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Mature Haploids and their Reproductive Capacity in *Rana rugosa*

By

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(With 7 Text-figures)

INTRODUCTION

Experimentally induced haploids have been reported in many species of amphibians (G. HERTWIG, 1913; FANKHAUSER, 1937; KAWAMURA, 1939a; PORTER, 1939; BRIGGS, 1949; BEETSCHEN, 1963; NISHIOKA and KONDO, 1978). However, all investigators agree that haploid amphibians usually reveal characteristic abnormalities such as shortened neural plates, edema, ascites, dwarfing, and microcephaly and die sooner or later. No mature haploids have been produced up to the present.

However, there have been a few haploids which could attain or pass the critical period of metamorphosis. BALTZER (1922) obtained a haploid newt from an egg of *Triturus vulgaris* by merogony. FISCHBERG (1948) also reported on a haploid newt produced from an egg of *Triturus alpestris* by cold treatment. In anurans, MIYADA (1960) produced 18 gynogenetic haploids from *Rana nigromaculata* eggs by insemination with sperm treated with toluidine blue. Nine of these completed metamorphosis, of which one lived for 7.5 months after metamorphosis. Thereafter, he (1977) obtained seven more haploid frogs by the same method in the same species. Although one of these haploids became three years old, it was a dwarf and far from sexual maturity.

In spite of repeated efforts to rear haploid amphibians, none of the haploids produced by various means have attained sexual maturity. Thus, the reproductive capacity as well as the gametogenesis of haploid amphibians have been completely unknown. The problem of whether haploid amphibians are able to yield healthy offspring or not is of a great interest from embryological, cytological, and genetical viewpoints.

The present author casually succeeded in producing mature haploid females in *Rana rugosa*. They were raised from among eggs inseminated with UV-irradiated sperm. The present paper describes the development, external and internal characters, sex differentiation and reproductive capacity of these haploid frogs.

MATERIALS AND METHODS

Rana rugosa SCHLEGEL used as material for the present study were collected in the vicinity of Hiroshima. Eggs were obtained from two females by injecting pituitaries of *Rana catesbeiana* into the body cavity. Gynogenetic haploids were produced by pseudofertilization with UV-irradiated sperm according to SELMAN's method (1958). This method was as follows: Fresh sperm suspension was made in Cl-free tap water. A vial with 0.5 ml of sperm suspension was placed 20 cm from the source of ultraviolet rays (Toshiba Electric Company, Tokyo) and exposed for 2 minutes under a current of 125 mA. Eggs were inseminated with the UV-irradiated sperm suspension.

Embryos were placed in Petri dishes, 17.5 cm in diameter, and allowed to develop until the early tadpole stage under the laboratory conditions. Tadpoles at the age of about 30 days were transferred into a cement tank, 95 cm in length, 65 cm in width and 20 cm in depth, placed outdoors. Tadpoles were fed on boiled spinach, while frogs were fed on crickets.

Chromosome preparations were made from the tail-tips of tadpoles by the squash method described by MAKINO and NISHIMURA (1952). Haploid frogs were fixed in NAVASHIN's fluid immediately after death or sacrifice and preserved in 70% alcohol for cytological observations. After embedding in paraffin, various organs and tissues were sectioned at 12 μ and stained with HEIDENHAIN's hematoxylin.

The description of developmental stages follows those of *Rana pipiens* established by SHUMWAY (1940) and TAYLOR and KALLROS (1946) for convenience sake.

OBSERVATION

I. Developmental capacity of eggs inseminated with irradiated sperm

A total of 1,612 eggs obtained from two females were inseminated with UV-irradiated sperm (Table 1). As a result, they were slightly higher than the controls in the rate of normally cleaved eggs; 1,564 eggs (97.0%) cleaved normally, while 74 (75.5%) of 98 control eggs did so. From the gastrula stage the embryos in the experimental series began to show delayed development. Striking differences in external features between the embryos of the experimental series and the controls appeared at the tail-bud stage, although the former were considerably normal in viability during the subsequent embryonic stage. Eventually, 1,220 embryos (75.7%) hatched and became normally swimming tadpoles. However, there were 310 (19.2%) abnormal tadpoles at the hatching stage in contrast with the controls that were all normal. The lethal tadpoles at this stage showed characteristic haploid syndromes, such as microcephaly, shortened neural plate, lordosis and edema in abdomen.

Most of the normally swimming tadpoles of the experimental series became

TABLE 1
Viability and ploidy of individuals developed from eggs inseminated with UV-irradiated sperm

Series	No. of eggs	No. of normally cleaved eggs	No. of normal neurulae	No. of hatched tadpoles		No. of normally shaped tadpoles at V~X	No. of tadpoles whose ploidy was examined			
				Normal	Abnormal		Total	n	2n	Mosaics
Control I	58	41 (70.7%)	41 (70.7%)	41 (70.7%)	0	41 (70.7%)	30	0	30	0
Experimental I	1130	1108 (98.1%)	1108 (98.1%)	862 (76.3%)	246 (21.8%)	33 (2.9%)	31	30	0	1
Control II	40	33 (82.5%)	33 (82.5%)	33 (82.5%)	0	32 (80.0%)	30	0	30	0
Experimental II	482	456 (94.6%)	422 (87.6%)	358 (74.3%)	64 (13.3%)	102 (21.2%)	90	88	1	1
Total:										
Control	98	74 (75.5%)	74 (75.5%)	74 (75.5%)	0	73 (74.5%)	60	0	60	0
Experimental	1612	1564 (97.0%)	1530 (94.9%)	1220 (75.7%)	310 (19.2%)	135 (8.4%)	121	118	1	2

edematous and died before reaching stages V~X, and 135 (8.4%) were living at these stages, while 73 (74.5%) controls were living. It was remarkable that experimental series I differed distinctly from experimental series II in the survival rate. In series II, there were 102 (21.2%) normally shaped tadpoles, while there were only 33 (2.9%) such tadpoles in series I.

Control tadpoles at the early stage were light brown in dorsal color and somewhat transparent. Thereafter, they gradually became yellowish brown and revealed numerous dark brown spots on the back. In contrast, a considerable number of tadpoles of the experimental series were dark brown in dorsal color and revealed no dark spots on the back, owing to the darkness of the dorsal coloration. The tadpoles of the experimental series could be distinguished from the controls by the shape of tails; they had a shorter and broader tail. Some of them had a tail which was bent vertically or laterally. In the other respects, the tadpoles of the experimental series were very similar to the controls. The tadpoles at the late stage were relatively vigorous and swam normally. Most of them were not remarkably retarded in development.

II. Identification of ploidy

The ploidy of tadpoles at stages V~X in the two experimental series as well as the controls was determined by examining the chromosomes of epidermal cells from the tail-tips (Table 1). These tadpoles were 30 days old. Chromosome counts were made in all the metaphase plates with well spread chromosomes. Each tadpoles possessed about 5 to 30 analyzable figures. All 60 controls were diploids, consisting of 5 pairs of large chromosomes and 8 pairs of smaller ones. In contrast with these controls, 118 out of 121 tadpoles in the experimental series

were haploids, whose metaphase plates consisted of 5 large chromosomes and 8 smaller ones (Fig. 1). One of the remaining three tadpoles was a diploid with 26 normal chromosomes and two were mosaics consisting of a mixture of haploid and diploid cells. These three non-haploid tadpoles were killed and preserved. The haploids and the control diploids were continuously reared together with 13 other controls which were believed to be diploids in order to produce metamorphosed frogs.

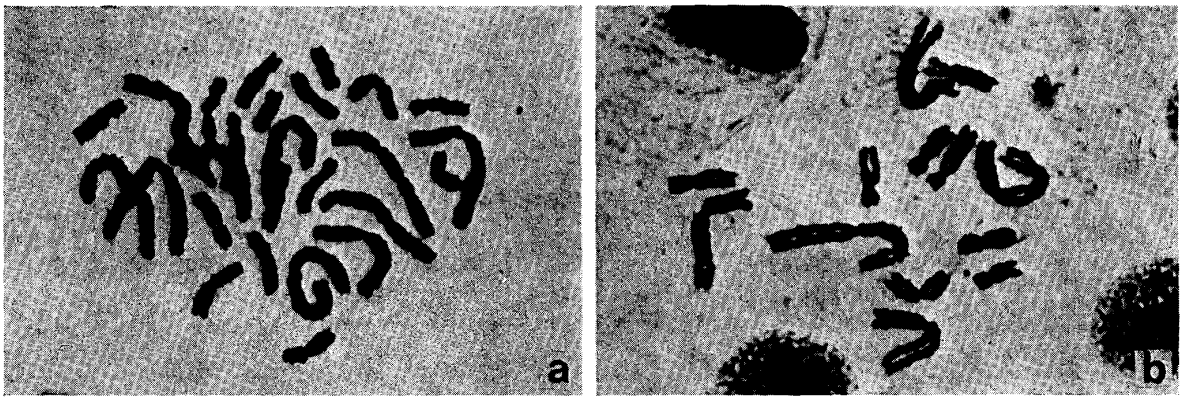

Fig. 1. Metaphase plates of epidermal cells in the tail-tips of tadpoles. ×2800
a. Diploid tadpole b. Haploid tadpole

III. Metamorphosis of haploid tadpoles

Of 118 haploid tadpoles, 82 (69.5%) climbed out of water at the age of 70～130 days, while 69 (94.5%) of 73 controls did so at the age of 60～100 days. They were delayed approximately 10 to 30 days in climbing out of water as compared with the diploids. The haploids were remarkably inferior in viability to the

TABLE 2
Body length and sex of metamorphosed haploids

Series	Tadpoles at the time of climbing land		Frogs immediately after metamorphosis		No. of frogs whose sex was examined						
					Total	Within one month after metamorph.				2 months after metamorph.	10 months after metamorph.	
	Number	Age (days)	Number	Body length (mm)		♀	♀	♂R	♂	♂	♀	♀
Control I	37	60～100 (m 83.1)	37	18.8±0.6	37	16	0	0	18	0	3	0
Experimental I	23	70～130 (m 105.4)	21	15.7±1.4	21	1	5	1	11	0	2	1
Control II	32	60～100 (m 82.4)	32	19.9±0.6	32	14	0	0	15	1	2	0
Experimental II	59	70～110 (m 88.5)	48	14.2±1.2	48	0	4	1	40	2	1	0

♂R, Male with rudimentary testes m, Mean

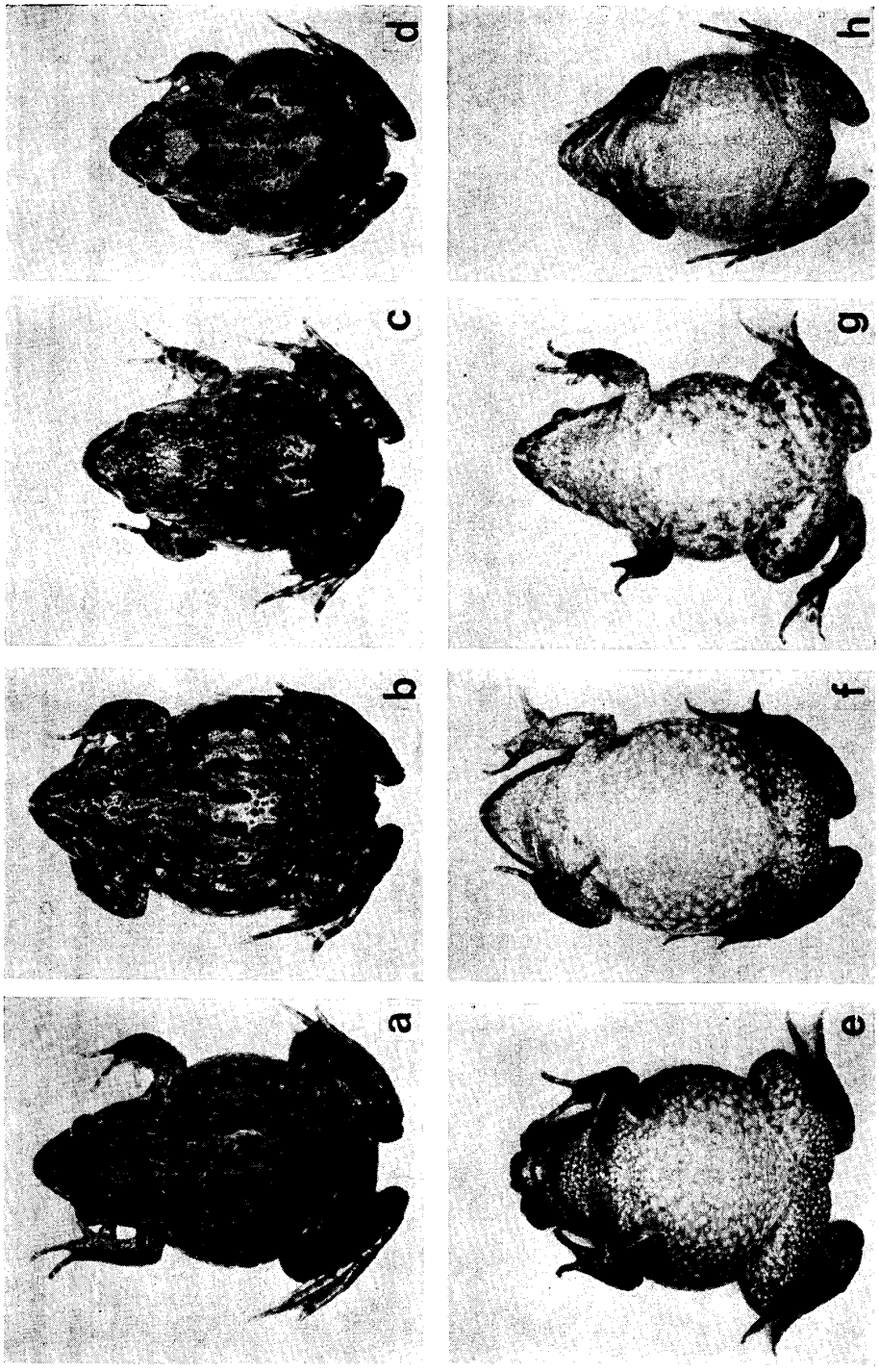

Fig. 2. Mature haploid frogs and a control diploid. ×0.9

a, e. Dorsal and ventral views of a diploid frog, Dipl. I, No. 36, 290 days after metamorphosis
b, f. Dorsal and ventral views of a haploid frog, Hapl. I, No. 21, 284 days after metamorphosis
c, g. Dorsal and ventral views of a haploid frog, Hapl. I, No. 22, 279 days after metamorphosis
d, h. Dorsal and ventral views of a haploid frog, Hapl. II, No. 52, 274 days after metamorphosis

diploids during the critical period of metamorphosis. Of the haploid tadpoles which could climb land, 69 completed metamorphosis in contrast with that none of the diploids died during this period. The tails of the diploid tadpoles were completely absorbed approximately 4 or 5 days after the forelimbs protruded. In contrast, absorption of the tails of the haploids was delayed a few days. Immediately after metamorphosis, the haploid frogs were distinctly smaller in mean body length than the control diploids (Table 2). The majority of the haploids

failed to feed after the completion of metamorphosis and died within one month. Eventually, only 6 haploids survived for two months after metamorphosis. Of these frogs, Hapl. II, No. 50 and Hapl. II, No. 51, died at the age of 152 days and 157 days, respectively. The remaining four were living in the breeding season of the following year (Fig. 2). While Hapl. I, No. 20 was underdeveloped, being 21.5 mm in body length, and died at the age of one year, the other three, Hapl. I, No. 21, Hapl. I, No. 22 and Hapl. II, No. 52, ate well and grew almost normally; they were 36~45 mm body length at the age of one year. While two of them were slightly smaller than the control diploids, the other was slightly larger than the latter (Table 3).

TABLE 3
Measurements of mature haploid frogs and the control diploids

Individual no.	Ploidy	Body length (mm)	Head length (mm)	Head width (mm)	Diameter of eye (mm)	Interval of eyes (mm)	Diameter of tympanic membrane (mm)	Hind limb length (mm)
Dipl. I, No. 35	2n	44	11	17	R 5	4	R 3	R 68
					L 5		L 4	L 68
Dipl. I, No. 36	2n	43	11	17	R 5	4	R 4	R 68
					L 5		L 4	L 66
Dipl. II, No. 31	2n	43	11	15	R 5	4	R 4	R 68
					L 5		L 4	L 68
Hapl. I, No. 21	n	45	11	17	R 5	5	R 5	R 59
					L 5		L 4	L 56
Hapl. I, No. 22	n	40	10	16	R 5	4	R 4	R 58
					L 4		L 4	L 57
Hapl. II, No. 52	n	36	10	15	R 4	3	R 4	R 50
					L 4		L 4	L 50

R, right L, left

IV. External characters

Nearly all the haploid frogs were dwarfs and of stocky body shortly after metamorphosis. The snouts were more roundish than those of the controls and the hind limbs were abnormally short in proportion to body length. In a considerable number of haploid frogs, the dorsal skin was blackish, and the dark spots on the back were indistinguishable. The dorsal skin had a reduced number of short longitudinal ridgers.

A few haploid frogs were slightly edematous. The more normal the haploids were in appearance, the longer they could generally survive. Actually, the haploid frogs that could mature were scarcely distinguishable from the control diploids in appearance, except that their hind limbs were abnormally smaller as compared with those of the control diploids.

V. Internal characters

The kidneys of 63 haploid frogs which died within one month after metamor-

phosis were compared with those of the control diploids. They were normal in shape, although they were small in proportion to the body size. The cross sections of nephric tubules were grossly normal. However, their walls consisted of a larger number of smaller cells than those of the controls. The nuclei of epithelial cells were distinctly small and roundish; each nucleus had a single nucleolus. The renal corpuscles in the haploids were normal in number and arrangement, but somewhat larger than those of the controls. The size of the

TABLE 4

Size of the nuclei of epithelial cells of nephric tubules in the kidneys of haploid frogs and the control diploids. Measurements were made on 200 nuclei in each individual

Individual no.	Days after climbing land	Ploidy	Mean diameter (μ)	Mean volume (μ^3)
Dipl. I, Nos. 1~5	4~35	2n	9.1±0.1	755.5±18.2
Hapl. I, Nos. 1~5	1~35	n	7.6±0.1	442.6±13.7
Dipl. II, No. 30	60	2n	8.8±0.1	685.7±19.6
Hapl. II, No. 50	60	n	6.5±0.1	309.0±16.7
Hapl. II, No. 51	65	n	6.7±0.1	283.4±11.7
Hapl. I, No. 20	290	n	6.4±0.1	260.5± 7.5

TABLE 5

Size of nuclei of six kinds of cells from three mature haploid frogs and the control diploids. Measurements were made on 200 nuclei in each kind of cells

Kind of cells	Indiv. no. / Item	Dipl. I, Nos. 35~37 Dipl. II, Nos. 31~32 (2n)	Hapl. I, No. 21 (n)	Hapl. I, No. 22 (n)	Hapl. II, No. 52 (n)
Epidermal	D	8.5± 0.1	7.0± 0.1	7.3± 0.1	7.3± 0.1
cells	V	637.9±25.4	350.1±13.5	388.2±12.5	401.9±15.2
	R	1	0.55	0.61	0.63
Epithelial	D	8.2± 0.1	7.1± 0.1	7.7± 0.1	7.3± 0.1
cells of	V	545.0±14.2	376.6±19.5	457.9±16.0	389.5±12.3
duodenum	R	1	0.69	0.84	0.71
Acinar	D	7.6± 0.1	6.3± 0.1	6.8± 0.1	6.6± 0.1
cells of	V	430.5±15.4	255.0± 9.5	314.6± 7.8	288.7± 8.4
pancreas	R	1	0.59	0.73	0.67
Hepatic	D	7.6± 0.1	6.6± 0.1	6.9± 0.1	7.0± 0.1
cells of	V	445.2±12.4	287.6± 8.2	337.7± 8.7	344.6± 8.9
liver	R	1	0.65	0.76	0.77
Epithelial	D	8.6± 0.1	7.4± 0.1	7.0± 0.1	6.4± 0.1
cells of	V	626.1±20.9	421.7±17.1	350.9±12.0	276.3±10.3
mesonephric tubules	R	1	0.67	0.56	0.44
Epithelial	D	7.5± 0.1	6.7± 0.1	7.0± 0.1	7.1± 0.1
cells of	V	426.8±12.9	306.7±14.1	345.0± 7.1	376.1± 7.5
oviduct	R	1	0.72	0.81	0.88

D, Mean diameter measured in μ V, Mean volume measured in μ^3

R, Ratio of mean volume of haploid nuclei to mean volume of diploid nuclei

nuclei of epithelial cells of nephric tubules from 8 haploids and 6 control diploids is shown in Table 4. Two haploid frogs, Hapl. II, No. 50 and Hapl. II, No. 51, that died two months after metamorphosis were dwarfs and had correspondingly small kidneys. These kidneys were very similar in internal structure to those of haploid frogs immediately after metamorphosis. Another haploid frog, Hapl. I, No. 20, that was reared for ten months after metamorphosis, was also noticeably smaller than the control diploids. The kidney of this haploid showed a conspicuous reduction in the number of nephric tubules and renal capsules.

Eight kinds of organs of the three sexually mature haploid frogs were microscopically examined and compared with those of five control diploids. The mature haploids were compared with the control diploids in nuclear size of six kinds of cells in six of those eight organs (Table 5). The nuclei of the mature haploids were definitely smaller than those of the control diploids. However, the haploid: diploid ratios of nuclei remarkably varied between 0.44: 1.00 and 0.88: 1.00 in the six kinds of cells.

a. Skin. The skin generally seemed to be thicker than that of the control diploids (Fig. 3a, b). While there was no apparent difference in the thickness of the outermost layer (stratum corneum) between the haploids and the diploids, the epidermis proper was definitely thicker and consisted of a larger number of cell layers than that of the diploids. The epidermal papillae were not so conspicuous as those of the diploids. The nuclei of epidermal cells in the three mature haploids were about half the size of those in the five control diploids (Table 5). The two kinds of cutaneous glands, mucous and poisonous, in the haploids were larger and more numerous. This seemed to indicate that these cutaneous glands of the haploids were more active in secretory function.

b. Duodenum. The duodenum of each haploid frog was normal in appearance except its reduced size. The villi of the duodenum were well developed and similar in shape to those of the controls, while the epithelial cells were distinctly smaller and more numerous than those of the latter (Fig. 3c, d). The glands of BRUNNER seemed to be normal in size and shape.

c. Pancreas. The pancreas of each haploid frog was approximately normal in size. The pancreatic ducts were slightly slender, although they consisted of more numerous cells than the controls. The acinar cells were smaller and more numerous than those of the controls (Fig. 3e, f).

d. Liver. The liver of each haploid frogs was nearly normal in size, although the hepatic ducts were comparatively slender. The liver and hepatic ducts consisted of smaller cells which seemed to be twice in number as those of the controls (Fig. 4a, b).

e. Spleen. The spleen of the three haploids was normal in position and appearance except that it was smaller than that of the control diploids. It was also similar in inner structure to that of the controls. However, the cells found in the white and red pulps were generally smaller than those of the controls.

f. Lung. The lungs of the haploid frogs were largely swollen and their walls were conspicuously simple in structure. The lining cells of alveoli were smaller

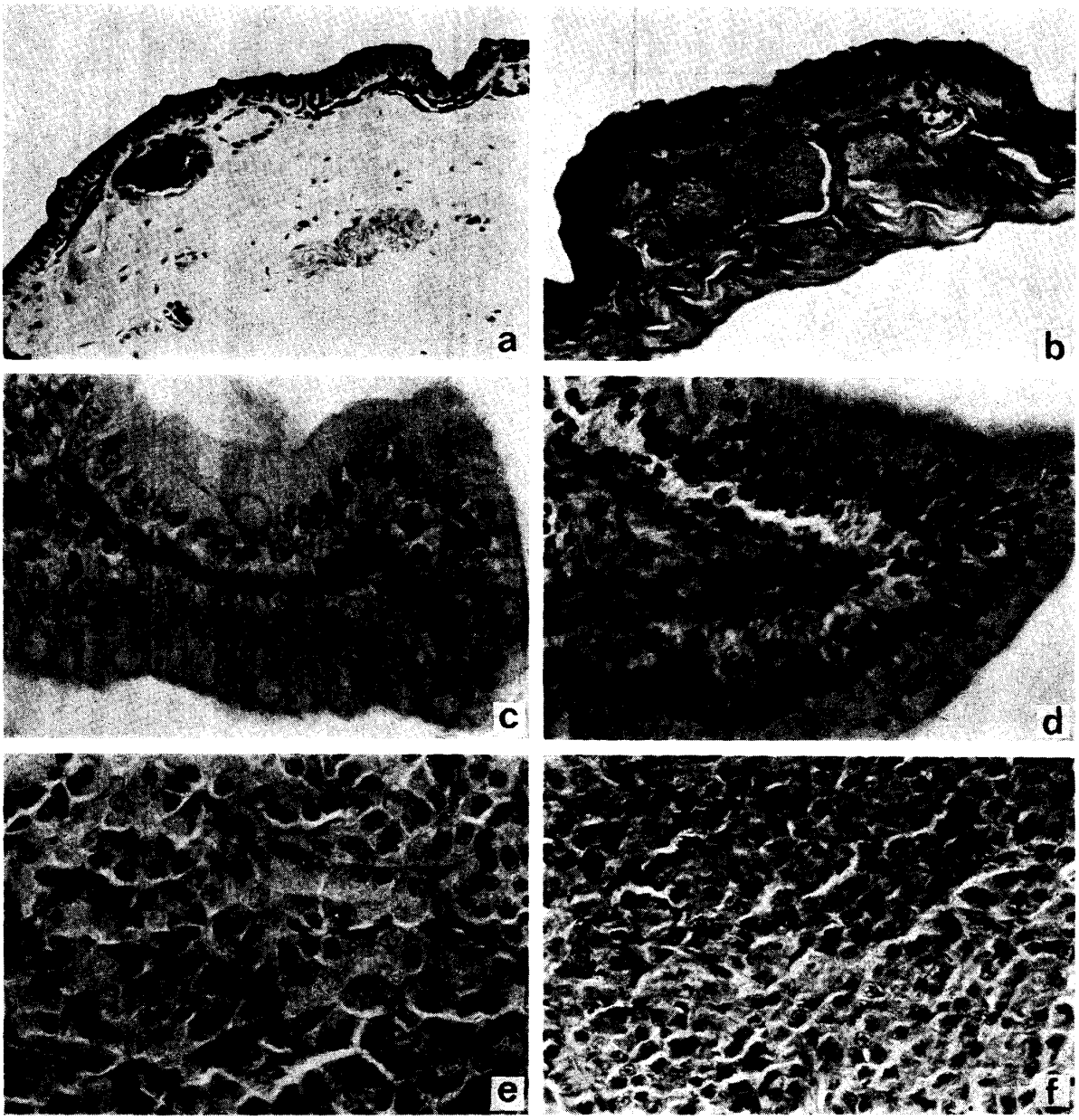

Fig. 3. Cross sections of three kinds of organs of a mature haploid frog, Hapl. I, No. 21 and a control diploid, Dipl. I, No. 35. Photographs on the left side are of the diploid. Those on the right side are of the haploid.

a, b. Skin ×200 c, d. Villus of duodenum ×780
e, f. Pancreas ×780

and more numerous than those of the controls.

g. Kidney. The kidneys of the three haploid frogs were somewhat larger than those of the control diploids for their body size (Table 6). However, the three mature haploids considerably differed from one another in internal structure of the kidneys. The nephric tubules of the haploid frogs, Hapl. I, No. 21, were remarkably swollen, although they were nearly the same in number as those of the controls. The epithelial cells of nephric tubules were distinctly smaller and

TABLE 6
Measurements of the kidneys of mature haploid frogs and the control diploids

Individual no.	Size of kidneys (mm)		Thickness of the walls of nephric tubules (μ)	Renal capsules	
				Number	Diameter of the largest capsule (μ)
Dipl. I, No. 35	R	9×3	15.0~22.5	13	122.5
	L	10×3	12.5~20.0	15	150.0
Dipl. II, No. 31	R	9×2	15.0~22.5	13	112.5
	L	10×2	15.0~22.5	12	157.5
Hapl. I, No. 21	R	11×3	15.0~22.5	18	157.5
	L	11×3	15.0~20.0	25	222.5
Hapl. I, No. 22	R	9×3	12.5~20.0	42	187.5
	L	9×3	12.5~20.0	30	230.0
Hapl. II, No. 52	R	10×3	15.0~20.0	18	237.5
	L	10×3	12.5~20.0	15	250.0

R, right L, left

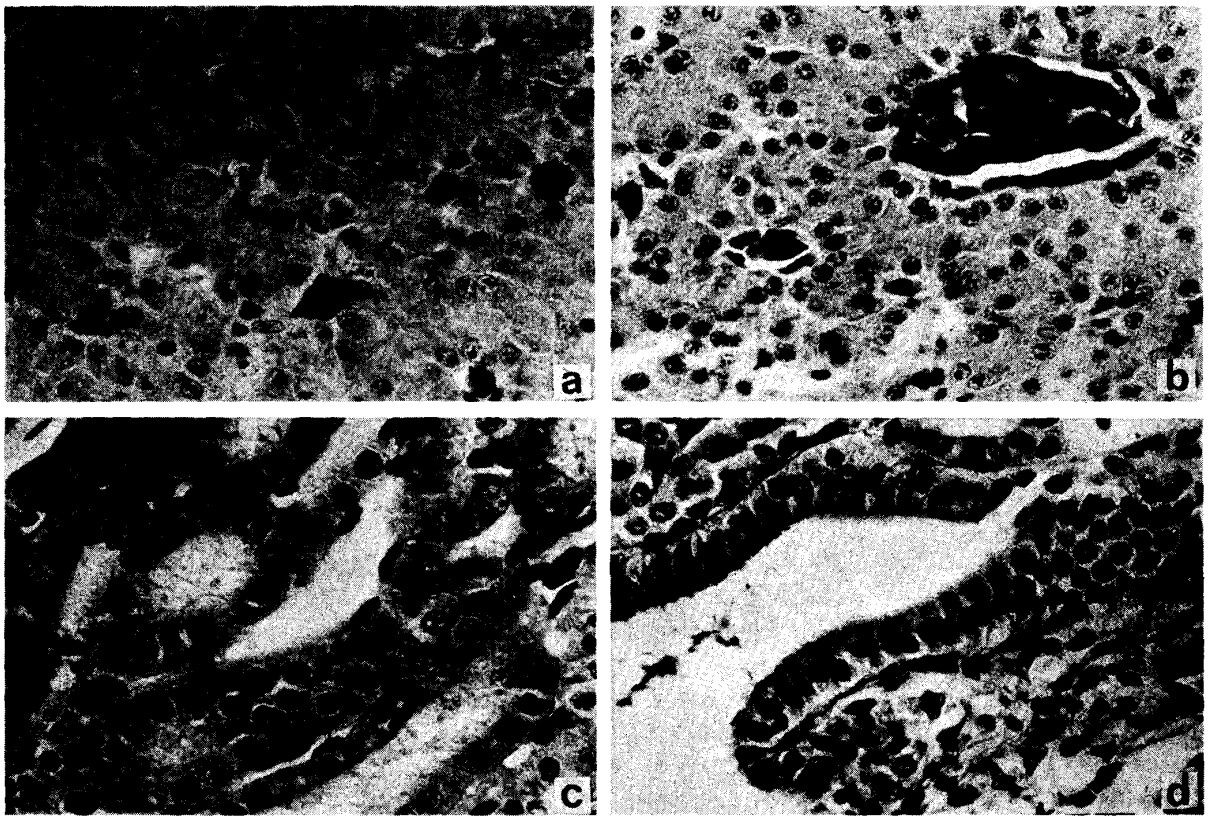

Fig. 4. Cross sections of two kinds of organs of a mature haploid frog, Hapl. I, No. 21 and a control diploid, Dipl. I, No. 35. Photographs on the left side are of the diploid. Those on the right side are of the haploid. ×780
a, b. Liver c, d. Kidney

more numerous (Fig. 4c, d). The renal corpuscles were remarkably larger and more numerous than those of the controls and irregular in arrangement. In the haploid animal, Hapl. I, No. 22, the nephric tubules were nearly normal in number and appearance. The renal corpuscles were abnormally large and

TABLE 7

Size of the gonads of haploid frogs and the control diploids

Series	No.	Days after climbing land	Sex	Body length (mm)	Size of gonads (mm)			
					Right		Left	
					Length	Width	Length	Width
Control I	16	4～35	♀	16.5～19.0	2.5～4.0	1.0～2.0	3.0～4.0	1.0～2.0
	18		♂	17.0～20.0	0.5～1.0	0.5～1.0	1.0～1.5	0.5～1.0
Experimental I	1	1～35	♀	15.0	2.0	1.0	3.0	1.0
	5		♀	14.0～18.0	0.5～2.5	0.5～1.0	0.5～2.0	0.5～1.0
	1		♂R	12.0	0.5	0.5	0.5	0.5
	11		♂	14.0～17.0	0.5～1.0	0.5～1.0	0.5～1.0	0.5～1.0
	1	290	♀	21.5	3.0	2.0	3.0	2.0
Control II	14	4～35	♀	17.0～21.0	3.0～3.5	1.5～2.5	3.0～4.0	1.5～2.0
	15		♂	17.5～22.0	1.0～1.5	0.5～1.0	1.0～1.5	0.5～1.0
	1	60	♂	24.5	2.5	1.0	2.0	1.0
Experimental II	4	1～35	♀	11.5～14.0	1.0～1.5	0.5～1.0	1.0～1.5	0.5～1.0
	1		♂R	15.5	0.5	0.5	0.5	0.5
	39		♂	11.5～19.0	0.5～1.0	0.5～1.0	0.5～1.0	0.5～1.0
	2	60～65	♂	15.0～16.0	0.5～1.0	0.5～1.0	0.5～1.0	0.5～1.0

♂R, Male with rudimentary testes

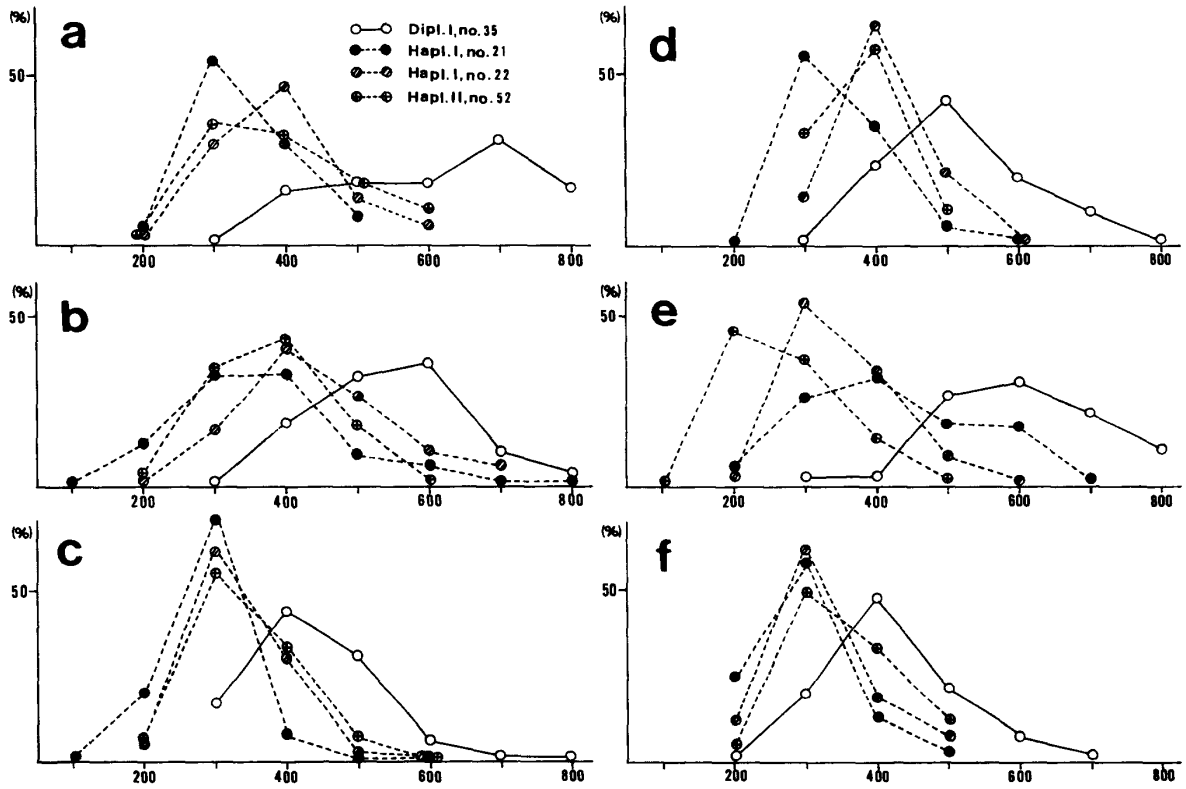

Fig. 5. Distribution of the volumes of nuclei of six kinds of cells from three mature haploid frogs and a control diploid.

a. Epidermal cells
b. Epithelial cells of the duodenum
c. Hepatic cells of the liver
d. Acinar cells of the pancreas
e. Epithelial cells of mesonephric tubules
f. Epithelial cells of the oviduct

irregular in arrangement. In the kidneys of the haploid, Hapl. II, No. 52, the renal corpuscles were extraordinary large as compared with those of the other two haploid frogs. They were equal in number to those of the controls.

h. Oviduct. The oviducts of the haploid frogs were normal in size and appearance. The walls of oviducts were also normal in thickness. However, the epithelial cells were somewhat smaller and more numerous than those of the controls.

The distribution of the nuclear volume of six kinds of cells from the three mature haploids and a control is shown in Figure 5. The peaks of the curves of the three haploids were shifted toward the left in each kind of cells in comparison to those of the control. The most distinctive shifts found in epidermal cells and epithelial cells of mesonephric tubules are most likely explained by the differences in size of nuclei between the haploids and the control. The peaks of the curves of the three haploids were similar in position to one another.

VI. Sex differentiation

Of 69 control diploid frogs produced in the present study, 35 were females with normal ovaries and 34 were males with normal testes. There were no hermaphrodites. In contrast with this, there were four females with normal ovaries, ten hermaphrodites, two males with rudimentary testes and 53 males with normal testes among 69 haploid frogs, as presented in Table 2. The ten hermaphrodites were transforming from females to males. While there were one female, nine hermaphrodites and 51 males among 63 juveniles which died within one month after metamorphosis, three frogs which died two months after metamorphosis were males. Of the remaining four frogs which were killed ten months after metamorphosis, only one was a hermaphrodite and the other three were females. These three females were sexually mature haploids.

When the three mature haploids were excluded, all the haploid frogs were smaller than those of the control diploids in proportion to the body length (Table 7). The single haploid female which died shortly after metamorphosis had extremely small ovaries in contrast with the ovaries of the control diploids. However, they contained a comparatively small number of growing oocytes which did not differ in size from those of the controls (Fig. 6a, b). These oocytes were 120 μ in maximum diameter. The testes of haploid males which died within one month after metamorphosis somewhat differed from male to male in size and differentiation. Although the testes were generally smaller than those of the controls, they were nearly the same as the latter in internal structure (Fig. 6, c, d). The inner part of each testis consisted of radially arranged rete cells and primary spermatogonia. Each spermatogonium was surrounded by rete cells and isolated from the others. In the testes of some males, the formation of seminiferous tubules was observed. The testes of two males were rudimentary; they were very small and had only a few spermatogonia (Fig. 6e). The nine hermaphrodites which died within one month after metamorphosis were in the early stage of sex transformation (Fig. 6f). While their gonads were basically ovaries and contained

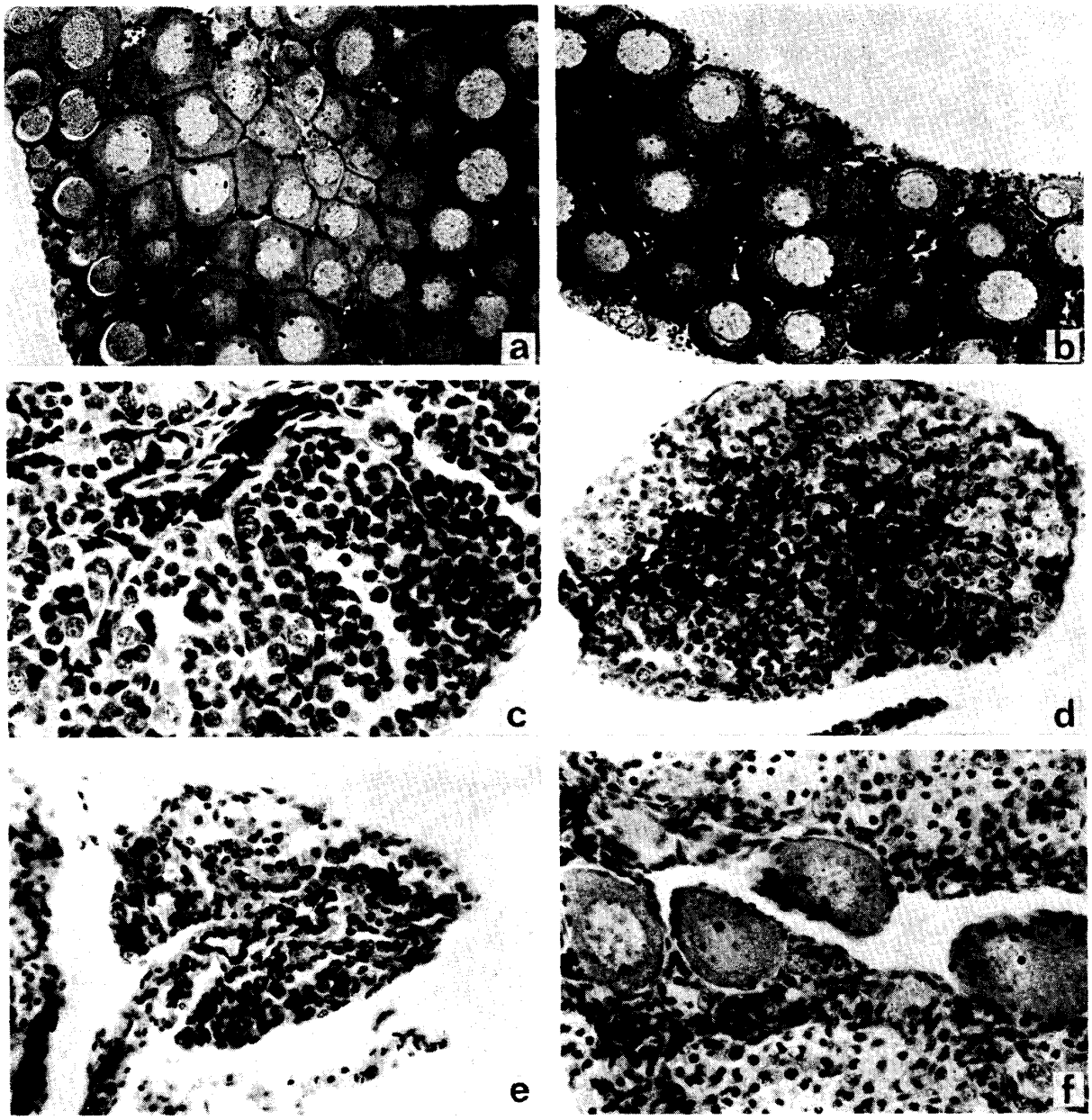

Fig. 6. Cross sections of the gonads of haploid frogs which died within one month after metamorphosis and the control diploids. ×400

a. Ovary of a control diploid female
b. Ovary of a haploid female
c. Testis of a control diploid male
d. Testis of a haploid male
e. Rudimentary testis of a haploid male
f. Gonad of a hermaphrodite in the early stage of sex transformation

a number of growing oocytes, there were small masses of increased rete cells along the wall of ovarian cavities. A small number of germ cells were already surrounded by these rete cells. The testes of the two frogs, Hapl. II, Nos. 50 and 51, which died about two months after metamorphosis were nearly the same in differentiation as those of the control diploids which were killed one month after metamorphosis, that is, formation of sperm was not yet begun. In contrast, the

control diploid males of the same age already had some sperm in the testes. These findings showed that the differentiation of testes in the haploid males was definitely retarded as compared with that in the control diploids. The gonads of the single hermaphrodites, Hapl. I, No. 20, which died ten months after metamorphosis were very small and appeared to be ovaries at a glance, as there were a considerable number of growing oocytes. However, the walls of ovarian cavities were remarkably hypertrophied and there were masses of increased number of rete cells surrounding the germ cells.

VII. Reproductive capacity

Two of the three mature haploid females laid eggs. In one female, Hapl. II, No. 52, ovulation occurred spontaneously without amplexus. Although the exact time of spawning was unknown, a total of 302 eggs were laid. This number of eggs corresponded to about one-third of the eggs laid by a normal diploid female. The appearance of the eggs showed that the spawning took place several hours before.

TABLE 8
Developmental capacity of eggs from a haploid female

Parents		Size of eggs (mm)	No. of eggs	No. of cleaved eggs		No. of uncleaved eggs	No. of blastulae at st. 9	
Female	Male			Normal	Abnormal		Normal	Abnormal
Dipl. I, No. 35	Untreated	1.1±0.1	248	198 (79.8%)	1 (0.4%)	49 (19.8%)	163 (65.7%)	35 (14.1%)
	UV-irradiated		203	188 (92.6%)	0	15 (7.4%)	158 (77.8%)	30 (14.8%)
Hapl. I, No. 21	Untreated	1.1±0.1	267	233 (87.3%)	18 (6.7%)	16 (6.0%)	0	233 (87.3%)
	UV-irradiated		207	79 (38.2%)	47 (22.7%)	81 (39.1%)	0	79 (38.2%)

The other haploid female, Hapl. I, No. 21, which was the largest in body length, was injected with pituitary suspension of *Rana catesbeiana* to accelerate ovulation (Fig. 7). As a result, this haploid female laid 474 eggs, while a control diploid female laid 451 eggs. These eggs were indistinguishable in appearance from those of the control female. They were light brown and 1.1 mm in mean diameter. The jelly membranes surrounding the eggs were normal in nature and amount. The eggs were divided into two groups. One was inseminated with untreated sperm of a normal male collected from the field, while the other was inseminated with UV-irradiated sperm from the same male.

The remaining mature haploid female, Hapl. I, No. 22, could not be induced to ovulate by pituitary injection and died after a short time.

The eggs laid spontaneously by the haploid female, Hapl. II, No. 52, failed to develop by insemination with sperm of a normal male. This was probably attributable to a change that had occurred in the eggs after spawning.

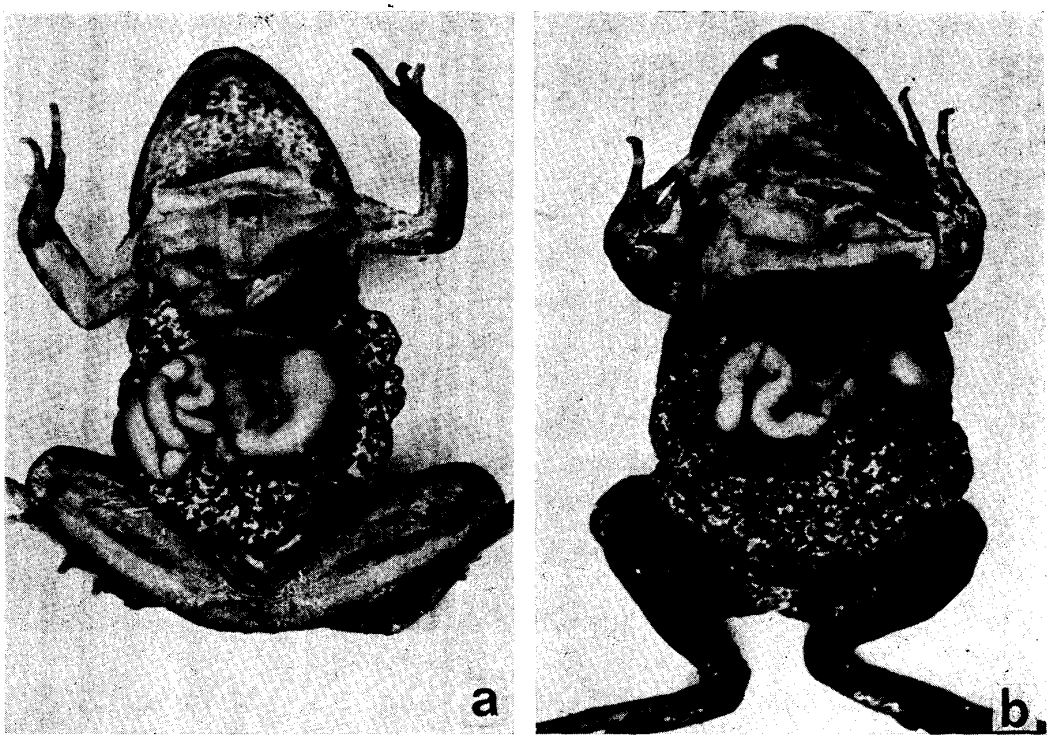

Fig. 7. Ovaries of a haploid frog, Hapl. I, No. 21, and a control diploid, Dipl. I, No. 35, after ovulation occurred by pituitary injection. ×1.5

a. Diploid frog b. Haploid frog

The developmental capacity of the eggs obtained from the other haploid female, Hapl. I, No. 21, is presented in Table 8. Eggs inseminated with untreated sperm from a diploid male began to cleave normally. At the 4-cell stage 233 (87.3%) out of 267 eggs were normal and 18 (6.7%) cleaved abnormally, while 198 (79.8%) out of 248 eggs from a control diploid female were normal, and only one egg cleaved abnormally. On the other hand, after insemination with irradiated sperm, 79 (38.2%) out of 207 eggs obtained from the haploid female cleaved normally and 47 (22.7%) cleaved abnormally, while 188 (92.6%) out of 203 eggs obtained from the control diploid female cleaved normally and there were no abnormally cleaved eggs. The normally cleaved eggs of the haploid female subsequently become blastulae. However, all of them were arrested simultaneously at the late blastula stage and died. In contrast, the eggs from the control diploid female mostly developed normally.

DISCUSSION

1. The production of haploid frogs

Various methods have been used in producing haploids in amphibians. The stimulation of unfertilized *Rana* eggs by pricking with a needle results in production of parthenogenetically developed haploids (PARMENTER, 1933; KAWA-

MURA, 1939a, b). Gynogenetic haploids are produced by inseminating *Rana* eggs with sperm treated with trypaflavin or toluidine blue (G. HERTWIG, 1924; BRIGGS, 1952; MIYADA, 1960; NISHIOKA and KONDO, 1978). Androgenetic haploids are obtained from eggs whose nuclei were damaged or removed, by insemination with normal sperm (G. HERTWIG, 1911; KAYLOR, 1940; GURDON, 1960). Elimination of the egg nucleus together with a fragment of the egg immediately after insemination permits production of an androgenetic haploid (FANKHAUSER, 1937).

According to P. HERTWIG (1923), haploid embryos in urodeles usually attain more advanced stages in development than those in anurans. It seemed likely that a similar difference in development exists between different species of newts (FANKHAUSER, 1937; KAYLOR, 1940). MIYADA (1960, 1977) produced some haploids which were at the most advanced stages among those obtained by many other investigators. He has described that about 500 out of about 10,000 gynogenetic haploid embryos obtained from 87 *Rana nigromaculata* and *R. brevipoda* females developed into swimming tadpoles, and only nine completed metamorphosis (MIYADA, 1960). Of about 100 viable haploid tadpoles obtained by his later experiments, seven completed metamorphosis and only one of them became three years old, although it was an immature dwarf male (MIYADA, 1977).

In the present study, 1,530 (94.9%) normal neurulae raised from 1,612 eggs inseminated with UV-irradiated sperm seemed to be haploids with a few exceptional individuals on the basis of the haploid syndrome at the hatching stage as well as the chromosome number counted in 121 normally shaped tadpoles. All these eggs were obtained from only two female *Rana rugosa*. Moreover, 69 tadpoles completed metamorphosis and three of them sexually matured. Two of them laid eggs. Thus, *Rana rugosa* seems to be a peculiar species, in which haploids develop until the mature stage. These results furthermore show that *Rana rugosa* is an extraordinarily excellent animal for the study of haploidy in amphibians. It seems to be evident that haploid frogs raised from *Rana rugosa* eggs by insemination with UV-irradiated sperm will greatly contribute in the future to the progress of various fields in amphibian biology.

Various hypotheses have been proposed to account for the cause of abnormalities and poor viability of haploid embryos. MIYADA (1960) has supported DARLINGTON's hypothesis (1937) that recessive lethal factors or genes are expressed in haploids and has noted that viable haploids are derived from special females which are almost free from such factors or genes. In the present study, it is also very probable that the three mature haploids were raised from eggs having no lethal factors or genes. At the same time, it seems reasonable to consider that *Rana rugosa* have comparatively few lethal or semilethal factors or genes, as the overwhelming majority of haploid embryos which were raised from eggs that had been inseminated with UV-irradiated sperm became normally shaped tadpoles.

2. Size of haploid cells and nuclei

It is evident that the size of nuclei or cells in amphibians varies in proportion

to the number of chromosome sets (FANKHAUSER, 1952). Several investigators compared the volume of nuclei in haploids with that in diploids. In this case, the nuclear volumes were usually estimated from measurements of diameters of nuclei. The haploid: diploid ratio was generally found to be close to the expected 0.5: 1.0 ratio in the cells of various organs (O. HERTWIG, 1913; G. HERTWIG, 1913, 1918, 1927). However, G. HERTWIG (1927) obtained a ratio of 0.31: 1.00 for nuclei of the cartilage of *Triturus vulgaris*. A similar ratio of 1.00: 3.24 was given by MUTO (1952, 1957) in nerve cells of *Bufo bufo formosus*. On the other hand, O. HERTWIG (1913) and BÖÖK (1941) found a higher ratio of 0.60: 1.00 in medulla cells of larvae of *Triturus vulgaris*. In various kinds of cells of metamorphosed frogs, MIYADA (1960) found the ratios varying between 1.00: 1.67 and 1.00: 2.74.

In the haploid *Rana rugosa* which completed metamorphosis or attained sexual maturity, the nuclei were definitely smaller than those in the diploid controls. The haploid: diploid ratios of nuclei varied between 0.44: 1.00 and 0.88: 1.00 in various kinds of cells. The actual cause for such a great range of the haploid: diploid ratios may be related to the difference in shape between haploid and diploid cells in various kinds of organs and tissues. Although the haploid: diploid ratios were estimated on the assumption that all the nuclei were spheres, they were always more or less irregular in shape. The degrees of divergence from the presumed sphere differed greatly with the kinds of organs and tissues. A reduction in size of nuclei with age of individuals was observed by MUTO (1952) in various kinds of diploid and triploid cells of *Bufo bufo formosus* tadpoles and by MIYADA (1960) in various kinds of haploid and diploid cells of *Rana nigromaculata* and *R. brevipoda*. In the present study, the same was observed in size of the nuclei of epithelial cells of nephric tubules in the kidneys of haploid and diploid frogs of *Rana rugosa* (Table 4).

3. Body size of haploid frogs

Haploids are usually smaller than diploids in amphibians. O. HERTWIG (1913) described that in *Triturus vulgaris* the bodies of haploid larvae were smaller than diploids. A similar observation was made by BÖÖK (1941) in a larva of the same species. FANKHAUSER (1938) reported that a haploid newt of this species which died immediately after metamorphosis was a dwarf. PORTER (1939) reported in *Rana pipiens* that various organs of haploid tadpoles were smaller than those of the control diploids in proportion to their body size except the notochord. This embryonic organ was nearly the same in dimensions of cross sections as that in the controls. MIYADA (1960) found that the organs of haploid frogs of *Rana nigromaculata* and *R. brevipoda* were mostly of normal size in proportion to the body length with the exception of the gonads, thyroid glands and retinae.

The majority of haploid frogs of *Rana rugosa* at the stage of metamorphosis were smaller than the control diploids. Above all, the hind limbs were characteristically shorter. However, three mature haploids reached the normal size except that the hind limbs were definitely shorter than those of the control diploids.

Various kinds of organs considerably varied in size. The duodenum and spleen were smaller than those of the controls. In contrast, the liver, pancreas and oviducts were approximately normal in size. The normal size of these organs in the haploid frogs seemed to be adjusted by an increase in number of cells. The kidneys of the mature frogs were somewhat larger than those of the control diploids for the body size. Moreover, they were not normal in inner structure. This seemed to indicate that these haploid frogs were not always normal in some metabolic function.

4. Differentiation of the gonads of haploid frogs

GALLIEN (1967) described meiosis in the testes of haploid *Pleurodeles waltlii*, joined in parabiosis with diploids of the same species. When the testes of a haploid newt were the best in development, the meiosis could proceed up to the anaphase of the first division, although bivalents were not formed, and spermatogenesis failed to proceed any further. When a haploid male received an intense effect from the diploid female partner, the testis was feminized into ovotestis and produced oocytes.

In the present study, two mature haploid females laid eggs which were normal in appearance. Moreover, eggs obtained from one of the haploid females cleaved normally by insemination with sperm of a normal male and became normal blastulae. However, all of them died simultaneously at the late blastula stage. The inviability of these blastulae may be attributable to disturbance by the chromosomes derived from abnormal meiosis in the oocytes. It was noteworthy that there were no mature haploid males in spite of the abundance of juvenile haploid males. All these males died within one month after metamorphosis. At the same stage, there was only one female among 69 haploids. As *Rana rugosa* is believed to be of male heterogamety on the basis of the sex of gynogenetic diploids produced by the present author (cf. KAWAMURA and NISHIOKA, 1977), all the haploid frogs produced gynogenetically were certain to have one X chromosome, that is, to be genetic females. Thus, it is very probable that the haploid males are sex-reversed genetic females. The fact that there were nine hermaphrodites and two males with rudimentary testes among the haploid juveniles in contrast with the finding of the controls firmly supports this assumption. The cause for sex reversal in the haploid frogs seems to be abnormal metabolism that is attributable to the haploidy. When injurious factors or genes born in the haploid set of chromosomes are powerful, the haploids will die sooner or later. When they are not so powerful and permit the haploids to reach the metamorphosis stage with difficulty, sex reversal will occur in nearly all the individuals. When the injurious factors or genes are so feeble that the haploids can attain the sexually mature stage, the genetic sex will be realized. However, it does not seem impossible to obtain mature haploid males in future, because sex-reversed genetic females can attain sexual maturity without difficulty in diploid *Rana*.

SUMMARY

1. Sexually mature haploid frogs were produced from *Rana rugosa* eggs by insemination with UV-irradiated sperm.

2. Of 1,612 eggs inseminated with UV-irradiated sperm, 135 became normally shaped tadpoles at TAYLOR and KALLROS' stages V~X. Examination of the chromosomes in the tail-tips of 121 out of these tadpoles revealed that 118 were haploids, one was diploid and two were haploid-diploid mosaics. Of the haploid tadpoles, 82 climbed land 10 to 30 days later than the control diploids did, and 69 completed metamorphosis. Among 63 haploid frogs which died within one month after metamorphosis, there were 51 males with normal testes, two males with rudimentary ovaries, nine hermaphrodites and one female. Two haploids which died two months after metamorphosis were dwarf males with underdeveloped testes, while one haploid which died at the age of one year was a dwarf hermaphrodite.

3. The remaining three haploids were one-year-old females. While two of them were slightly smaller than the control diploids, the other was slightly larger than the latter. These three females were normal in appearance except that their hind limbs were abnormally shorter. They had slight abnormalities in structure of various organs and tissues, especially of kidneys.

4. The nuclei of cells in various organs and tissues were definitely smaller than those of the control diploids. The smallness of cells was compensated by a larger number of the cells.

5. While one of the three mature haploid females laid eggs spontaneously, ovulation of another female was accelerated by pituitary injection. The eggs of the females were normal in appearance. Those obtained after pituitary injection cleaved normally and became blastulae. However, all of them died at the late blastula stage.

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