out of operated larvae, 84 pairs of gonads were available for examination. The larvae having been operated in different stages and reared for various lengths of time, their gonads displayed

different conditions. The gonads of larvae, in which the operation was executed at relatively early stages and fixed in rather a short period, were found to be all more or less modified, but some of them fixed near the metamorphosis seemed to be scarcely affected.

The striking modification of the gonads through the testicular grafts is marked by the reduction of the number of cortical germ cells and their distal migration and then by the growth of the medullary portion, composed mostly of many active interstitial cells which sometimes include several germ cells. Along with the atrophy of the cortical portion, the concomitant development of the medulla causes the transformation of the gonads towards the male type. On the other hand, when the medullary growth does not progress after the cortical degeneration, the gonads come to be reduced to the free-martin condition. In the writer's preparations are found all these cases. There were also found some peculiar gonads which are provided with a distinct ovarian cavity but contain a few inactive germ cells sparsely distributed on the cortical portion. Although these gonads have been influenced by the testicular grafts, they are inferred to have a genetical constitution with a strong tendency towards female differentiation. On the other hand, there can be found some gonads which are distinctly of the male type, composed of a solid mass of active interstitial cells containing only a few germ cells.

The effect of testicular grafts upon the development of gonads of hosts thus seems to be different according to individuals, stages of larvae at which the operation was executed, and duration of grafting. The results of the effect will be broadly divided into the following groups. There are, of course, intermediate stages between these groups and many combined forms of them; e. g. as shown in table II, one can often encounter with cases in which the cephalic or caudal ends of gonads display the state under the free-martin effect (=free-martin gonads), while the middle portion is highly masculinized.

1) *True female gonads,* ♀ : gonads with a distinct ovarian cavity and germ cells arranged on the cortical portion.

2) *Degenerated female gonads,* ♀ (*d*) : gonads with a distinct ovarian cavity but germ cells arranged on the cortical portion, meagre in number.

3) *Free-martin gonads, f;* gonads with germ cells only on the terminal portion, with or without rete cords, ovarian cavity packed with interstitial cells. When active rete cords are predominant, the

TABLE II.

Gonads of host	Duration of grafting	Sizes at the time of operation & fixation	Graft	Effect
A 1	20 days	30 mm, 34 mm	I. GC.	f + ♂ (♀)
A 3	20 days	30 mm, ? (tail partially torn off)	I.	f (♀)
A 4	20 days	30 mm, ? (tail partially torn off)	I. GC.	f
B 3	25 days	31 mm, 35 mm	I. GC.	♂ (♀) + f
B 4	25 days	31 mm, 35 mm	Lost	♀ (♂) + ♀ (d)
B 5	25 days	31 mm, 37 mm	I. GC.	♂ + ♂ (♀)
B 6	30 days	31 mm, 37 mm	I. GS.	f (♂)
B 7	30 days	31 mm, 37 mm	I. GC.	f + ♂ + ♀ (♂)
C 1	30 days	31 mm, 36 mm	I. GC.	♀ (d) + f (♂)
C 2	30 days	31 mm, 39 mm	?	♂ + ♂ (t)
C 3	30 days	31 mm, 38 mm	I.	f + f (♀)
C 4	35 days	31 mm, 40 mm	I. GC.	f + ♂ + f (♀)
C 5	35 days	31 mm, 32 mm	I. GC.	♀ (d) + ♂ (t)
D 1	35 days	32 mm, 48 mm	?	♀
D 2	35 days	32 mm, 44 mm	I. GC.	f + ♀ (♂)
D 3	35 days	32 mm, 34.5 mm	I. GC.	f (♂) + ♂
D 4	40 days	32 mm, 45 mm	I. GC.	♀ + ♀ (♂)
D 5	40 days	32 mm, 42 mm	I.	♀ + ♀ (♂)
D 6	40 days	32 mm, 40 mm	?	♂ + ♀ (♂)
D 7	40 days	32 mm, 40 mm	I. GC.	f + ♀ (d ♂)
D 8	40 days	32 mm, 40 mm	I. GC.	♂ (t) + ♀ (♂ d)
E 1	45 days	37 mm, 38 mm	I.	♀
E 3	45 days	37 mm, 45 mm	I.	♂ (t) + ♂
G 3	50 days	27 mm, 44 mm	Lost	♂
G 4	50 days	27 mm, 43 mm	I. GC.	♂
G 11	55 days	27 mm, 43 mm	I.	♂
G 12	55 days	27 mm, 31 mm	I. GC.	f + ♂ (t)
H 1	55 days	27 mm, 42 mm	I. GS.	♀
H 2	55 days	27 mm, 43 mm	I. GC.	♂ (t)
H 3	60 days	27 mm, 41 mm	I. GC.	♂
H 4	60 days	27 mm, 39 mm	I. GC.	♀ (d)
H 5	60 days	27 mm, 40 mm	I.	♀ + ♀ (♂)
I 1	60 days	33 mm, 40 mm	I. GC.	♂
I 3	60 days	33 mm, 38 mm	Lost	♂

TABLE II. (*Continued*)

Gonads of host	Duration of grafting	Sizes at the time of operation & fixation	Graft	Effect
I 4	65 days	33 mm, 41 mm (In metamorphosis)	I. GC.	♂ + ♀ (♂)
I 5	65 days	33 mm, 40 mm	I. GC.	♂ + f
I 6	65 days	33 mm, 40 mm	I. GC.	♂ + ♀ (d ♂)
J 1	72 days	33 mm, 40 mm	?	♀ (♂)
J 2	72 days	33 mm, 37 mm	?	♀
J 3	77 days	33 mm, 40 mm (Metamorphosed)	I. GC.	♂
J 4	77 days	33 mm, 41 mm (Nearly metamorphosed)	I. GC.	♂
J 5	77 days	33 mm, 45 mm (In metamorphosis)	I. GC.	♂
J 7	77 days	33 mm, 45 mm (At the beginning of metamorphosis)	I. GC.	♂ + ♀ (♂ d)
K 1	22 days	38.5-45 mm, 49 mm	I. GC.	♀ & ♀ (♂)
K 2	30 days	38.5-45 mm, 47 mm (Nearly metamorphosed)	I.	♀
K 4	32 days	38.5-45 mm, 46 mm (Nearly metamorphosed)	I ?	♀ + ♀ (♂)
K 5	37 days	38.5-45 mm, 48 mm (Nearly metamorphosed)	I. GC.	♀
K 7	22 days	38.5-45 mm, 45 mm (Nearly metamorphosed)	I. GC.	♂ (t)
K 10	32 days	38.5-45 mm, 47 mm (Nearly metamorphosed)	I. GC.	♀ (♂) & ♀
K 12	37 days	38.5-45 mm, 46 mm (Nearly metamorphosed)	?	♂ + ♂ (♀)
K 13	27 days	38.5-45 mm, 45 mm (Nearly metamorphosed)	I. GC.	♂ + ♂ (♀)
L 2	20 days	21 mm, 31 mm	I. GC.	f (♂)
L 3	20 days	21 mm, 31 mm	I. GC.	f + ♀ (♂)
L 6	25 days	21 mm, 34 mm	I. GC.	f
L 7	25 days	21 mm, 37 mm	I. GS.	♀ (♂)
L 11	30 days	21 mm, 34 mm	I. GS.	♀ (♂)
L 12	30 days	21 mm, 34 mm	Lost	♀ (♂)
M 13	50 days	21 mm, 47 mm	I. GC.	♂ (t)
M 14	50 days	21 mm, 43 mm	I. GC.	f (♂)
M 16	50 days	21 mm, 30 mm	I. GC.	f + ♀ (♂)
M 17	50 days	21 mm, 40 mm	I. GC.	♂ (t)

TABLE II. *(Continued)*

Gonads of host	Duration of grafting	Sizes at the time of operation & fixation	Graft	Effect
N 1	50 days	23 mm, 36 mm	I. GC.	f(♀)+ ♀(♂)
N 2	50 days	23 mm, 31 mm	I. GC.	f(♂)+ ♂
N 3	55 days	23 mm, 51 mm	I. GC.	f(♂)+ ♂
N 4	55 days	23 mm, 46 mm	I.	f+ ♀(♂)
N 5	55 days	23 mm, 40 mm	I. GC.	♂ +f(♂)
N 6	55 days	23 mm, 36 mm	I. GC.	f+ ♀(d)
N 7	55 days	23 mm, 31 mm	?	♀
N 8	60 days	23 mm, 47 mm	I. GC.	f(♂)
N 9	60 days	23 mm, 48 mm	I. GS.	♀ +f
N 10	60 days	23 mm, 47 mm	I. GC.	♂ +f(♂)
N 11	60 days	23 mm, 45 mm	I. GC.	♂ +f(♂)
N 12	60 days	23 mm, 45 mm	I. GC.	♂ (oc)
N 13	63 days	23 mm, 42 mm	I. GC.	♀(d)
N 14	35 days	23 mm, 35 mm	I. GC.	♂
N 15	63 days	23 mm, 41 mm	I. GC.	♂ (small)
N 16	63 days	23 mm, 40 mm	Lost	♀
N 19	63 days	23 mm, 42.5 mm	I. GC.	♂
N 20	63 days	23 mm, 42 mm	I. GC.	(♂ +f)+f
N 21	63 days	23 mm, 31 mm	I. (Minunte in size)	♀
P 3	27 days	30–35 mm, 47 mm	I. GC.	♂(t)+f
P 9	27 days	30–35 mm, 44 mm (Nearly metamorphosed)	I. GC.	♀ + ♀(♂)
P 10	23 days	30–35 mm, 43 mm (Metamorphosed)	?	♂

gonads are designated as *f* (♂); while the cortical germ layer is still neatly observable, the gonads are marked as *f* (♀).

4) *Bisexual (indifferent) gonads, n:* gonads with germ cells arranged on the cortical portion, the medullary part being packed with interstitial cells. The demarcation of the cortical and medullary portions is distinct. From these gonads can be derived the two following modifications: Gonads with germ cells arranged on the cortical portion, but with the medullary part packed with interstitial cells and germ cells. The demarcation of the cortical and medullary portion is distinct..... ♀(♂). The gonads of the other type are in general

masculine in character, having rete cords or sex-cords in the medulla but bear remnants of cortical germ cells..... ♂(♀).

5) *True male gonads*, ♂: gonads with distinct sex-cords, remnants of the ovarian cavity (*oc*) rarely present.

6) *Transformed male gonads*, ♂(*t*): gonads without the ovarian cavity, composed of many interstitial cells and a few germ cells situated in or near the medullary portion. These gonads have been probably transformed from free-martin gonads.

In the preceding pages has been given a table indicating conditions of the gonads of hosts affected by the testicular grafts, some of which were lost during manipulation and some have been probably absorbed by the host tissue. It will be noted that the operated animals are often provided with better differentiated gonads than in the controls in relation to the body length. It is due to the fact that the operation arrests the development of the larvae, at least for the first few months.

In the following pages a general survey of the host gonads will be made. Implantations of testicular grafts were generally made during the "ovarian stage." The grafts belonging to the group were fixed at three different stages; 1) the stage corresponding to the indifferent stage in the normal development, 2) the stage comparable to the "ovarian stage" in the normal development, 3) the stage after sexual differention in the normal larvae.

The gonads fixed in the indifferent stage are A 1, A 3, A 4, B 3, B 4, B 5, B 6, B 7, C 1, C 2, C 3, C 4, C 5, D 1, D 2, D 3, L 2, L 3, L 6, L 7, L 11, L 12. These gonads were found to have been distinctly transformed from those of the controls. The transformation is characterized by the proliferation of the medullary interstitial cells and the concomitant degeneration of the cortical germ cells. The gonads of this series were found to have the medullary portion not only packed with a larger and thicker mass of rete cords than in the controls but also often containing several germ cells (Figs. 4–11). On the other hand, the cortical germ cells were generally decreasing in number and located only on the terminal end. The gonad shown in Fig. 6 has a highly degenerated cortical portion and proliferating rete cords into the medulla from the hilar portion. The degeneration of cortical germ cells seems mostly to occur first from the proximal portion (Figs. 4, 10, 11) but sometimes from other peripheral parts (Figs. 7, 8). In some cases the germ cells tend to migrate on account of the active growth of the rete cords towards the distal portion and sometimes consequently

become aggregated there in a mass (Fig. 9). In the gonad of a host (Fig. 13), slightly longer harboured a graft than those above mentioned, is observable a germ cell on the thin cortical layer at the distal end, together with an exuberant growth of the medullary portion, which contains highly developed rete cords with a few germ cells. The gonad of this kind is seen in other gonads fixed after the same duration of grafting. They seem to be in the early stage of masculinized gonads from the free-martin condition. In Fig. 12 showing an affected female gonad a small ovarian cavity is seen in this section through the middle portion. The cortical germ cells are rather inactive and arranged on one lateral part of the cortex. In sections through the anterior and posterior portions is seen no indication of the ovarian cavity.

The following hosts; D 4, D 5, D 6, D 7, D 8, M 13, M 14, M 16, M 17, N 1, N 2, N 3, N 4, N 5, N 6, N 7, were fixed during the "ovarian stage." The gonads of these hosts can be roughly classified into three types, 1) ovarian, 2) free-martin, 3) masculinized. These phases are frequently mixed in a gonad: e. g. the cephalic part might be in the free-martin condition while the middle portion is in the masculinized condition, or the cephalic and middle parts might be of the degenerated ovarian type while the caudal portion is in the free-martin condition. The gonads belonging to the ovarian type are furnished with cortical germ cells and a distinct ovarian cavity, but are different from the normal ovary in several points. In these gonads the cortical germ cells were frequently partially degenerated and the ovarian cavity became more or less narrowed by the rapid proliferation of interstitial cells (Fig. 14). In some ovarian gonads both the terminal portions are solid with the rete cord in the medullary portion. In a few cases, furthermore, sex-cords were observed to have developed in the medulla. For example, Fig. 15 shows a gonad still having a part of the cortical layer of germ cells and rete cords developing from the hilar part. Several gonads in this group are distinctly classifiable as free-martin gonads; Fig. 16 shows the typical gonad in this state. In this gonad the medulla is small and is packed with interstitial cells, but the terminal periphery is bordered by several germ cells which will perhaps gradually atrophy. The gonads M 13, M 14, M 17, N 2, N 3, N 5, etc. are in the larger part masculinized but some still retain a few cortical germ cells. It is remarkable that these masculinized gonads contain rather few germ cells, especially in M 17. Sex-cords are not so well-developed in all these gondas as in the normal

male gonads, because these gonads have probably been newly re-
generated from a free-martin condition. Furthermore, remnants of
the ovarian cavity are often observed in them. The gonads; D 7,
D 8, N 2, N 6, are mixed as regards their characteristics. They are
all small in size, and have few germ cells, remnants of the ovarian
cavity and a few cortical germ cells.

The gonads of the hosts, G 3, G 4, G 11, G 12, H 1, H 2, H 3,
H 4, H 5, N 8, N 9, N 10, N 11, N 12, N 13, N 14, N 15, N 16,
N 18, N 19, N 20, and N 21, were fixed at stages correponding to
the stage at the beginning of sex-differentiation. In these gonads the
following ones; G 3, G 4, G 11, H 3, N 9, N 10, N 11, N 12, N 14,
N 15, N 19, are of the male type. Out of them, some gonads as
pictured in Fig. 17 have well-developed sex-cords and show the features
of the typical male gonad, while some are surmised to have been
modified from ovarian gonads (Fig. 18), in which a remnant of ovarian
cavity is seen, and the arrangement of germ cells and interstitial cells
is in disorder. These gonads are somewhat ragged in outline. A
section from a gonad, represented in Figs. 19 & 20, undoubtedly indi-
cates a male gonad. This gonad contains two cavities as shown in
Fig. 20. The cavities seem to be different from the ovarian cavity,
because they are not clearly surrounded by interstitial cells and these
cavities are observable, though not frequently, in the normal develop-
ment of the male gonad. Among these gonads, N 9, N 10 and N 11
are masculinized (Fig. 21) in the middle portion, but in the free-martin
condition at both the terminal ends (Fig. 22). The gonads classifiable
as female gonads are seen in the hosts, H 1, H 2, H 4, H 5, N 13,
N 16, N 18, N 21. Among them the gonads of N 18 and N 21 appear
to be normal females in most characteristics. In other gonads, how-
ever, the female characteristics are slightly modified, though variable
in degree. The gonads pictured in Fig. 23, though small in size, seem
to be of the normal female type in this middle section, but towards
both the extremities the ovarian cavity becomes relatively small and
the cortical germ cells are slightly degenerated. Besides these charac-
teristics, the rete cord develops from the basal to the middle portion.
In Fig. 24, a section of a gonad, the cortical germ cells are partially
atrophied and few in number. The section of the other part of this
gonad represented by Fig. 25 indicates invasion of the medullary portion
by the interstitial cells. Through Figs. 26 and 27, showing two sections
of a single degenerated ovarian gonad, can be surmised the dis-

integration of the germinal epithelium and modification towards the free-martin condition. Sections of the posterior portion of this gonad express distinctly the free-martin condition. The gonad repersented by Figs. 28 & 29 is a nearly normal female gonad in the middle portion (Fig. 28), but obviously a free-martin gonad in the posterior end (Fig. 29). In the host (H 5) the left gonad having well-developed ova is slightly masculinized on the upper side and with a narrowed ovarian cavity (Fig. 30), but the right gonad is evidently masculinized on the distal margin, represented by the thick mass of interstitial cells containing a few germ cells (Fig. 31). Moreover, the cortical germ cells are relatively meagre in number in these gonads. The gonads G 12, N 8, N 20 are small in size and in the free-martin condition mostly represented by the solid medulla containing a few or no germ cells and by the presence of a few distal germ cells. Out of them, the gonads of N 20 are small in size and masculinized in the anterior part but rather of a degenerated female type in the posterior part. Furthermore, they are represented in places by combined gonads as shown in Fig. 32; the terminal part of the gonad is represented by a mass of inactive cortical germ cells but the proximal part is clearly of the male type, having sex cords.

The gonads of the I- and J- series belong to the group just described but these larvae were operated on slightly later than the latter and fixed for a longer period after implantation. The gonads, I 1, I 3, I 4, I 5, I 6, J 3, J 4, J 5, J 7, are all of the male type, but are small in size and often retain in the cephalic and caudal portions a degenerated cortical layer. They have been probably regenerated from a free-martin condition. This case is the most remarkable in I 6. The anterior half of the gonads is distinctly of the male type (Fig. 33) but the posterior half is modified as shown in Fig. 34, and the part near the posterior end recalls the free-martin gonad having a few degenerated germ cells on the peripheral portion (Fig. 35). In these sections one can see a vigorous development of interstitial cells. The gonads J 1, J 2, are modified females, with ova and a small ovarian cavity like the gonad shown in Fig. 30.

The gonads of the hosts, E 1 and E 3, in which the testicular pieces were implanted just before the appearance of the ovarian cavity, were fixed after 40 days at the stage of sex-differentiation. The gonads of E 1 can hardly be distinguishable from the normal female gonads. The gonads of E 3 are specially noticeable; because the graft was found

fused to the anterior portion of the left gonad (Fig. 36). The graft, having remnants of seminiferous tubules, is vesiculated and contains only interstitial cells but no germ cells in the larger part. Some germ cells from the gonad of the host are however invading the graft and making rete cords there (Fig. 37), The left gonad is large in size and long in the anterior portion to which the graft is fused, and contains several sex-cords, and in the middle portion is of the typical male gonad with many well-developed sex-cords and no cortical germ cell (Fig. 38). On the other hand, the right gonad, considerably smaller than the left gonad, is provided with well-developed sex-cords but also with a remnant of the ovarian cavity of a fair size (Fig. 39). There are no cortical germ cells, except a few occurring in posterior sections. It is a remarkable fact that the left gonad attached to the testicular graft is far larger and more masculinized in its characteristics than the right gonad. It may be plausible that the left gonad has been more influenced by the graft than the right one. But the fact cannot be easily concluded that the hormonal impulse is more effective in the left than in the right. In Hanaoka's unpublished material is found well-developed ovary attached to the testicular graft, germ cells being found in both the ovary and the testicular graft, though the male cells are inactive.

In the series of K- hosts the implantation was made in the "ovarian stage" and fixation took place after sexual differentiation. The hosts were fixed nearly after the metamorphosis. Out of eight hosts, the following hosts, K 1, K 2, K 4, K 5, K 10 were found to bear female gonads. Among them, K 2 and K 5 do not seem to be different from the normal female gonad as is shown in Fig. 40. Three other gonads seem to be slightly affected female gonads, with the ovarian cavity relatively small or already packed with interstitial cells. The cortical germ layer is well-formed in all gonads. The gonads of the hosts K 7, K 12, K 13 are gonads of the male type. Out of them K 12 is the typical male, but two other pairs of gonads are more or less modified from the normal male. The gonads of K 7, though distinctly of the male type, contain very few germ cells and well-developed sex-cords (Fig. 41). The gonad has probably regenerated from a free-martin gonad after the degeneration of the cortical germ cells. This fact has been clearly demonstrated by the high-temperature-experiment which will be published in another paper. The gonads K 13 also display the masculinization from the ovarian gonads. The gonads are for the most

part masculinized, with active interstitial cells and germ cells forming
sex-cords (Fig. 42). The posterior portion of the gonads is of a mixed
type, having an ovarian cavity surrounded by germ cells in the distal
half, and active rete cords and sex-cords in the proximal half (Fig. 43).
The gonads show clearly the process of the modification from a female
gonad to a male, but the similar phase is also seen in the normal
transformation from the ovarian stage of the male gonad in this
semidifferentiated species, so it cannot be decided whether these gonads
have been actually affected by the testicular graft or not.

6. Discussion

In 1927 Witschi using the undifferentiated race of the European
frog, *Rana temporaria*, engrafted testicular pieces of the adult upon
tadpoles still possessing indifferent gonads, and found that the gonads
of the recipients developed uninfluenced in spite of the presence of the
testicular grafts. In the writer's experiment on this Urodelan, on the
contrary, the effect of the grafts is in general clearly observable,
though different in degree and extent. The reason why these two
results were in conflict with each other cannot be explained for the
present time,[1] but may be attributable to differences in the species as in
the case of the inductive substances pointed out by Witschi (see page
36). The writer previously (1935) stated that the gonads of well-deve-
loped larvae, in which the gonads were in the " ovarian stage ", were
found to be scarcely affected by testicular grafts. On closer examina-
tion, however, with the exception of a few cases, these gonads have
been generally modified more or less by the grafts as shown on pages
44–46. The transformation of the gonads of the hosts is due to the
degeneration of cortical germ cells and the concomitant development
of the medullary cells with which the ovarian cavity is consequently
filled. The transformation seems to be regularly pursued according to
stages of modification. When the implantation was made before or at
the same time as the appearance of the gonads and the hosts were
fixed in the indifferent stage, the gonads of the hosts were all chara-
cterized by the development of the medullary portion and the degenera-
tion of cortical germ cells. At the beginning of the modification, some
cortical germ cells are still active and there can be found no germ

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1) A similar experiment on the Japanese *Rana temporaria* is now being carried
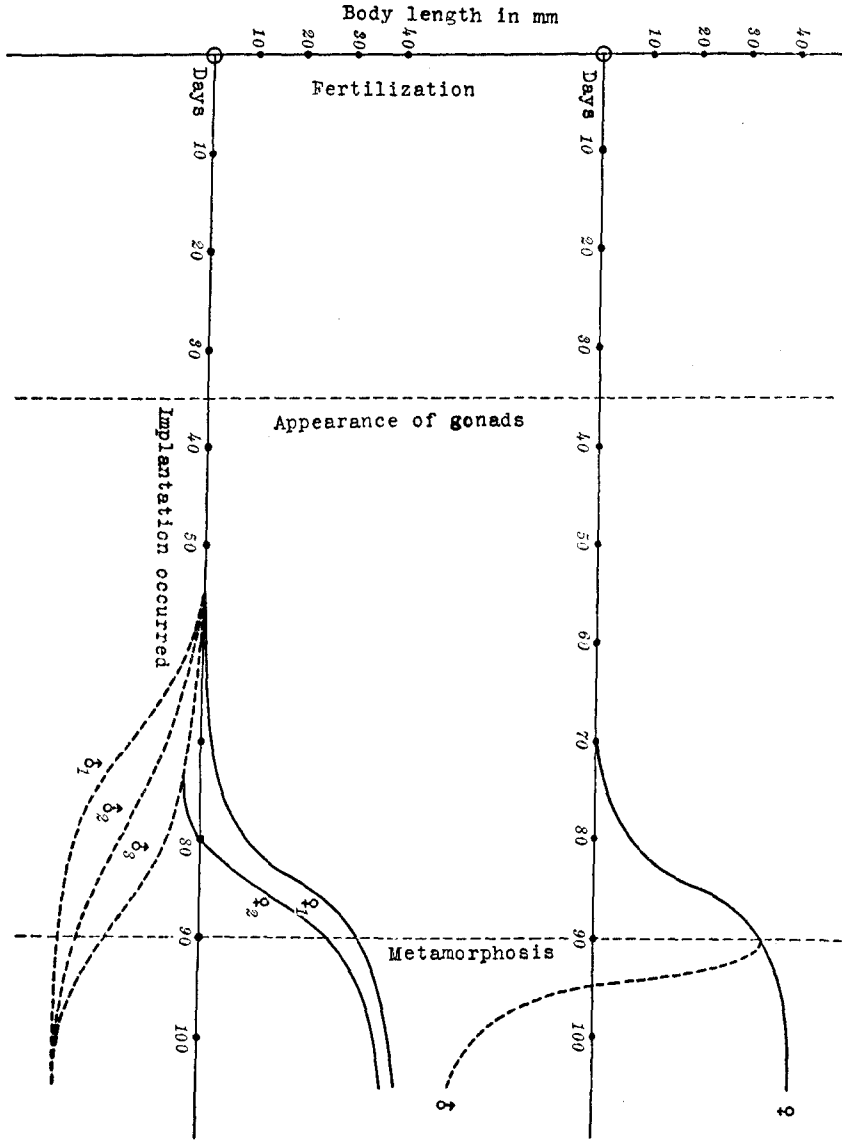out in our Institute.

Fig. 3. a. Graph illustrating the sex differentiation in the semidifferentiated species, *Hynobius retardatus*, in respect to the body length. The dash-line corresponds to the genetic female, while the dotted one to the genetic male. b. Graph showing variation of the sex differentiation in the same species influenced by testicular grafts.

cell in the medulla. In the hosts fixed during the "ovarian stage", the above mentioned modifications become more pronounced and the gonads can be broadly divided into the following three groups : 1) gonads of the free-martin type, 2) masculinized gonads, 3) slightly affected female gonads. In the free-martin gonads of this stage some cortical germ cells are found aggregated in the distal portion. The masculinized gonads generally show the free-martin condition in the caudal or in the cephalic portion. The affected female gonads also indicate the free-martin condition in the caudal or in the cephalic portion. When the hosts were fixed during the differentiated stage, the gonads are similarily grouped into the three groups as mentioned above, but the degrees of modification are different in the above mentioned gonads. In the free-martin gonads of this stage the cortical germ cells are very meagre and the proximal half is often masculinized. These free-martin gonads are small in size compared with gonads belonging to the other two groups. Some masculinized gonads of this stage are typical male gonads but some bear the free-martin part in the terminal localized portion. There were found a few masculinized gonads having meagre germ cells. These gonads seem to have been regenerated as mas-culine gonads from the free-martin gonads, after the cortical germ cells had utterly atrophied. In the female gonads some are generally normal, though slightly affected, with cortical germ cells partially atrophied and with the ovarian cavity slightly diminished in size. In some female gonads cortical germ cells were mostly inactive and greatly degenerated, though the ovarian cavity is distinct on account of the poor immigration of rete cells. The different conditions of the gonads do not seem to depend only upon the different degrees of the function of the grafts, because the grafts, though variable in condition, were found mostly to contain germ cells in a localized part. The difference in the results seems to be due to the genetical difference of the larvae ; for example one may suppose that the genetical males accelerate on their way to male modification through the rapid degeneration of the cortical germ cells, while the genetical females, though more or less affected, proceed on their regular development. Therefore, there are found several female gonads with a distinct ovarian cavity but partially (or largely) degenerated germinal epithelium. Though some free-martin gonads might be later converted into the male gonads, most of the free-martin gonads probably belong to the genetical females of a somewhat undiffe-rentiated nature. When the effect of testicular grafts is removed, they

will perhaps develop as females. So there seems to occur no perpetual sex-reversal at least in this grafting experiment.

When the implantation was made in the ovarian stage and hosts were fixed after the metamorphosis, some females were found to be quite normal but other females were slightly affected. Some male gonads of this group were normal but some were found to have the female characteristics in the localized part, but they may be in the process of the normal transformation from the ovarian stage towards the male condition. The occurrence of a few ova as seen in the gonads, H 5, J 1, J 2, seems to be attributable to a pathological growth of ovocytes in the affected female gonads, because the normal female gonads in this stage are not furnished with such large ova. The sexual induction in the Amphibians has been hitherto demonstrated by means of parabiosis or by the grafting of germinal preprimordium. In these former experiments the interaction of the opposite sex is due to the sexual induction of primitive larval gonads. The writer's experiment indicates the influence of the adult gonads upon the larval gonads. Judging from the similarity of the results obtained from the former experiments with those from the present experiment, it can be concluded that the effect of the primitive larval gonads on the opposite gonads is almost similar to the direct influence of the adult gonads upon the larval gonads of the other sex.

7. Summary

1. The testicular pieces of the adult salamander, *Hynobius retardatus* were implanted in the larvae prior to sexual differentiation.

2. Influenced by the testicular pieces, the sexually semidifferentiated characteristics of the hosts have always been modified, though different in degree mainly according to the genetical constitution, indicating several scala of the free-martin condition.

3. Perpetual sex-reversal cannot be expected in this experiment. Some of the genetical females, if not all, differentiate towards the female, despite the presence of grafted testes.

4. The sex hormone of the adult male seems to have a similar effect on the larval gonads of the hosts as in the case of the inductive substance of the male larva.

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Explanations of Plate II

Fig. 4.   Cross section through the middle part of the right gonad (L 3), showing immigration of interstitial cells with a germ cell into the medullary portion.   ×320.

Fig. 5.   Section through the middle part of the right gonad (A 1) having germ cells in the medulla.   ×320.

Fig. 6.   Cross section through the anterior half of the left gonad (B 3), in which the cortical part has degenerated and the rete cord is invading the medulla.   ×320.

Fig. 7.   Section through the middle portion of the right gonad (B 4), having a few cortical germ cells and proliferating interstitial cells.   ×320.

Fig. 8.   Section through the middle part of the right gonal (B 5), the cortical germ layer partially degenerated but medullary rete cells proliferating. ×320.   The posterior portion of the gonad is more masculinized, having a few germ cells in the medulla.

Fig. 9.   Section through the middle part of the left gonad (B 6).   Germ cells are in clumps in the distal crest.   Rete cords and two medullary germ cells are visible in the proximal half.   ×320.

Fig. 10.   Cross section through the anterior portion of the left gonad (B 7), indicating degenerating distal germ cells and slightly enlarged rete-cell-mass.   ×320.

Fig. 11.   Section through the posterior portion of the left gonad (C 3) showing vigorous proliferation of rete cells in the medullary portion.   Germ cells are sparsely arranged on the periphery.   ×320.

Fig. 12.   Section through the middle part of the right gonad (L 11) bearing a few germ cells on the cortex.   The ovarian cavity, though small, is present in the medulla.   ×320.

Fig. 13.   Section through the middle part of the left gonad (C 5), indicating a plentiful growth of rete cords which have a few germ cells and fill the ovarian cavity.   Cortical germ layer nearly degenerated, having a germ cell in this section.   ×320.   This gonad is nearly of the male type.

Fig. 14.   Section through the middle portion of the left gonad (D 4).   This gonad is generally of the female type ; but the ovarian cavity is relatively small and immigration of rete cells toward the medulla is observable.   ×320.

Fig. 15.   Section through the middle part of the left gonad (D 6), furnished with the remnant of the cortical germ layer in the distal half.   The proximal half of the gonad is masculinized.   ×320.

Fig. 16.   Section through the middle portion of the right free-martin gonad (M 16). Cortical germ cells are arranged only on the distal part and the medulla contains a core of interstitial cells.

Fig. 17.   Section through the middle portion of the male gonad (H 3).   The gonad has distinct male characteristics in this part, composed of many sex-cords.   ×320.

Fig. 18.   Section through the middle portion of the masculinized gonad (G 3).   The gonad is masculinized but still has remnants of the ovarian cavity.   Some germ cells are inactive.   ×320.   This gonad has probably modified from the female to the male form.

Fig. 19.   Section through the middle part of the masculine gonad (N 12).   This gonad is distinctly of the male type, having many sex cords.   ×320.

Fig. 20.   Section throught the middle part of the gonad on the opposte side of the same salamander (N 12).   This gonad is also of the male type but has a distinct cavity.
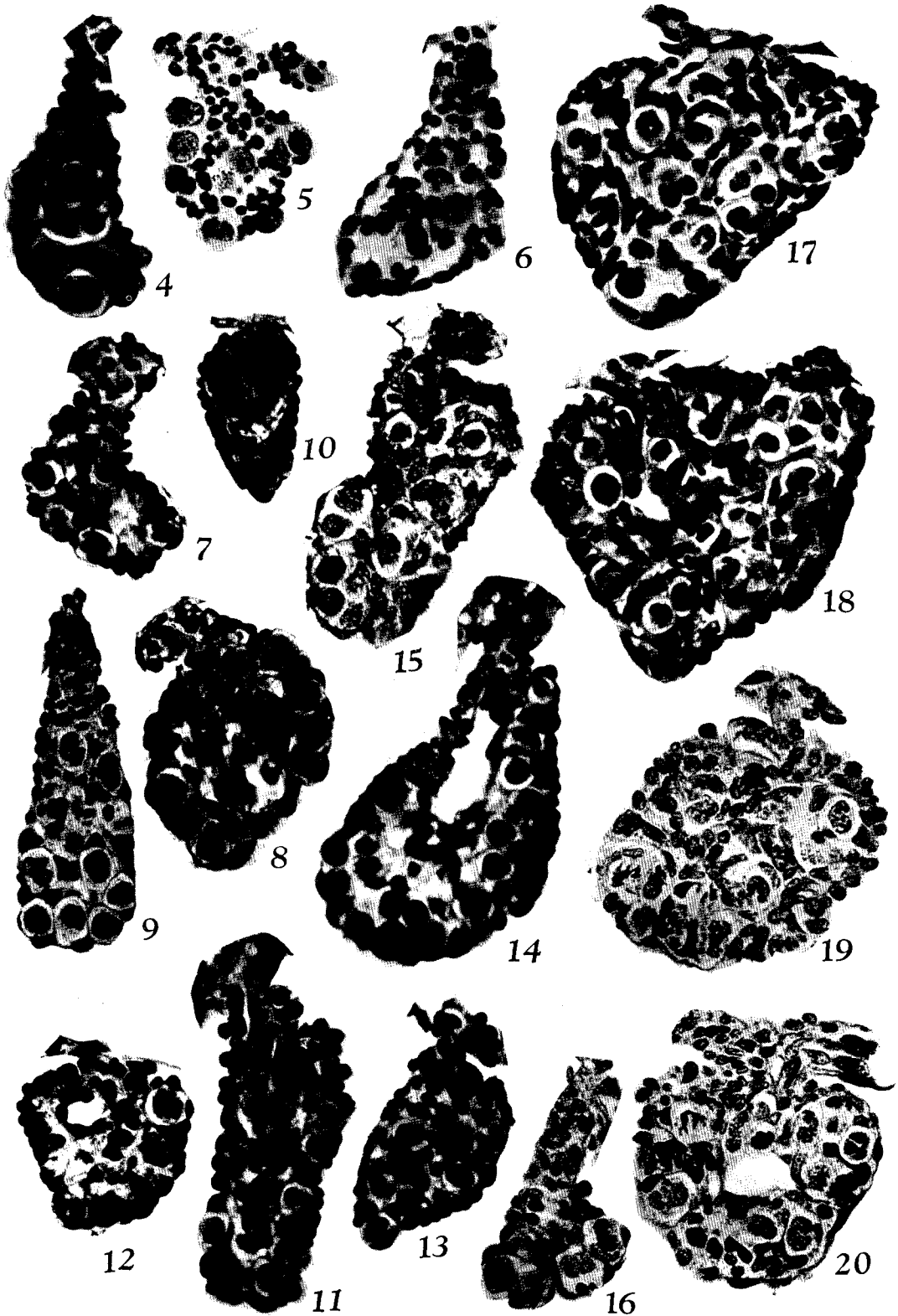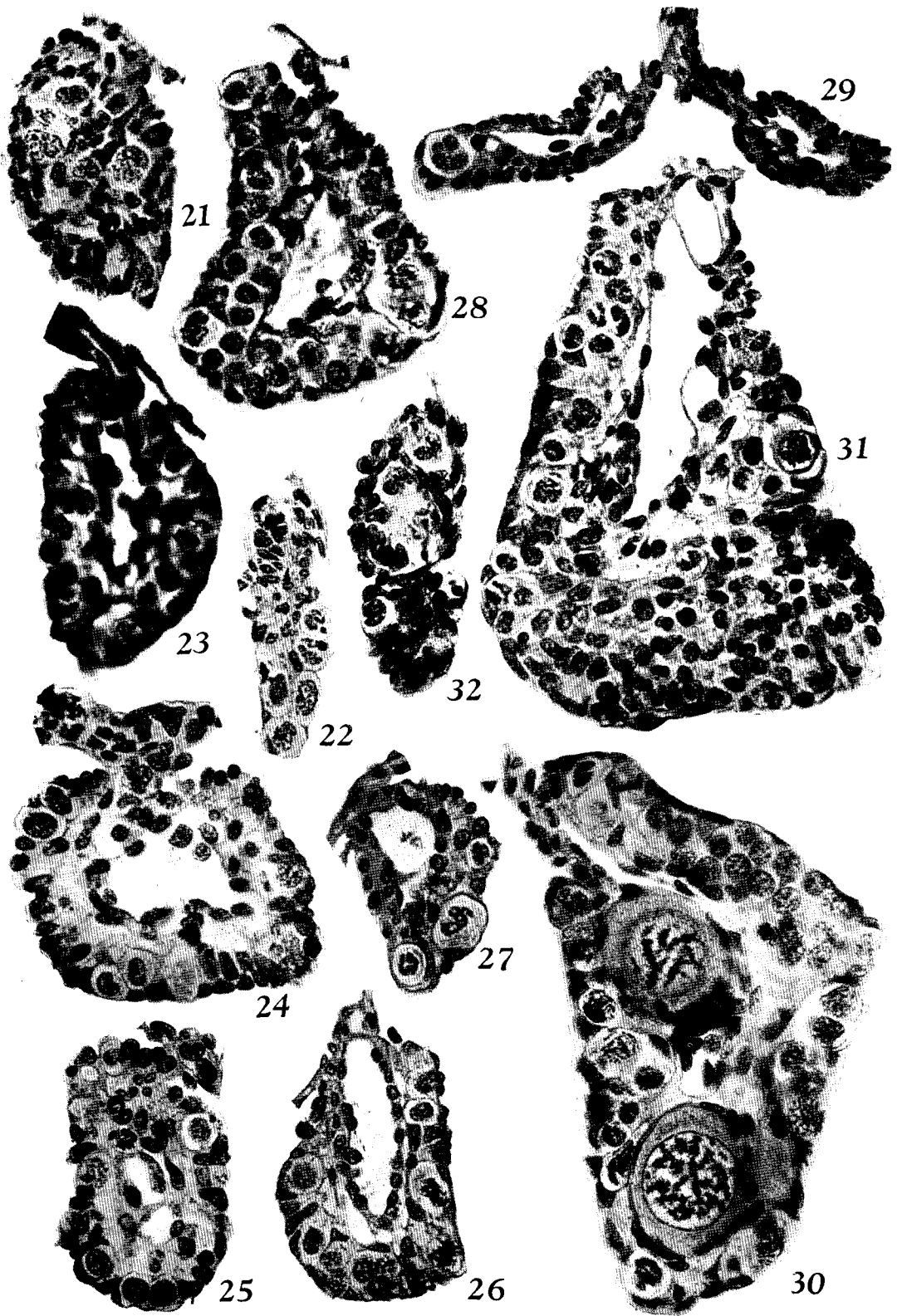
T. Uchida : Sexual induction in a salamander

Explanations of Plate III

Fig. 21.   Section through the middle portion of the right gonad (N 11). This portion is nearly of the male type but bears a few cortical germ cells. ×320.

Fig. 22.   Section through the caudal part of the same gonad (N 11). This part shows distinctly a free-martin condition, with inactive cortical germ cells on the distal crest and a rete cord developed in the medulla. ×320.

Fig. 23.   Section through the middle portion of the right gonad (H 2). The gonad is of the female type, but relatively small in size and bears few germ cells in the greater part. The figure is representative of the most feminized portion. ×320.

Fig. 24.   Section through the middle portion of the right gonad (N 13). The gonad is of the female type but cortical germ cells are partially degenerated. ×320.

Fig. 25.   Section through the caudal portion of the same gonad (N 13). In this part the ovarian cavity is somewhat occluded by the interstitial cells developed from the basal portion. ×320.

Fig. 26.   Section through the middle portion of the left gonad (H 4). The gonad is a somewhat degenerated female gonad. Cortical germ cells are not active and few in all sect ons. ×320.

Fig. 27.   Section through the cephalic portion of the same gonad (H 4). Cortical germ cells few in number. ×320.

Fig. 28.   Section through the middle part of the right gonad (N 16). This is the best develope feminine part. Other parts are also feminine but bear relatively few germ cells. ×320.

Fig. 29.   Section through the caudal part of gonads of the same host (N 16). The right gonad shows a distinct free-martin condition (compare with Fig. 28). ×320.

Fig. 30.   Section through the middle port on of the left gonad (H 5). This gonad is of the female type, with a narrowed ovarian cavity and two eggs, but is slightly masculinized on the proximal portion on the outer side. ×320.

Fig. 31.   Section through the middle portion of the right gonad of the same host (H 5). This gonad has the ovarian cavity and a few cortical germ cells, but the distal portion is highly tra sformed to the male condition. ×320.

Fig. 32.   Section through the middle portion of the right gonad (N 20). This gonad is composed of the female distal half and the male proximal half. The distal female part has inactive cortical germ cells, while the proximal male half consists of sex-cords. ×320. The cephalic part of this gonad is of the male type but the caudal part is of a degenerated female type.

H. Yamaguchi photo.

T. Uchida :   Sexual induction in a salamander

Explanations of Plate IV

Fig. 33. Section through the anterior portion of the right gonad (I 6). This portion is of the male type, with sex-cords and rete cords. ×320.

Fig. 34. Section through the middle portion of the same gonad (I 6). This portion indicates a modified condition from the female to the male gonad. ×320.

Fig. 35. Section through the posterior portion of the same gonad (I 6). This portion is composed of an active medullary part and a degenerated cortical layer. A single germ cell is found on the cortex. This portion shows modification from a free-martin gonad to the male gonad. ×320.

Fig. 36. Section through the cephalic part of the left gonad (E 3) to which the graft is fused. In the gonad sex-cords are well developed. ×80.

Fig. 37. Section through the part just anterior of the above section, showing immigration of rete cords from the gonad to the graft. ×320.

Fig. 38. Section through the middle portion of the same gonad (E 3). ×320. This part is typical of the male type, bearing many sex cords.

Fig. 39. Section through the largest part of the right gonad of the same larva (E 3). ×320. This gonad, though also of the male type, is smaller in size compared with the left gonad and has a remnant of the ovarian cavity. In the caudal part a few cortical germ cells were observed.

Fig. 40. Section through the middle portion of the female gonad (K 5). ×320.

Fig. 41. Section through the middle part of the right gonad (K 7). ×320. This gonad has been probably regenerated from the free-martin condition. In this gonad rete cords are well-developed but germ cells are scarcely found.

Fig. 42. Section through the middle portion of the left gonad (K 13). ×320. This gonad, generally of the male type, seems to have beed modified from the female gonad. The outline of the gonad is ragged and rete cords are developing from the hilar portion.

Fig. 43. Section through the more caudal portion of the same gonad (K 13). ×320. This section shows the female characteristics having the cortical germ layer and the ovarian cavity in the distal half but the active invasion of rete cords in the proximal half.

H. Yamaguchi photo.

T. Uchida : Sexual induction in a salamander