Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ. 12: 37–53 (1993)

Artificially Produced Chimeras between Haploids and Diploids in Japanese Anurans

By

Keiko KASHIWAGI and Akihiko KASHIWAGI

Laboratory for Amphibian Biology, Faculty of Science, Hiroshima University, Higashihiroshima 724, Japan

ABSTRACT

An investigation was undertaken to test the validity of the hypothesis that edema is the major cause of the low viability of haploid embryos and tadpoles. Chimeras were produced by exchanging the anterior and posterior parts of haploid embryos (H) with those of diploid embryos (D) by joining them after cutting transversely at the site immediately before and behind the pronephros (p).

It was found that severe edema usually found in haploid embryos at the early stage is remarkably diminished in the Dp·H and H·pD chimeras which have diploid pronephroi in the anterior and posterior diploid halves, respectively. Especially, the Dp·H chimeras which consist of anterior diploid halves with pronephroi and posterior haploid halves are comparatively good in development. Of the Dp·H chimeras, 18 (9.7%) in *Rana nigromaculata* and 15 (10%) in *Rana japonica* attained the stage immediately before or after the completion of metamorphosis.

INTRODUCTION

Many reports have been published on the development, viability, external and internal characters and sex of artificially produced haploids in amphibians (PARMENTER, 1933; KAWAMURA, 1939; PORTER, 1939; HUMPHREY and FANKHAUSER, 1957; MIYADA, 1960, 1977; NISHIOKA and KONDO, 1978; KASHIWAGI, 1980; KAWAMURA and NISHIOKA, 1981; NISHIOKA, OKUMOTO and KONDO, 1981 and others). All these authors have shown that haploids are greatly reduced in viability when compared with diploids. The mortality at the embryonic and tadpole stages is extremely high. The characteristic abnormalities found in haploids are edema, microcephaly, abortive development of the gills, ill-development of the heart and gut, *etc.* It is well-known that edematous swelling of the body appears sooner or later in the great majority of haploids. DALCQ (1932) has reported that malfunction of the excretory system of haploids results in edema which in turn may be severe enough to cause their death.

The present author made longitudinal chimeras which consisted of haploid and diploid halves joined at the site shortly before or behind the pronephros. Such chimeras would elucidate the relationship between the defective kidney and the reduced viability in haploids, and also the developmental ability and sex dif-

ferentiation of haploids, if the chimeras could survive longer.

MATERIALS AND METHODS

Adult frogs of *Rana nigromaculata* HOLLOWELL and *Rana japonica* GÜNTHER used as material in the present study were collected from the suburbs of Hiroshima City.

Ovulation was induced by injection of crushed pituitaries of *Rana nigromaculata* into the body cavity. Haploid embryos were produced by pseudofertilization of eggs with UV-irradiated spermatozoa (NISHIOKA, OKUMOTO and KONDO, 1981; NISHIOKA and TANAKA, 1981). Chimeras were made between haploid and diploid embryos. The operation was carried out in a petri dish whose bottom was covered with soft paraffin. Embryos at SHUMWAY's stage 17 were divided into halves across the middle of the body in HOLTFRETER's solution containing 50,000 units of penicillin and 5 mg of streptomycin per liter in addition to 0.1% globulin. Anterior and posterior halves were brought into contact in a small hollow made on the paraffin bed and held tightly together by building a paraffin bank around the half embryos in such a manner that they were not distorted with excessive pressure. As the wound was healed from two to three hours after operation, the

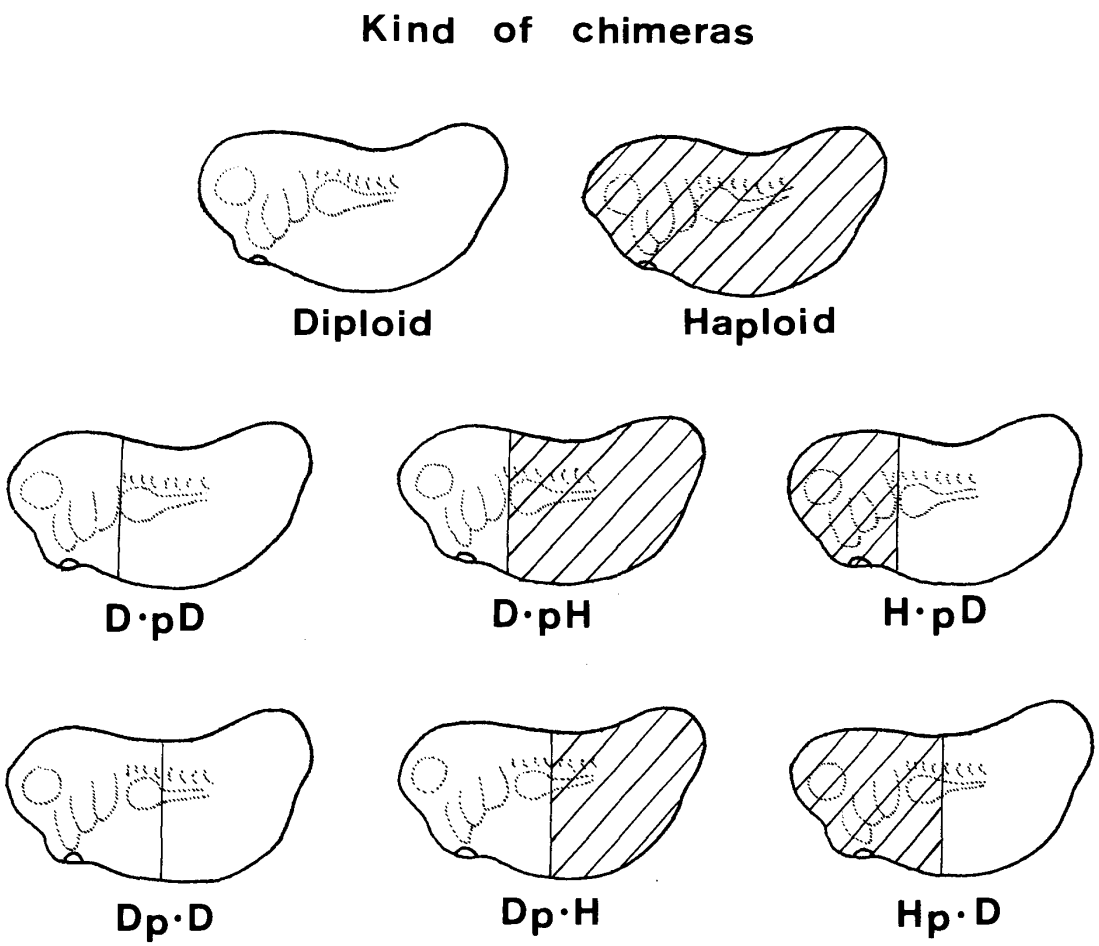

Fig. 1. Diagrams showing the four kinds of chimeras and the controls. Vertical line shows the site of adhesion between diploid (white) and haploid (oblique lines) halves.

chimeras were then transferred to 50% HOLTFRETER's solution and kept there for 24 hours. Thereafter, they were returned to Cl-free tap water. Four kinds of chimeric combinations are illustrated in Fig. 1. The chromosome number of tadpoles was counted in tail-tips by the water-pretreatment squash method of NISHIOKA (1972). The kidneys and gonads of metamorphosed frogs were fixed in NAVASHIN's fluid shortly after death, embedded in paraffin, sectioned at 12 μ, and stained with HEIDENHAIN's hematoxylin.

OBSERVATION

I. Production of chimeras and their development

The production and developmental capacity of chimeras are shown in Figs. 2 and 3. The success rate in the operations was 61.4% in *Rana nigromaculata* and 73.0% in *Rana japonica*. Incompatibility was not observed in the contact zone between the anterior and posterior halves. The kind of chimeras is shown by the following abbreviations.

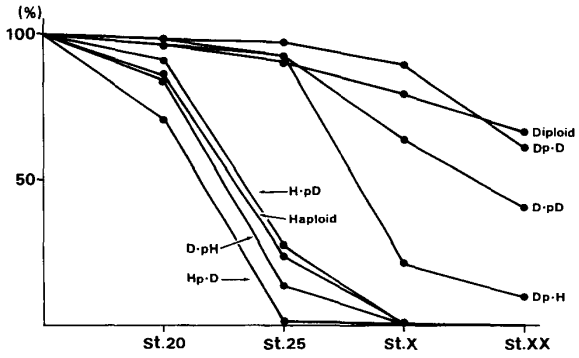

Fig. 2. Survival rates of the four kinds of chimeras, haploids, diploids and the two kinds of controls in *Rana nigromaculata*. The axis of ordinates indicates the percentages of survivals. The abscissa shows the stage of development according to SHUMWAY, 1940, and TAYLOR and KOLLROS, 1946.

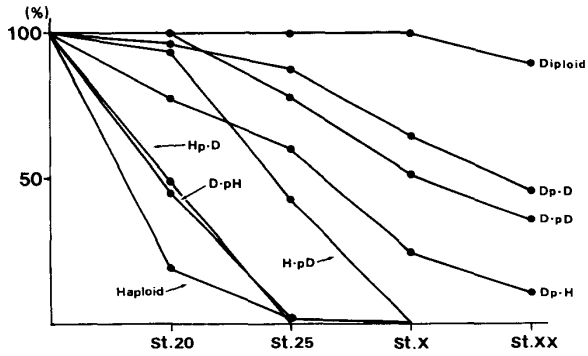

Fig. 3. Survival rates of the four kinds of chimeras, haploids, diploids and the two kinds of controls in *Rana japonica*.

H·pD: Adhesion of an anterior haploid half to a posterior diploid half after dividing haploid and diploid embryos into halves transversely at the site immediately before the pronephros.

D·pH: Adhesion of an anterior diploid half to a posterior haploid half after dividing haploid and diploid embryos into halves transversely at the site immediately before the pronephros.

Hp·D: Adhesion of an anterior haploid half to a posterior diploid half after dividing haploid and diploid embryos into halves transversely at the site immediately behind the pronephros.

Dp·H: Adhesion of an anterior diploid half to a posterior haploid half after

dividing haploid and diploid embryos into halves transversely at the site immediately behind the pronephros.

1. Haploids

Most of the haploid tadpoles died between gill circulation and completion of the operculum. They exhibited haploid syndrome, such as ill-development of gills, manifestation of edema, lordosis, microcephaly and abnormal development of the heart and gut. Of the 413 normally cleaved eggs, 99 (24.0%) hatched normally and became haploid tadpoles in *Rana nigromaculata*, while seven (2.0%) of the 357 normally cleaved eggs hatched normally and became haploid tadpoles in *Rana japonica*.

2. D·pH and H·pD chimeras

The development of the H·pD embryos was slightly better at the early stages than that of the D·pH embryos. Most of the H·pD embryos were normal at the hatching stage. At the beginning stage of feeding activity, they were divided into two groups; the first group made normal development, while the second group displayed haploid syndrome. Of the 87 H·pD chimeras in *Rana nigromaculata* and of the 193 H·pD chimeras in *Rana japonica*, 24 (27.6%) and 82 (42.5%) were normal at stage 25, respectively. However, the surviving H·pD chimeras became progressively feeble with advancing age and all died within one month after operation. In contrast to the H·pD chimeras, nine (13.8%) of the 65 D·pH chimeras in *Rana nigromaculata* and three (1.7%) of the 173 D·pH chimeras in *Rana japonica* reached stage 25, but died before stage X. While the capillary circulation in the gills of the D·pH embryos was normal, their tails became abnormal and showed haploid syndrome.

3. Dp·H and Hp·D chimeras

The Dp·H chimeras developed best among the four kind of chimeras. They were almost normal in appearance except for short tails. Of the 185 Dp·H chimeras in *Rana nigromaculata* and of the 150 Dp·H chimeras in *Rana japonica*, 171(92.4%) and 90(60.0%) developed into normally swimming tadpoles, respectively. In contrast, all but one of the 82 Hp·D chimeras in *Rana nigromaculata* and all the 144 Hp·D chimeras in *Rana japonica* died of edema or cytolysis before stage 25.

Of the Dp·H chimeras, only 18 in *Rana nigromaculata* and 15 in *Rana japonica* survived during the tadpole stage and attained the time of metamorphosis. The days required by the Dp·H chimeras to complete metamorphosis after the operation were somewhat longer than those required by the normal diploids. This tendency was particularly prominent in *Rana japonica*. Many of the Dp·H chimeras became edematous at the metamorphosing stage. Six of the 18 Dp·H chimeras in *Rana nigromaculata* became edematous after protrusion of the forelimbs. Only three Dp·H chimeras of this species attained the stage immediately before the completion of metamorphosis. In *Rana japonica*, the forelimbs appeared in 15

Dp·H chimeras, of which five became edematous, while five other chimeras developed beyond metamorphosis. Three of these metamorphosed frogs survived approximately two months after completion of metamorphosis. Forty of the Dp·D controls of *Rana nigromaculata* and 52 of the Dp·D controls of *Rana japonica* survived beyond metamorphosis. In each kind of chimeras, the haploid and diploid parts showed their own characters in developmental rate and external characters, and did not show any intermediate feature due to interaction between the two parts.

The ploidy of the posterior parts of the Dp·H chimeras was determined by counting chromosomes in their tail-tips clipped within one month after operation. Table 1 shows the results of counting chromosomes in the chimeras and the controls. The chromosome numbers of both $2n=26$ and $n=13$ were confirmed in the diploid and haploid parts of the Dp·H chimeras, respectivety. As an exceptional case, a single Dp·H chimera contained only one diploid mitosis among many haploid ones in the haploid part.

TABLE 1

Numbers of haploid and diploid mitoses in the tail-tips of Dp·H chimeras and haploid and diploid tadpoles at stage V in *R. nigromaculata* and *R. japonica*

Species	Kind	Female no.	No. of tadpoles	No. of mitoses		
				Total	Haploid	Diploid
R. nigromaculata	Dp·H	N_1	4	36	36	0
		N_2	4	17	17	0
		N_3	6	39	39	0
		N_4	13	73	73	0
		N_5	7	37	37	0
	Diploid	N_5	5	19	0	19
	Haploid	N_5	2	10	10	0
R. japonica	Dp·H	J_1	3	36	36	0
		J_2	18	71	70	1
		J_3	14	92	92	0
	Diploid	J_3	5	11	0	11

II. Characters of metamorphosed chimeras

1. External features

Metamorphosed chimeras are shown in Figs. 4 and 5. They were Dp·H chimeras in *Rana nigromaculata* or *Rana japonica*. All of them were considerably small and weak. The posterior halves of their bodies were especially underdeveloped. Measurements were made on various body parts of the chimeras and controls (Table 2; Figs. 4, 5). While the anterior part of each chimera was diploid on the basis of the measurements of the head width, head length and the diameter of the eyes, the hindlimbs seemed to be haploid. The hindlimbs of the

TABLE 2

Measurements of haploid-diploid chimeras (Dp·H), normal diploids (D) and the control diploids (Dp·D) at the stage immediately before or after completion of metamorphosis

Kind	No. of frogs	Body length mm	Head length mm	Head width mm	Eye diameter mm	Hindlimb length mm	Hindlimb length / Body length
N_1Dp·H	3	13.8~14.0 (13.9)	5.8~6.0 (5.9)	6.1~6.9 (6.5)	2.0~2.5 (2.3)	11.0~11.2 (11.1)	0.80
N_2Dp·H	3	12.8~14.4 (13.7)	5.2~6.2 (5.8)	6.1~7.0 (6.5)	2.0~2.5 (2.2)	10.4~11.4 (10.9)	0.79
N_3Dp·H	1	14.0	5.2	6.0	2.0	9.1	0.65
N_4Dp·H	4	13.8~14.0 (13.9)	5.2~7.1 (6.2)	6.1~6.9 (6.5)	2.8~3.0 (2.9)	10.0~11.7 (10.9)	0.78
N_5Dp·H	7	14.1~18.5 (16.3)	5.8~7.0 (6.2)	7.0~7.8 (7.4)	2.0~3.0 (2.8)	10.9~14.6 (12.4)	0.77
N_5D	20	16.0~19.1 (18.8)	6.8~7.5 (7.2)	7.0~8.0 (7.4)	2.2~3.2 (2.7)	21.2~21.8 (21.6)	1.15
N_5Dp·D	20	16.7~18.0 (17.4)	6.2~8.0 (7.1)	7.0~8.0 (7.5)	2.8~3.0 (2.9)	16.9~17.1 (17.0)	0.98
J_1Dp·H	3	10.9~12.2 (11.5)	4.3~5.5 (4.9)	5.1~6.0 (5.6)	2.0~2.1 (2.0)	7.0~11.2 (9.1)	0.77
J_2Dp·H	4	8.9~12.6 (10.6)	3.4~5.0 (4.5)	4.5~6.2 (5.2)	1.8~2.2 (2.0)	4.2~ 9.0 (6.9)	0.65
J_3Dp·H	8	10.0~12.1 (11.2)	4.3~5.8 (4.7)	5.0~6.0 (5.5)	1.8~2.1 (2.0)	4.1~10.1 (9.3)	0.82
J_3D	20	12.2~16.0 (14.6)	6.0~6.6 (6.2)	5.6~6.8 (6.2)	2.0~2.2 (2.1)	10.1~17.8 (13.8)	0.94
J_3Dp·D	20	12.0~14.2 (13.8)	5.0~6.8 (5.9)	5.5~6.5 (6.0)	1.9~2.5 (2.3)	11.2~14.5 (13.6)	0.99

N, *Rana nigromaculata* Parentheses show the mean.
J, *Rana japonica*

Dp·H chimeras were shorter than those of the Dp·D controls. The ratios of the hindlimb length to the body length were 0.65~0.82 in the Dp·H chimeras, while they were 0.98 and 0.99 in the Dp·D controls. The color and pattern of the body in the Dp·H chimeras were very similar to those of the Dp·D controls.

2. Cellular constitution of the mesonephros

The mesonephroi of the Dp·H chimeras were presumed to consist of haploid tissues, as these organs are established in the posterior half of the body. Actually, these organs were extremely small and underdeveloped. Most of the Dp·H chimeras were evidently edematous and had abnormal mesonephros. Therefore, there seemed to be a close correlation between the abnormal mesonephros and the production of edema. Tables 3 and 4 show a comparison in the number of cells and the nuclear size of the mesonephros between haploid and diploid areas. These two areas were readily distinguished from each other. In an area of 110 μm$\times$310 μm, 0~4.4% and 0~7.2% of the total number of cells in the haploid

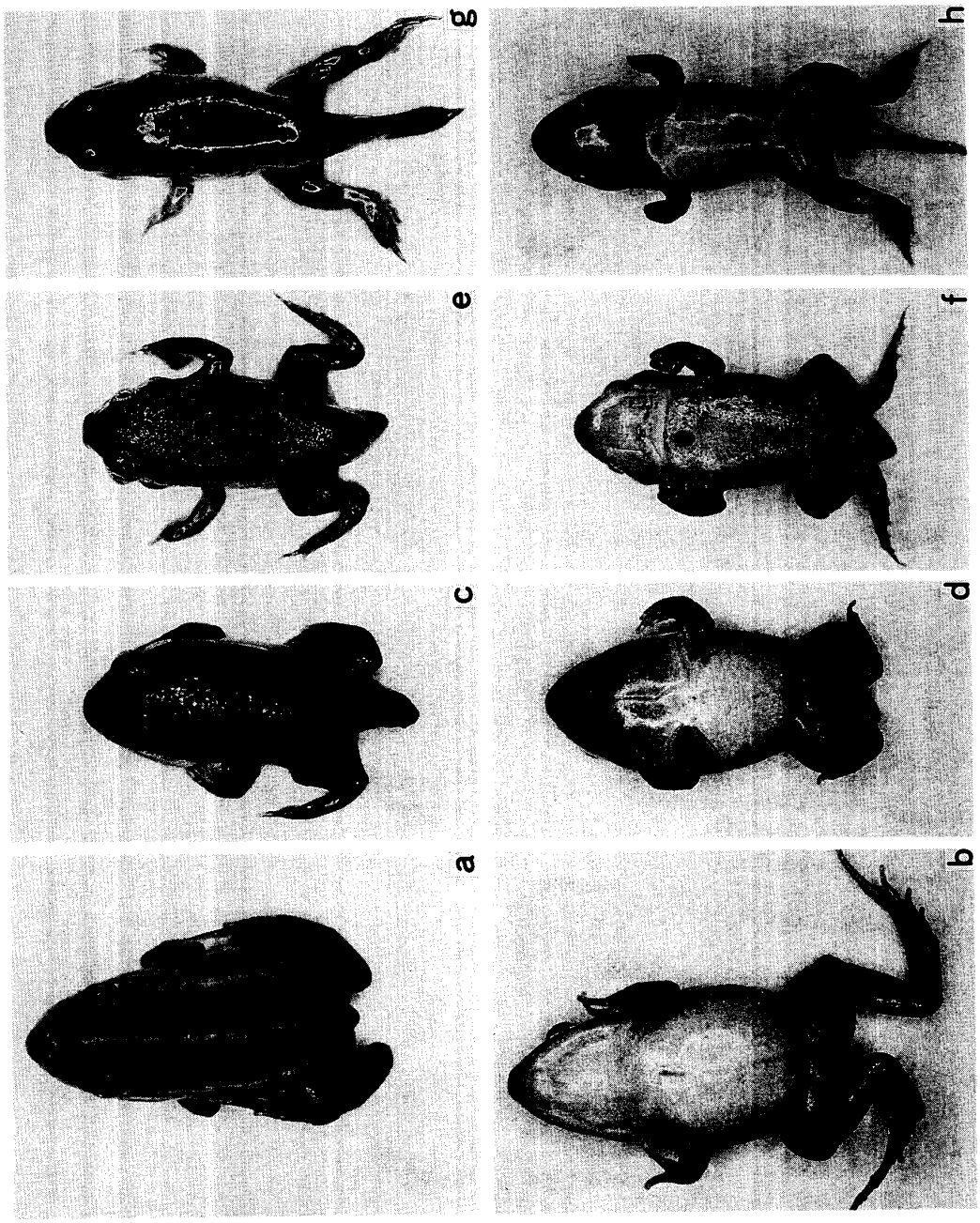

Fig. 4. Dorsal and ventral views of three haploid-diploid chimeras at the stage shortly before completion of metamorphosis and the control diploid juvenile in *Rana nigromaculata*. ×2.3
a, b. A normal diploid juvenile, N_5D, No. 1 e, f. Haploid-diploid chimera, $N_1Dp{\cdot}H$, No. 2
c, d. Haploid-diploid chimera, $N_4Dp{\cdot}H$, No. 11 g, h. Haploid-diploid chimera, $N_5Dp{\cdot}H$, No. 18

areas of the Dp·H chimeras of *Rana nigromaculata* and *Rana japonica* were diploid cells, respectively. The diameters of 90 nuclei of mesonephros were measured in the Dp·H chimeras and the Dp·D controls by making use of a camera lucida. Each nucleus was assumed to be a sphere for convenience' sake. The diameter of each nucleus was shown by the mean of the longest and shortest diameters. It was found that the volume of nuclei of the Dp·H chimeras in *Rana nigromaculata* was 220.75 μm^3 on the average, while that of the Dp·D controls was 344.19 μm^3. The Dp·H/Dp·D ratio in volume of nuclei was 0.64 : 1. In *Rana japonica*, the volume of nuclei of the Dp·H chimeras was 211.89 μm^3 on the average, while that of the Dp·D controls was 341.24 μm^3. The Dp·H/Dp·D ratio in volume of nuclei was 0.62 : 1. While only large and elliptical erythrocytes were found in the

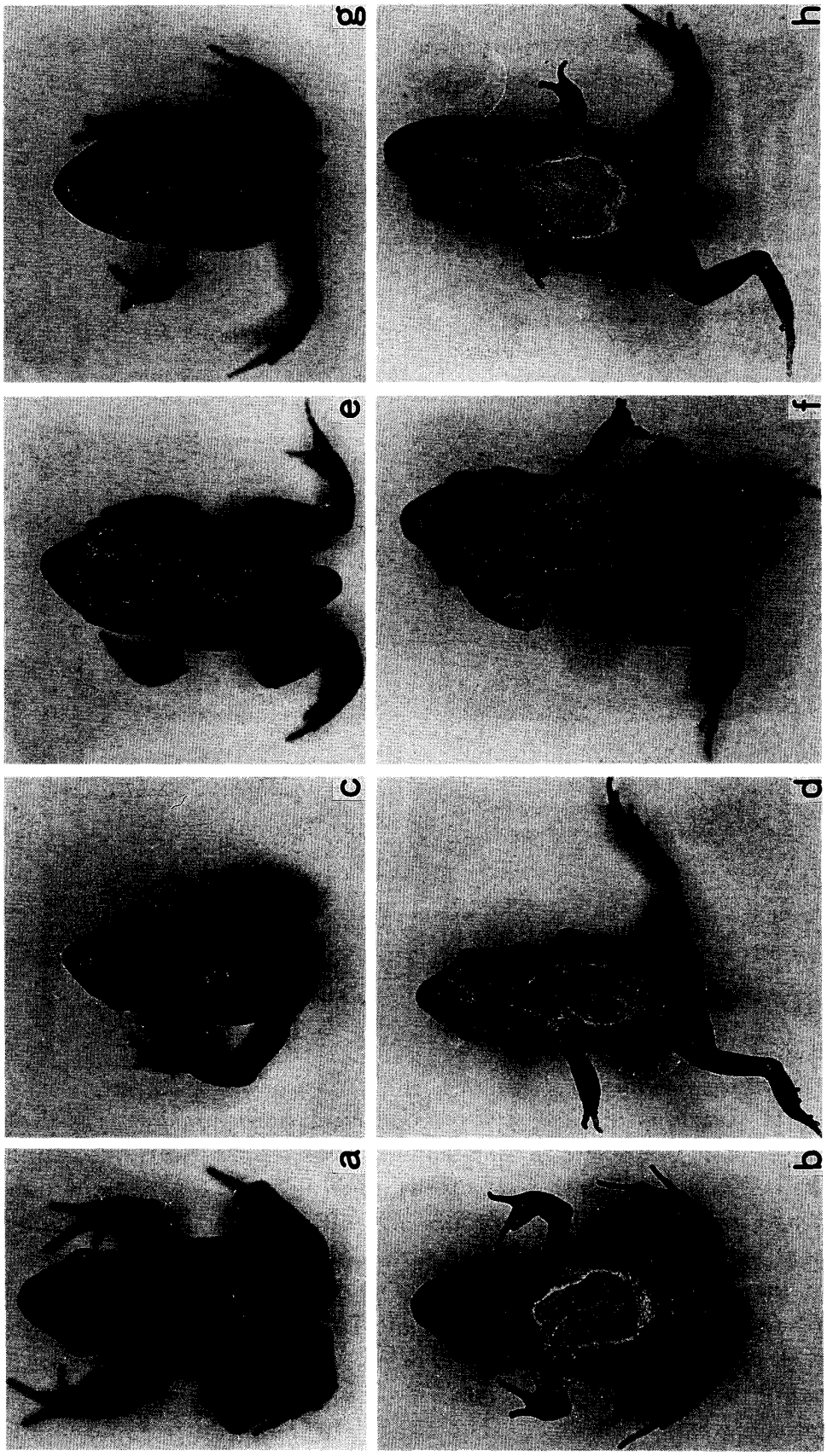

Fig. 5. Dorsal and ventral views of three haploid-diploid chimeras at the stage shortly before or after completion of metamorphosis and the contorl diploid juvenile in *Rana japonica*. ×3.2

a, b. A normal diploid juvenile, J_3D, No. 1
c, d. Haploid-diploid chimera, $J_1Dp{\cdot}H$, No. 3
e, f. Haploid-diploid chimera, $J_3Dp{\cdot}H$, No. 13
g, h. Haploid-diploid chimera, $J_3Dp{\cdot}H$, No. 12

TABLE 3

Number and nuclear volume of the mesonephric cells in haploid-diploid chimeras and the controls of *Rana nigromaculata* at the stage immediately before or after completion of metamorphosis

Individual number		Number of all the cells in an area, 110 μ×310 μ	Number of diploid cells in an area, 110 μ×310 μ	Mean nuclear volume of all the mesonephric cells
N_1Dp·H,	No. 1	125	0	208.65±4.80
	No. 2	91	4(4.4%)	237.06±6.39
	No. 3	112	0	256.07±6.40
N_2Dp·H,	No. 4	95	1(1.1%)	252.19±6.40
	No. 5	100	1(1.0%)	246.44±6.90
	No. 6	114	3(2.6%)	224.33±6.43
N_3Dp·H,	No. 7	106	0	212.07±5.79
N_4Dp·H,	No. 8	100	4(4.0%)	184.16±3.73
	No. 9	121	0	205.26±4.42 *(0.64)
	No. 10	75	3(4.0%)	215.52±4.75
	No. 11	87	1(1.1%)	195.33±3.86
N_5Dp·H,	No. 12	85	3(3.5%)	219.02±4.85
	No. 13	131	4(3.1%)	261.96±8.00
	No. 14	126	0	181.05±3.31
	No. 15	57	2(3.5%)	205.26±3.78
	No. 16	120	0	248.35±5.35
	No. 17	116	4(3.4%)	205.26±4.24
	No. 18	109	1(0.9%)	215.52±4.79
Dp·D,	No. 1	87	87(100%)	356.62±8.45
	No. 2	107	107(100%)	359.07±9.09
	No. 3	104	104(100%)	316.88±8.59

Parentheses show the rate of diploid cells to all the mesonephric cells. Parentheses with an asterisk show the rate of the mean nuclear volume of the mesonephric cells in 18 Dp·H chimeras to that of the nuclei in three control Dp·D frogs.

mesonephros of the Dp·D controls, small and spherical erythrocytes were observed together with large and elliptical ones in the Dp·H chimeras. It was evident that haploid cells were mingled with diploid cells in the mesonephros of Dp·H chimeras.

3. Gonads

The external forms and inner structures of the gonads of metamorphosed chimeras were examined. The results are shown in Tables 5 and 6. Among 29 metamorphosed frogs of *Rana nigromaculata* in the control series, there were 16 males with normal testes (♂N), four juvenile hermaphrodites (⚥) and nine females with normal ovaries (♀N). Of the 28 Dp·D controls at the stage shortly before the completion of metamorphosis, 18 were normal males (♂N) and 10 were normal females (♀N). In the experimental series, the 18 Dp·H chimeras included five males, four females with underdeveloped ovaries, five females with degenerating or degenerated ovaries, three juvenile hermaphrodites and one chimera with

TABLE 4

Number and nuclear volume of the mesonephric cells in haploid-diploid chimeras and the controls of *Rana japonica* at the stage immediately before or after completion of metamorphosis

Individual number		Number of all the cells in an area, 110 μ×310 μ	Number of diploid cells in an area, 110 μ×310 μ	Mean nuclear volume of all the mesonephric cells
J_1Dp·H,	No. 1	154	0	213.79±5.88
	No. 2	118	0	174.93±3.30
	No. 3	135	0	170.43±2.72
J_2Dp·H,	No. 4	83	6(7.2%)	258.02±4.88
	No. 5	115	0	170.43±3.79
	No. 6	114	3(2.6%)	220.78±4.80
	No. 7	127	2(1.6%)	182.60±3.31
J_3Dp·H,	No. 8	102	4(3.9%)	224.33±4.94 *(0.62)
	No. 9	67	0	203.59±4.35
	No. 10	74	0	248.35±6.99
	No. 11	107	0	190.49±4.23
	No. 12	121	0	212.07±5.90
	No. 13	64	4(6.3%)	248.35±7.01
	No. 14	104	1(1.0%)	200.26±4.75
	No. 15	91	5(5.5%)	260.00±7.43
Dp·D,	No. 1	135	135(100%)	307.97±8.49
	No. 2	60	60(100%)	394.37±8.57
	No. 3	66	66(100%)	321.39±7.92

Parentheses show the rate of diploid cells to all the mesonephric cells. Parentheses with an asterisk show the rate of the mean nuclear volume of the mesonephric cells in 15 Dp·H chimeras to that of the nuclei in three control Dp·D frogs.

indifferent gonads (Table 5). Of the five males, one (N_4Dp·H, No. 10) had testes which were remarkably small but normal in inner structure. The other four males had testes which were nearly the same as those of the control frogs in size but contained extremely small spermatogonia (Fig. 6e). In the four females with underdeveloped ovaries, the auxocytes were very scarce, while the oogonia were abundant in the cortical parts. Of the remaining five females, two (N_1Dp·H, No. 1 and N_4Dp·H, No. 8) had no oogonia in their ovaries. In one female (N_4Dp·H, No. 11), the ovaries were very slender and contained narrow ovarian cavities. There were a few oogonia, but no oocytes. In another female (N_5Dp·H, No. 12; Fig. 6c), the ovaries contained a few degenerating auxocytes and some hypertrophied follicular cells, indicating traces of degenerated auxocytes, but they contained no oogonia. In the remaining female (N_5Dp·H, No. 15), the gonads resembled testes in appearance and there were no cavities in the sections. However, as there were no hypertrophied rete cells, the gonads of this frog were considered to be degenerated ovaries.

Of the three juvenile hermaphrodites, one (N_1Dp·H, No. 2; Fig. 6d) had evident ovarian cavities. There were no auxocytes, but some of the oogonia were

TABLE 5

Size and structure of the gonads of haploid-diploid chimeras and the control and normal individuals in *Rana nigromaculata* at the stage shortly before completion of metamorphosis

Individual number		Age (days)	Sex	Size of gonads (mm)	
				Right	Left
N_1Dp·H,	No. 1	94	♀D	0.7×0.3	0.5×0.2
	No. 2	118	⚥	—	1.3×1.0
	No. 3	97	ID	1.0×0.2	1.0×0.2
N_2Dp·H,	No. 4	71	♀U	1.2×0.6	1.8×0.5
	No. 5	80	♂N	1.2×0.3	1.0×0.3
	No. 6	79	♂N	1.1×0.5	1.0×0.5
N_3Dp·H,	No. 7	74	♀U	2.1×0.4	2.3×0.4
N_4Dp·H,	No. 8	61	♀D	1.0×0.5	0.9×0.5
	No. 9	68	♀U	1.2×0.8	1.0×0.9
	No. 10	65	♂N	0.6×0.3	0.7×0.2
	No. 11	77	♀D	1.0×0.2	0.9×0.2
N_5Dp·H,	No. 12	84	♀D	1.2×0.7	1.0×0.7
	No. 13	60	♂N	0.9×0.4	1.0×0.4
	No. 14	77	⚥	1.0×0.4	1.0×0.5
	No. 15	57	♀D	1.0×1.0	1.1×1.0
	No. 16	62	♂N	1.1×0.6	1.0×0.7
	No. 17	71	♀U	2.0×0.9	2.0×0.7
	No. 18	75	⚥	1.2×0.8	2.0×1.0
Diploid (9 females)		66～95	♀N	1.0～2.8 ×0.7～1.9	1.0～3.0 ×0.5～1.3
Diploid (16 males)		66～95	♂N	0.5～1.2 ×0.3～0.7	0.8～1.1 ×0.2～0.9
Dp·D (10 females)		75～95	♀N	1.0～2.9 ×0.7～1.2	1.1～3.3 ×0.8～1.2
Dp·D (18 males)		75～95	♂N	0.8～1.2 ×0.3～0.9	1.0～1.1 ×0.3～0.8

♀N, Normal ovary
♀U, Underdeveloped ovary
♂N, Normal testis
⚥ , Hermaphrodite
♀D, Degenerating or degenerated ovary
ID , Indifferent gonad

surrounded here and there with rete cells. In another juvenile hermaphrodite (N_5Dp·H, No. 14), the gonads had well-developed cortical portions, although there were no cavities. In the cortical portions, there were no auxocytes, in spite of the presence of abundant oogonia. Some of the oogonia were surrounded by hypertrophied rete cells. The remaining juvenile hermaphrodite (N_5Dp·H, No. 18) had gonads, which had well-developed cortical portions. There were no cavities. While there were numerous oogonia, auxocytes could be found nowhere. Some oogonia were surrounded by hypertrophied rete cells here and there. There were no females having normal auxocytes in their ovaries. The auxocytes seemed to degenerate, even if they were produced.

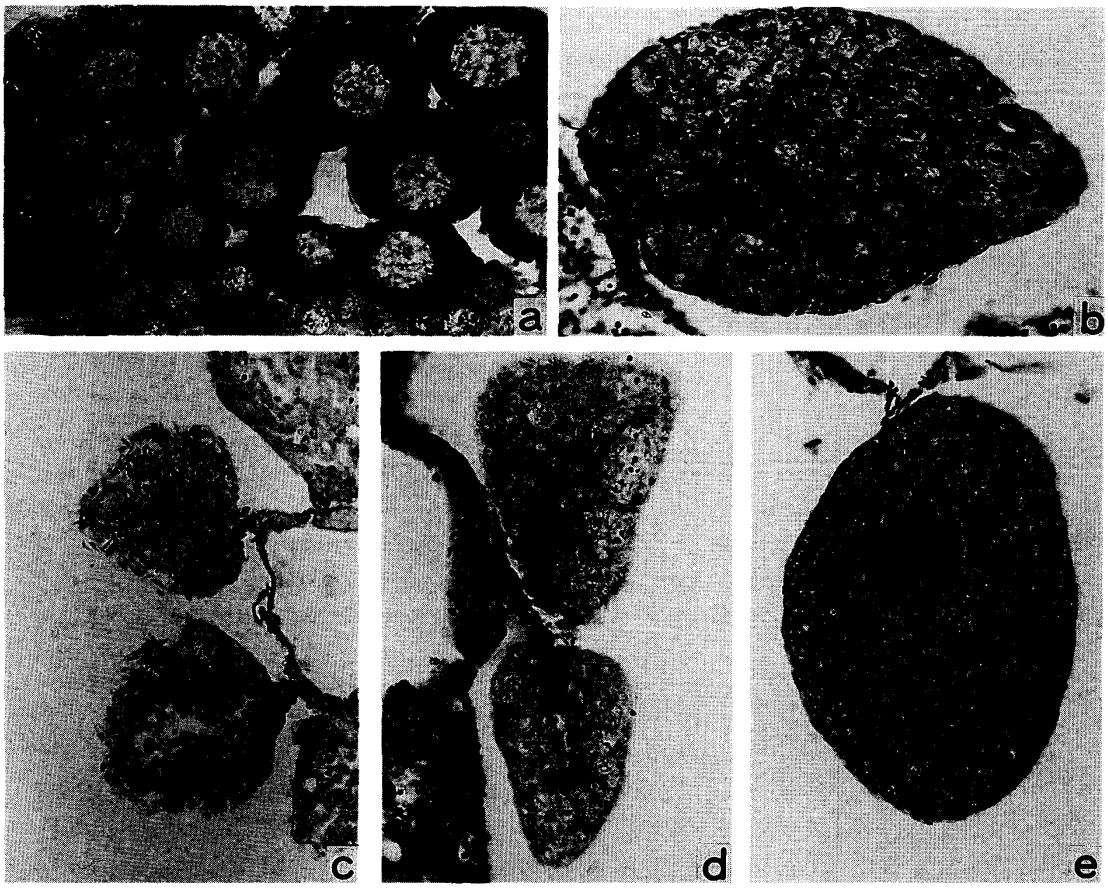

Fig. 6. Cross-sections of the gonads of haploid-diploid chimeras at the stage shortly before or after completion of metamorphosis, and the control diploids in *Rana nigromaculata*. ×180

a. Normal ovary (♀N) of the control diploid female
b. Normal testis (♂N) of the control diploid male
c. Degenerating ovary (♀D) of a haploid-diploid chimera, N_5Dp·H, No. 12
d. Hermaphroditic gonad (⚥) of a haploid-diploid chimera, N_1Dp·H, No. 2
e. Normal testis (♂N) of a haploid-diploid chimera, N_2Dp·H, No. 5

The gonads of *Rana japonica* were generally more advanced in differentiation when compared with those of *Rana nigromaculata*. There were 27 males and 34 females among the metamorphosed frogs of *Rana japonica* in the control series. Of the Dp·D controls, 25 were males and 18 were females. In contrast, eight of the 15 chimeras were nearly normal females (Fig. 7c), three were females with degenerating or degenerated ovaries, two were normal males (Fig. 7e) and the remaining two were juvenile hermaphrodites (Table 6). The ovaries of the eight females were filled with auxocytes which did not differ in size from those of the control diploid females, even if they were smaller and fewer in number of auxocytes. However, the ovaries of two (J_1Dp·H, No. 1 and J_2Dp·H, No. 6) of the eight females contained a few degenerating auxocytes. Of the three females with degenerating or degenerated ovaries, one (J_2Dp·H, No. 4) contained ovarian cavities but no oogonia. There were a few degenerating auxocytes and some traces of degenerated ones which were replaced with hypertrophied follicular cells. In another female (J_2Dp·H, No. 5), the ovaries had no cavities, while the auxo-

TABLE 6

Size and structure of the gonads of haploid-diploid chimeras and the control and normal individuals in *Rana japonica* at the stage shortly before or after completion of metamorphosis

Individual number		Age (days)	Sex	Size of gonads (mm)	
				Right	Left
J_1Dp·H,	No. 1	96	♀N	1.2×0.7	1.0×0.8
	No. 2	102	♀N	1.1×0.2	1.2×0.3
	No. 3	149	♂N	0.6×0.4	0.7×0.4
J_2Dp·H,	No. 4	86	♀D	1.0×0.5	0.9×0.5
	No. 5	115	♀D	1.0×0.4	0.7×0.4
	No. 6	99	♀N	1.2×0.6	0.9×0.5
	No. 7	90	♀N	1.0×0.6	1.2×1.0
J_3Dp·H,	No. 8	91	♀N	1.2×0.9	1.3×0.6
	No. 9	97	⚥	0.3×0.2	0.3×0.2
	No. 10	98	♀N	1.2×0.5	1.3×0.6
	No. 11	112	⚥	1.2×1.5	0.6×0.5
	No. 12	167	♀D	0.5×0.5	0.2×0.2
	No. 13	172	♂N	0.7×0.7	0.7×0.4
	No. 14	117	♀N	1.1×0.9	1.0×0.9
	No. 15	106	♀N	1.2×0.6	1.7×1.0
Diploid (34 females)		89～128	♀N	1.9～3.0 ×0.9～1.1	2.0～2.9 ×0.9～1.1
Diploid (27 males)		89～128	♂N	0.3～1.0 ×0.2～0.6	0.6～0.9 ×0.4～0.6
Dp·D (18 females)		95～130	♀N	2.0～3.2 ×0.7～1.5	2.0～3.1 ×0.9～1.3
Dp·D (25 males)		95～130	♂N	0.3～1.0 ×0.2～0.6	0.3～1.2 ×0.2～0.5

♀N, Normal ovary ♀D, Degenerating or degenerated ovary
♂N, Normal testis ⚥ , Hermaphrodite

cytes were degenerating or degenerated and replaced by hypertrophied follicular cells. The ovaries of the remaining female (J_3Dp·H, No. 12) were small and roundish for the age of two months after metamorphosis. Although they appeared to be testes, their inner structures were degenerated ovaries. While there were a small number of degenerating large auxocytes, no oogonia and also no multiplied rete cells were found. One (J_3Dp·H, No. 9; Fig. 7d) of the two juvenile hermaphrodites had extremely small roundish gonads. While there were narrow cavities and numerous oogonia, some of the latter were surrounded by somewhat multiplied rete cells. The other juvenile hermaphrodite (J_3Dp·H, No. 11) had gonads which were flat in shape and contained no cavities. While they mostly consisted of cortical portions, some oogonia were surrounded by rete cells. Two males were at the age of one or two months. The testes were quite normal in inner structure, although they were smaller than those of the control diploid males. The Dp·H chimeras of *Rana japonica* differed from those of *R. nigromaculata*

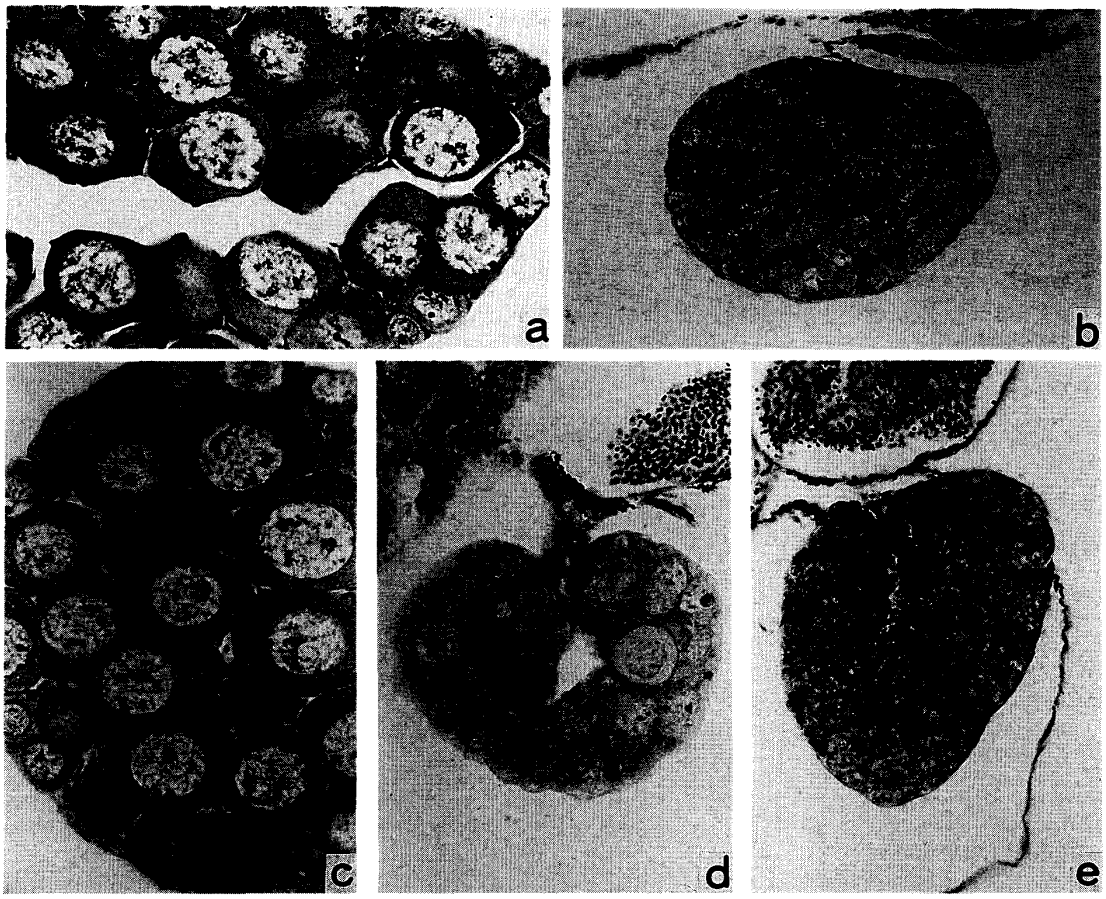

Fig. 7. Cross-sections of the gonads of haploid-diploid chimeras at the stage shortly before or after completion of metamorphosis, and the control diploids in *Rana japonica*. ×180

a. Normal ovary (♀N) of the control diploid female
b. Normal testis (♂N) of the control diploid male
c. Normal ovary (♀N) of a haploid-diploid chimera, $J_2Dp{\cdot}H$, No. 6
d. Hermaphroditic gonad (⚥) of a haploid-diploid chimera, $J_3Dp{\cdot}H$, No. 9
e. Normal testis (♂N) of a haploid-diploid chimera, $J_3Dp{\cdot}H$, No. 13

in that females contained auxocytes in their ovaries.

DISCUSSION

MIYADA (1960, 1977) showed that the appearance of viable haploid tadpoles is related to the genetic constitution of mother frogs which have few lethal or deleterious genes. According to him, all the viable haploids were produced from eggs of only eight of 87 *Rana nigromaculata* and *R. brevipoda* females. Nine of these haploid tadpoles completed metamorphosis and became young frogs. They were produced from four females (MIYADA, 1960). In 1977, he obtained nine haploid frogs from two females of *R. nigromaculata*. Of these haploid frogs, one three-year-old and six younger frogs were produced from one of the two females. KASHIWAGI (1980) obtained three sexually mature haploid frogs in *Rana rugosa*. These mature haploids seemed to have developed from eggs which had no lethal genes. This species was peculiar in having comparatively few lethal or semilethal genes,

because the overwhelming majority of the haploid embryos produced gynogenetically became normal tadpoles.

HAMILTON (1963) confirmed that the poor viability of haploid individuals was improved by addition of diploid tissues. When she made 24 haploid-diploid chimeras (CD-H) consisting of an anterior diploid half and a posterior haploid half in *Xenopus laevis*, two of them survived for five and a half months. One reached the middle tadpole stage, while the other attained the metamorphosing stage. In contrast, all the 20 control haploids died by 12 days after the operation producing chimeras was performed in the experimental series. According to HAMILTON (1963), the CD-H chimeras were much superior in development to the reverse CH-D chimeras which consisted of an anterior haploid half and a posterior diploid half.

The 33 haploid-diploid chimeras obtained in the present study in *Rana nigromaculata* and *R. japonica* could develop further than the chimeras of *X. laevis* obtained by HAMILTON. The Dp·H chimeras of *R. nigromaculata* and *R. japonica* survived until the stages shortly before or after the completion of metamorphosis. Of the four kinds of chimeras produced by the present author, the H·pD and Dp·H showed a remarkable diminution of the severe edema usually found in the haploid partners of the other kinds of chimeras at the early stage. This seems to suggest strongly that the dysfunction of the haploid kidney is closely related to the development of edema in haploid embryos. However, the H·pD chimeras remarkably differed from the Dp·H ones in development. While most of the H·pD chimeras were normal at the hatching stage, the number of tadpoles displaying the haploid syndrome increased progressively as the development proceeded. Eventually all the H·pD chimeras died by one month after operation. The Dp·H chimeras, on the other hand, were viable much more than the H·pD ones. These findings seemed to indicate that not only edema but also the other factors located in the anterior half, namely, small head, short gills, defective heart, *etc.* associated with haploidy, have a remarkable effect on the viability of the chimeras, too. Thus, it was very probable that the better development observed in the Dp·H chimeras was due to the removal of the above-mentioned syndromes including edema owing to the existence of diploid tissues in the anterior half.

GALLIEN (1967) examined the differentiation of the testes in haploid *Pleurodeles waltl* united in parabiosis with diploid individuals of the same species. In the testes showing the best differentiation, the spermatogenesis failed at the spermatocyte stage. Bivalents were not formed, although the meiosis proceeded up to anaphase of the first division. When haploid individuals were united with diploid females, the testes were feminized into ovotestis under the dominant influence of female partners. The gonads of haploid frogs have been reported by MIYADA (1960, 1977) in *Rana nigromaculata* and by KASHIWAGI (1980) in *Rana rugosa*. According to MIYADA, the haploid frogs of *R. nigromaculata* should be genetically females, as they are considered to have a single X chromosome. The result of his experiments showed that the 18 haploid frogs and old tadpoles obtained by gynogenesis comprised 14 females, three males and one juvenile hermaphrodite

with gonads transforming from ovaries into testes. Ten of the 14 females had underdeveloped or degenerating ovaries (MIYADA, 1960). The three-year-old haploid frog produced by MIYADA (1977) was a female whose ovaries were filled with a large number of growing auxocytes having no yolk granules and a considerable number of degenerating auxocytes. This haploid female seemed to have no reproductive ability, even if she lived longer.

In *Rana rugosa* distributed in Hiroshima district, the male heterogamety has been established on the basis of the studies both on the sex of the offspring of genetic females whose sex was reversed by treatment with testosterone propionate (NISHIOKA, MIURA and SAITOH, 1993) and on the sex of gynogenetic diploids (KASHIWAGI, 1993). KASHIWAGI (1980) obtained 69 haploid frogs gynogenetically from eggs of two females of *R. rugosa*. Among 63 of these haploid frogs which died within one month after metamorphosis, there were 51 males with normal testes, two males with rudimentary testes, nine juvenile hermaphrodites and one female with normal ovaries. Two of the other six haploids died two months after metamorphosis. They were dwarf males with underdeveloped testes. Another haploid died at the age of one year. This was a dwarf hermaphrodite. The remaining three haploids were also one-year-old females. One of them laid eggs spontaneously, and another female laid eggs after pituitary injection. These eggs cleaved normally and became blastulae, while all of them died at the late blastula stage. All the haploid males and hermaphrodites should be sex-reversed genetic females.

It is expected that the Dp·H chimeras of *R. nigromaculata* having the gonads in the posterior haploid halves are all genetically females. Among the 18 Dp·H chimeras, however, there were five males, four females with underdeveloped ovaries, five females with degenerating or degenerated ovaries, three juvenile hermaphrodites and one chimera with indifferent gonads. Of the 29 metamorphosed frogs in the control series, 16 were males with normal testes, four were juvenile hermaphrodites and nine were females with normal ovaries. Of the 28 Dp·D controls at the stage shortly before the completion of metamorphosis, 18 were normal males and 10 were normal females.

As the male heterogamety of *Rana japonica* was insisted by KAWAMURA and YOKOTA (1959) and KAWAMURA and NISHIOKA (1981), the Dp·H chimeras of this species are all expected to be females. In fact, eight of the 15 Dp·H chimeras were nearly normal females, three were females with degenerating or degenerated ovaries, two were normal males and the remaining two were juvenile hermaphrodites. In contrast, there were 27 males and 34 females in the control series. Of the Dp·D controls, 25 were males and 18 were females. While the Dp·H females in *Rana nigromaculata* did not contain normal auxocytes in the ovaries, the ovaries of the eight Dp·H females of *R. japonica* were filled with auxocytes which did not differ in size from those of the control diploid females. This seems to be attributable to species differences. The haploid partners in these eight *R. japonica* chimeras are assumed to have been raised from eggs which contained almost no deleterious genes.

ACKNOWLEDGMENTS

The authors wish to express their sincere thanks to Emeritus Professor Toshijiro KAWAMURA and Professor Midori NISHIOKA of Hiroshima University for their constant guidance in the course of the work as well as for their critical review of the manuscript.

LITERATURE

DALCQ, A. 1932. Contribution á l'analyse des fonctions nucléaires dans l'ontogénèse de la grenouille. IV. Modifications de la formule chromosomiale. Arch. Biol., **43**: 343–366.

GALLIEN, L. 1967. Développement d'individus haploïdes adultes élevés en parabiose chez le triton *Pleurodeles waltlii* MICHAH.: Syndrome de l'haploïdie et différenciation sexuelle. J. Embryol. exp. Morph., **18**: 401–426.

HAMILTON, L. 1963. An experimental analysis of the development of the haploid syndrome in embryos of *Xenopus laevis*. J. Embryol. exp. Morph., **11**: 267–278.

HUMPHREY, R. R. and G. FANKHAUSER 1957. The origin of spontaneous and experimental haploids in the Mexican axolotl. J. Exp. Zool., **134**: 427–447.

KASHIWAGI, K. 1980. Mature haploids and their reproductive capacity in *Rana rugosa*. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **4**: 217–237.

———— 1993. Gynogenetic diploids in *Rana rugosa* and their offspring. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **12**: 1–22.

KAWAMURA, T. 1939. Artificial parthenogenesis in the frog. I. Chromosome numbers and their relation to cleavage histories. J. Sci. Hiroshima Univ., Ser. B, Div. 1, **6**: 115–218.

KAWAMURA, T. and M. NISHIOKA 1981. Developmental capacity and sex of gynogenetic diploids in *Rana japonica* and *Rana tsushimensis*. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **5**: 325–358.

KAWAMURA, T and R. YOKOTA 1959. The offspring of sex-reversed females of *Rana japonica* GUENTHER. J. Sci. Hiroshima Univ., Ser. B, Div. 1, **18**: 31–38.

MIYADA, S. 1960. Studies on haploid frogs. J. Sci. Hiroshima Univ., Ser. B, Div. 1, **19**: 1–56.

———— 1977. A three-year-old haploid frog. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **2**: 213–227.

NISHIOKA, M. 1972. The karyotypes of the two sibling species of Japanese pond frogs, with special reference to those of the diploid and triploid hybrids. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **1**: 319–337.

NISHIOKA, M. and Y. KONDO 1978. Production of gynogenetic haploids by treatment of spermatozoa with toluidine blue in *Rana japonica*. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **3**: 385–398.

NISHIOKA, M., I. MIURA and K. SAITOH 1993. Sex chromosomes of *Rana rugosa* with special reference to local differences in sex-determining mechanism. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **12**: 55–81.

NISHIOKA, M., H. OKUMOTO and Y. KONDO 1981. Effects of ultraviolet rays on the sperm of *Rana japonica*. I. Production of gynogenetic haploids. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **5**: 47–67.

NISHIOKA, M. and K. TANAKA 1981. Effects of ultraviolet rays on the sperm of *Rana japonica*. II. Chromosome aberrations induced by UV-irradiation. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **5**: 69–87.

PARMENTER, C. L. 1933. Haploid, diploid, triploid, and tetraploid chromosome numbers, and their origin in parthenogenetically developed larvae and frogs of *Rana pipiens* and *R. palustris*. J. Exp. Zool., **66**: 409–453.

PORTER, K. R. 1939. Androgenetic development of the egg of *Rana pipiens*. Biol. Bull., **77**: 233–257.

SHUMWAY, W. 1940. Stages in the normal development of *Rana pipiens*. I. External form. Anat. Rec., **78**: 139–147.

TAYLOR, A. C. and J. J. KOLLROS 1946. Stages in the normal development of *Rana pipiens* larvae. Anat. Rec., **94**: 7–24.