Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ. 12: 1-22 (1993)

Gynogenetic Diploids in *Rana rugosa* and their Offspring

By

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ABSTRACT

Gynogenetic diploids were produced from eggs of 10 female *Rana rugosa* by refrigeration after insemination with UV-irradiated sperm of 10 males of the same species. They were compared with the control diploids and gynogenetic haploids in developmental capacity. When refrigerated at 0~2°C for two hours after insemination with UV-irradiated sperm, 73.1% of the eggs cleaved normally, and 65.1% and 20.9% of these normally cleaved eggs hatched normally and became normally metamorphosed frogs, respectively. The diploidy of the individuals in the experimental series was confirmed by counting chromosomes at the tadpole stage. Of the 457 gynogenetic diploids, 418 were females and 39 (8.5%) were males. There were 931 females and 25 (2.6%) males among the 956 offspring of six gynogenetic diploid males mated with normal females. As the great majority of gynogenetic diploids and the offspring of gynogenetic males mated with normal females were females,it is assumed that the male is heterogametic in *Rana rugosa* and that gynogenetic males are genetic females. In the gynogenetic diploid tadpoles, there were 26 (2.4%) black-eyed and 23 (2.1%) gray-eyed mutants.

INTRODUCTION

The present author has succeeded in producing three mature haploid females in *Rana rugosa* (KASHIWAGI, 1980). They were raised from less than 0.2% of the eggs inseminated with UV-irradiated sperm. Eggs from one of these haploid females inseminated with sperm of a normal male collected from the field cleaved normally and became blastulae. However, all of them died at the late blastula stage. The extremely low percentage of mature haploid amphibians is likely attributed to recessive lethal genes unmasked by haploidy. Parthenogenetic frogs which were probably diploids and homozygous for all genes have been artificially produced by GOLDSCHMIDT (1920), PARMENTER (1925, 1933), TCHOU SU, CHEN CHAO-HSI and CHANG KO (1938) and KAWAMURA (1939a, b). In these cases the diploid condition is usually considered to have resulted from doubling of the haploid chromosome set during the first cleavage which was not followed by cytoplasmic division. All these parthenogenetic frogs could not produce offspring, even if they attained sexual maturity. These findings appeared to indicate that a slight degree of heterozygosity is necessary for normal reproduction. Such a situation was established by MORIWAKI (1957, 1959, 1960) who refrigerated eggs of *Rana japonica*

after pricking with a needle. This process probably brought about union of the egg nucleus with the second polar body nucleus retained in the egg. A few male and female mature parthenogenetic diploids produced by MORIWAKI were quite or partially fertile. VOLPE and DASGUPTA (1962) obtained a similar genetic constitution by subjecting *Rana pipiens* eggs to heat shock after insemination with sperm of *Scaphiopus holbrooki*. KAWAMURA and NISHIOKA (1977, 1981) and NISHIOKA (1977) have reported that many gynogenetic diploids can be easily produced in several anuran species by refrigeration or heat shock after pseudofertilization. The method for producing diploid gynogenesis has been used to reveal various color mutations in *Rana nigromaculata* (NISHIOKA, 1977; NISHIOKA and UEDA, 1985a), *Hyla japonica* (NISHIOKA and UEDA, 1977, 1985b) and *Rana brevipoda porosa* (NISHIOKA and UEDA, 1985c) and also to understand the sex determining mechanism in several species of Japanese anurans.

The purpose of the present study is to investigate the viability, sex ratio, reproductive capacity and color mutations of gynogenetic diploids in *Rana rugosa* which is the only amphibian species producing mature haploids.

MATERIALS AND METHODS

Adult specimens of *Rana rugosa* SCHLEGEL were collected in and around Hiroshima City. The ovulation of mature females was accelerated by injection of pituitaries of *Rana catesbeiana* SHAW into the body cavity. Fertilization was done artificially. Ten mature females (W♀, Nos. 1~10) and 10 mature males (W♂, Nos. 1~10) were used in the present study. Gynogenetic diploids were produced by the following method. Sperm suspension was prepared in Cl-free tap water shortly after removing the testes from a male. A vial containing 0.5 ml of sperm suspension was placed 20 cm away from an ultraviolet source (GUL-5J type U-shaped mercury-vapor lamp, Toshiba Electric Company, Tokyo) and exposed for two minutes at 24 erg/mm^2/sec. The ultraviolet rays were 2537×10^{-1} nm in main wave length. Eggs were inseminated with UV-irradiated sperm. Twenty minutes after insemination, the eggs were refrigerated at 0~2°C for two hours (NISHIOKA, 1977).

When the eggs became tadpoles at stage V about 30 days after insemination, they were put in cement tanks, each of which was $95 \times 65 \times 20$ cm in size, and reared until metamorphosis. The tadpoles and frogs were fed on boiled spinach and crickets, respectively. Chromosome preparations were made by the squash method of NISHIOKA (1972) using the tail-tips of feeding tadpoles. The ploidy of each tadpole was determined by counting chromosomes in more than five well-spread metaphase figures. Metamorphosed frogs were fixed in NAVASHIN's solution and preserved in 70% alcohol. Gonads and kidneys were embedded in paraffin, sectioned at 12 μ and stained with HEIDENHAIN's hematoxylin. They were classified by the criteria described by KAWAMURA and NISHIOKA (1972). The developmental stages described in this paper follow those of *Rana pipiens* established by SHUMWAY (1940) and TAYLOR and KOLLROS (1946) for conven-

ience' sake.

OBSERVATION

I. Developmental capacity

1. Controls

Ten control matings were made between 10 females (W♀, Nos. 1~10) and 10 males (W♂, Nos. 1~10) as shown in Table 1. In these 10 matings, 48.7~100% of the 39~153 eggs used in the experiments, 757 (88.0%) eggs in total, cleaved normally. After 17 eggs died of abnormality by the hatching stage, 46.2~100%, 740 (86.0%) eggs in total, became normally hatched tadpoles. Thereafter, 140 tadpoles died of abnormality by the feeding V~X stages and one more tadpole died during metamorphosis. Eventually, 31.0~82.1% of the eggs of the 10 matings, 532 (61.9%) tadpoles in total, completed metamorphosis. These percentages corresponded to 49.1~82.1%, 70.3% on the average, of the normally cleaved eggs.

2. Gynogenetic haploids

Eggs of the 10 females (W♀, Nos. 1~10) were inseminated with UV-irradiated sperm of the 10 males (W♂, Nos. 1~10). In the 10 matings, 63.9~100%, 88.5% of the 1079 eggs in total, cleaved normally. These cleavage rates were almost the same as those of the control matings. Of the normally cleaved eggs, 212 eggs became abnormal and died by the hatching stage, while 43.7~100%, 743 (68.9%) eggs in total, hatched normally. Thereafter, various abnormalities of the haploid type appeared in the majority of tadpoles. Eventually, 98 (9.1%) and 25 (2.3%) became normally feeding tadpoles and completed metamorphosis, respectively (Table 1). Two of the feeding tadpoles at stage V were gray-eyed mutants. These mutants were produced from female W♀, No. 8, which was collected from Fuchu-cho near Hiroshima City. They failed to survive until metamorphosis.

3. Gynogenetic diploids

Eggs of the 10 females (W♀, Nos. 1~10) were inseminated with UV-irradiated sperm of the 10 males (W♂, Nos. 1~10) and refrigerated at 0~2°C for two hours in order to suppress extrusion of the second polar bodies. Although the normal cleavage rate was comparatively high, many eggs became abnormal at the embryonic stage. In the 10 matings, 43.7~97.9%, 4138 (73.1%) of the 5661 eggs in total, cleaved normally. After 1444 died of various abnormalities by the hatching stage, 31.3~65.5% of the eggs in the 10 matings, 2694 (47.6%) eggs in total, hatched normally, and 6.5~34.8%, 866 (15.3%) tadpoles in total, became normally metamorphosed frogs (Table 1). These percentages of the frogs corresponded to 6.6~43.7%, 20.9% on the average, of the normally cleaved eggs.

The ploidy of 969 of the 1086 tadpoles at stage V which had been raised from

TABLE 1

Developmental capacities of gynogenetic haploids and diploids in *Rana rugosa*

Parents		No. of eggs	No. of normal cleavages (%)	No. of normally hatched tadpoles (%)	No. of normally feeding tadpoles (%)	No. of metamorphosed frogs (%)
Female	Male					
W, No. 1	W, No. 1	87	55 (63.2)	49 (56.3)	40 (46.0)	27 (31.0)
W, No. 2	W, No. 2	113	113 (100)	113 (100)	108 (95.6)	85 (75.2)
W, No. 3	W, No. 3	84	84 (100)	84 (100)	71 (84.5)	69 (82.1)
W, No. 4	W, No. 4	110	110 (100)	110 (100)	79 (71.8)	72 (65.5)
W, No. 5	W, No. 5	82	75 (91.5)	74 (90.2)	65 (79.3)	55 (67.1)
W, No. 6	W, No. 6	76	75 (98.7)	73 (96.1)	45 (59.2)	41 (53.9)
W, No. 7	W, No. 7	52	41 (78.8)	38 (73.1)	29 (55.8)	28 (53.8)
W, No. 8	W, No. 8	153	145 (94.8)	142 (92.8)	120 (78.4)	116 (75.8)
W, No. 9	W, No. 9	64	40 (62.5)	39 (60.9)	26 (40.6)	26 (40.6)
W, No. 10	W, No. 10	39	19 (48.7)	18 (46.2)	17 (43.6)	13 (33.3)
Total		860	757 (88.0)	740 (86.0)	600 (69.8)	532 (61.9)
W, No. 1	UV-1	80	80 (100)	80 (100)	7 (8.8)	
W, No. 2	UV-2	99	98 (99.0)	80 (80.8)	15 (15.2)	
W, No. 3	UV-3	119	76 (63.9)	67 (56.3)	11 (9.2)	
W, No. 4	UV-4	95	93 (97.9)	93 (97.9)	6 (6.3)	
W, No. 5	UV-5	93	85 (91.4)	85 (91.4)	11 (11.8)	
W, No. 6	UV-6	70	69 (98.6)	66 (94.3)	5 (7.1)	
W, No. 7	UV-7	131	120 (91.6)	83 (63.4)	11 (8.4)	
W, No. 8	UV-8	119	110 (92.4)	52 (43.7)	14 (11.8)	
W, No. 9	UV-9	193	163 (84.5)	94 (48.7)	11 (5.7)	
W, No. 10	UV-10	80	61 (76.3)	43 (53.8)	7 (8.8)	
Total		1079	955 (88.5)	743 (68.9)	98 (9.1)	25 (2.3)
W, No. 1	UVR-1	492	215 (43.7)	156 (31.7)	69 (14.0)	46 (9.3)
W, No. 2	UVR-2	360	294 (81.7)	129 (35.8)	66 (18.3)	60 (16.7)
W, No. 3	UVR-3	606	483 (79.7)	395 (65.2)	262 (43.2)	211 (34.8)
W, No. 4	UVR-4	402	230 (57.2)	192 (47.8)	92 (22.9)	85 (21.1)
W, No. 5	UVR-5	801	784 (97.9)	515 (64.3)	76 (9.5)	52 (6.5)
W, No. 6	UVR-6	542	431 (79.5)	355 (65.5)	71 (13.1)	49 (9.0)
W, No. 7	UVR-7	686	467 (68.1)	216 (31.5)	112 (16.3)	103 (15.0)
W, No. 8	UVR-8	770	538 (69.9)	362 (47.0)	125 (16.2)	92 (11.9)
W, No. 9	UVR-9	521	314 (60.3)	163 (31.3)	115 (22.1)	95 (18.2)
W, No. 10	UVR-10	481	382 (79.4)	211 (43.9)	98 (20.4)	73 (15.2)
Total		5661	4138 (73.1)	2694 (47.6)	1086 (19.2)	866 (15.3)

W, Field-caught

UV-1, Eggs were fertilized with UV-irradiated sperm of W, No. 1.

UVR-1, Eggs were refrigerated after fertilization with UV-irradiated sperm of W, No. 1.

eggs refrigerated after insemination with UV-irradiated sperm was examined (Fig. 1). It was found that 934 (96.4%) were diploids, three (0.3%) were triploids and the remaining 32 (3.3%) were five kinds of mosaics, of which three were haploid-

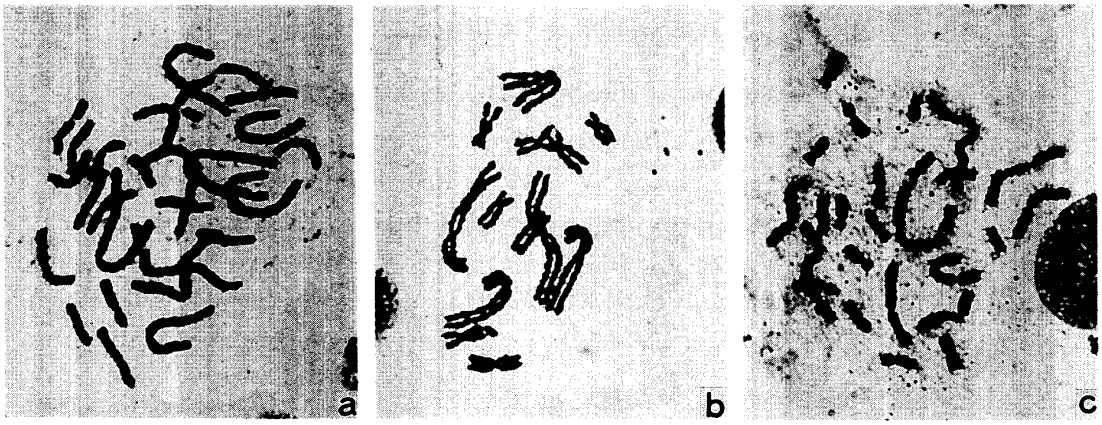

Fig. 1. Metaphase plates of epidermal cells in the tail-tips of tadpoles. ×1000
a. Control diploid tadpole
b. Gynogenetic haploid tadpole
c. Gynogenetic diploid tadpole

TABLE 2
Ploidy of the tadpoles produced from eggs inseminated with UV-irradiated sperm, those refrigerated after insemination with UV-irradiated sperm and the control eggs

Kind of eggs	No. of tadpoles				
	Total	n	2n	3n	Mosaics
Cont	84	0	82 (97.6%)	0	2 (2.4%)
UV	98	95 (96.9%)	2 (2.0%)	0	1 (1.0%)
UVR	969	0	934 (96.4%)	3 (0.3%)	32 (3.3%)

Cont, Control eggs
UV, Eggs inseminated with UV-irradiated sperm
UVR, Eggs refrigerated after insemination with UV-irradiated sperm

diploid, two were haploid-triploid, five were haploid-diploid-triploid, six were diploid-triploid and 16 were diploid-tetraploid mosaics (Table 2).

While all the 600 control tadpoles climbed out of water at the age of 61~110 days, 78.8 days on the average, 924 (98.9%) of 934 gynogenetic diploids climbed out of water at the age of 56~120 days, 76.2 days on the average. In contrast, 50 (52.6%) of 95 haploids climbed out of water at the age of 79~120 days, 95.1 days on the average.

Of the 1086 tadpoles at stage V, 26 were black-eyed and 23 were gray-eyed mutants. These two kinds of mutants were produced from females W♀, Nos. 10 and 8, respectively. They were collected from Fuchu-cho, near Hiroshima City. Of these mutants, 16 black-eyed and 13 gray-eyed tadpoles completed metamorphosis, together with 837 wild-type tadpoles.

II. Sex ratio and the structure of gonads

1. Structure of gonads in immature frogs

The gonads and kidneys of gynogenetic diploids, gynogenetic haploids, and their controls were observed at the stages of completion of metamorphosis and sexual maturity (Fig. 2a, b). In the gynogenetic diploids and haploids at immature stage, there were always a small number of juvenile hermaphrodites whose gonads were transforming from ovaries into testes. The juveniles were divided into the following six types by the structure of their gonads according to KAWAMURA and NISHIOKA (1972).

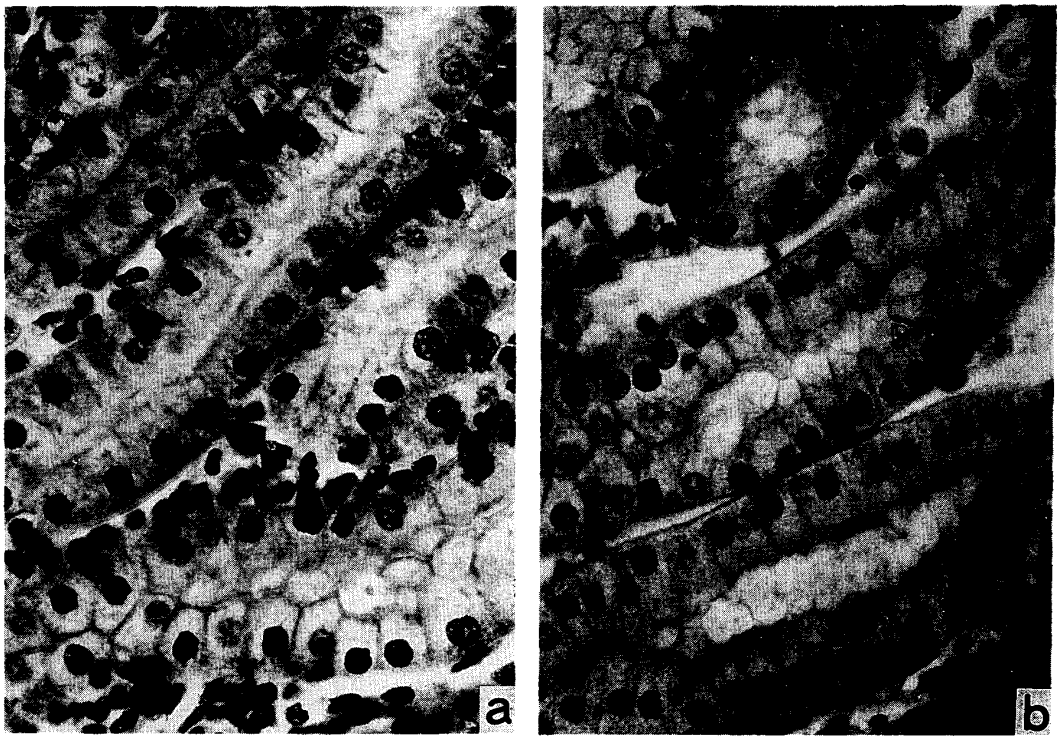

Fig. 2. Cross-sections of the kidneys of gynogenetic diploid and haploid frogs. ×840
a. Gynogenetic diploid frog
b. Gynogenetic haploid frog

(1) Normal female (♀). The gonads are normal ovaries filled with growing auxocytes.

(2) Hermaphrodite type 1 (⚣1). The gonads are at the beginning stage of sex reversal. Multiplication of rete cells is found in the medullary parts. In the cortical parts, there are abundant oogonia and young oocytes.

(3) Hermaphrodite type 2 (⚣2). The gonads are at the middle stage of sex reversal. Owing to distinct multiplication of rete cells, the inner parts of the gonads are testicular in structure, while the outer wide parts remain as an ovarian structure.

(4) Hermaphrodite type 3 (⚣3). The gonads are at the last stage of sex reversal. They are almost testicular in structure, as nearly all the germ cells are

surrounded with rete cells. There are ovarian cavities and some small groups of oocytes.

(5) Normal male (♂). The gonads are typical testes, although some males have a few testis-ova.

(6) Male with rudimentary testes (♂R). The testes are extremely small or have very few germ cells.

2. Sex ratio

a. Controls

Of the 488 juvenile frogs produced from 10 matings between 10 females (W♀, Nos. 1~10) and 10 males (W♂, Nos. 1~10), 255 were females (Fig. 3a) and 233

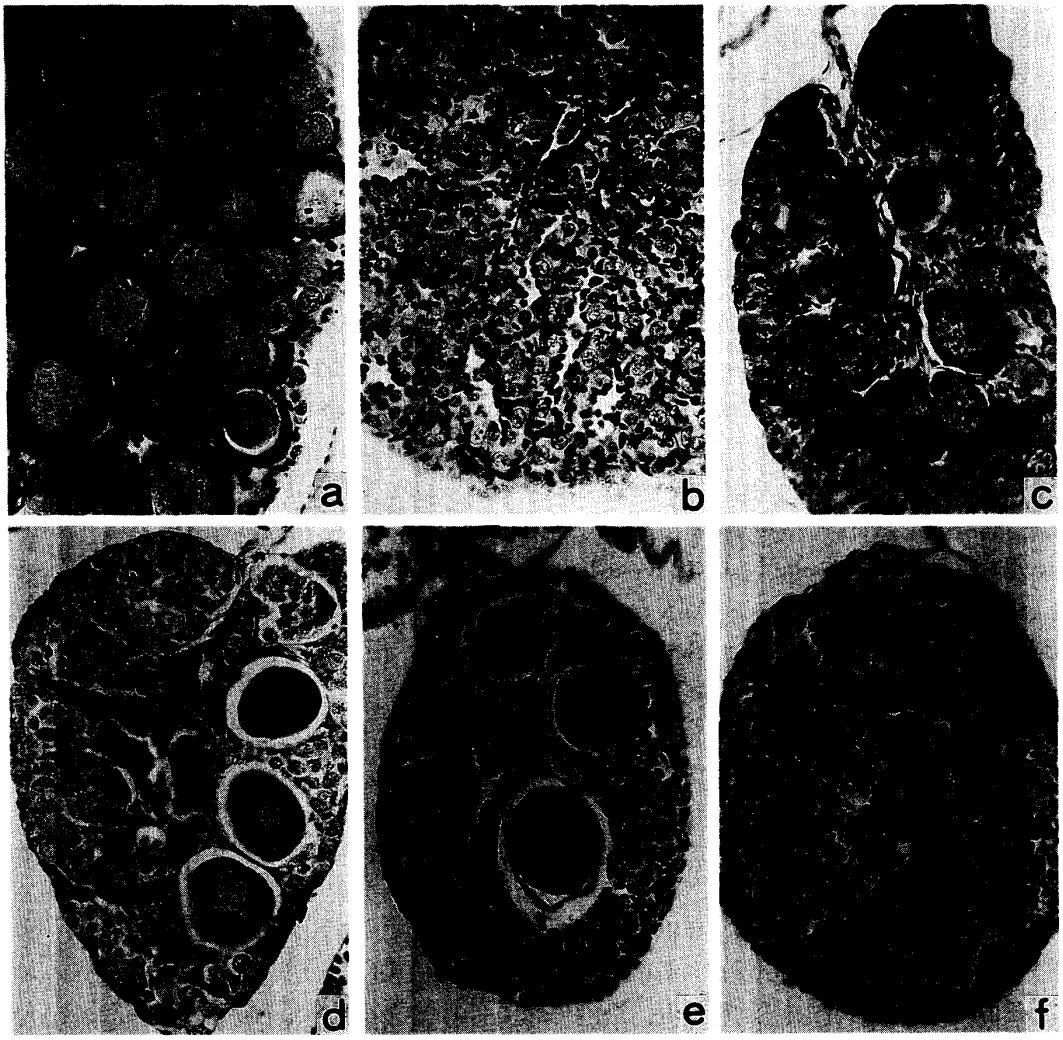

Fig. 3. Cross-sections of the gonads of gynogenetic diploid frogs immediately after metamorphosis and the controls. ×170

a. Normal ovary (♀N) of the control diploid female frog
b. Normal testis (♂N) of the control diploid male frog
c. Hermaphroditic gonad of type 1 (⚥1) of a gynogenetic diploid frog
d. Hermaphroditic gonad of type 2 (⚥2) of a gynogenetic diploid frog
e. Hermaphroditic gonad of type 3 (⚥3) of a gynogenetic diploid frog
f. Normal testis (♂N) of a gynogenetic diploid frog

TABLE 3
Sex of gynogenetic haploids, gynogenetic diploids and the controls

Kind	Juvenile frogs							Mature frogs			All frogs examined			
	Total	♀	⚣1	⚣2	⚣3	♂R	♂	Total	♀	♂	Total	♀	♂	(%)
Cont	488	255	0	0	0	0	233	21	11	10	509	266	243	(47.7)
GH	46*	4	11	2	3	1	25	—	—	—	46	4	42	(91.3)
GD	422	389	7	2	3	0	21	35	29	6	457	418	39	(8.5)

♂R, Male with rudimentary testes
⚣, Hermaphrodite with gonads transforming from ovaries into testes
*, Including 24 metamorphosing frogs
Cont, Controls
GH, Gynogenetic haploids
GD, Gynogenetic diploids

were males (Fig. 3b). Of the 21 mature frogs, 11 were females and 10 were males (Fig. 4a~d). Accordingly, there were 266 females and 243 (47.7%) males among 509 juvenile and mature frogs (Table 3).

b. Gynogenetic haploids

The haploid tadpoles produced gynogenetically from 10 females (W♀, Nos. 1~10) usually died during metamorphosis and could not survive until sexual maturity. The sex was examined in 24 metamorphosing and 22 juvenile frogs. Of the 24 metamorphosing tadpoles, one was a female, seven were hermaphrodites of type 1, two were hermaphrodites of type 2, three were hermaphrodites of type 3 and 11 were males. Of the 22 juvenile frogs, three were females, four were hermaphrodites of type 1, one was a male with rudimentary testes and 14 were males with normal testes. When hermaphrodites were counted as males as they would eventually become males, there were four females and 42 (91.3%) males among the 46 haploids (Table 3).

c. Gynogenetic diploids

Of the 422 juvenile gynogenetic diploid frogs produced from 10 females (W♀, Nos. 1~10), 389 were females, seven were hermaphrodites of type 1 (Fig. 3c), two were hermaphrodites of type 2 (Fig. 3d), three were hermaphrodites of type 3 (Fig. 3e) and 21 were males (Fig. 3f). Of the 35 mature gynogenetic diploids, 29 were females and six were males. Of these females, seven were black-eyed and five were gray-eyed mutants. While the dorsal surface of mature wild-type frogs was yellowish brown (Fig. 4e~h), that of the black-eyed mutants was dark brown. The iris was deep black and indistinguishable from the pupil in coloration. In the black-eyed mutants, the ventral skin was semitransparent and parts of the visceral organs were indistinctly seen (Fig. 5a, b). The ventral surface of the gray-eyed mutants was quite similar to that of the wild-type frogs, while the iris was gray, and the pupil was dark red (Fig. 5c, d). When hermaphrodites were counted as males, there were 418 females and 39 (8.5%) males among 457 juvenile

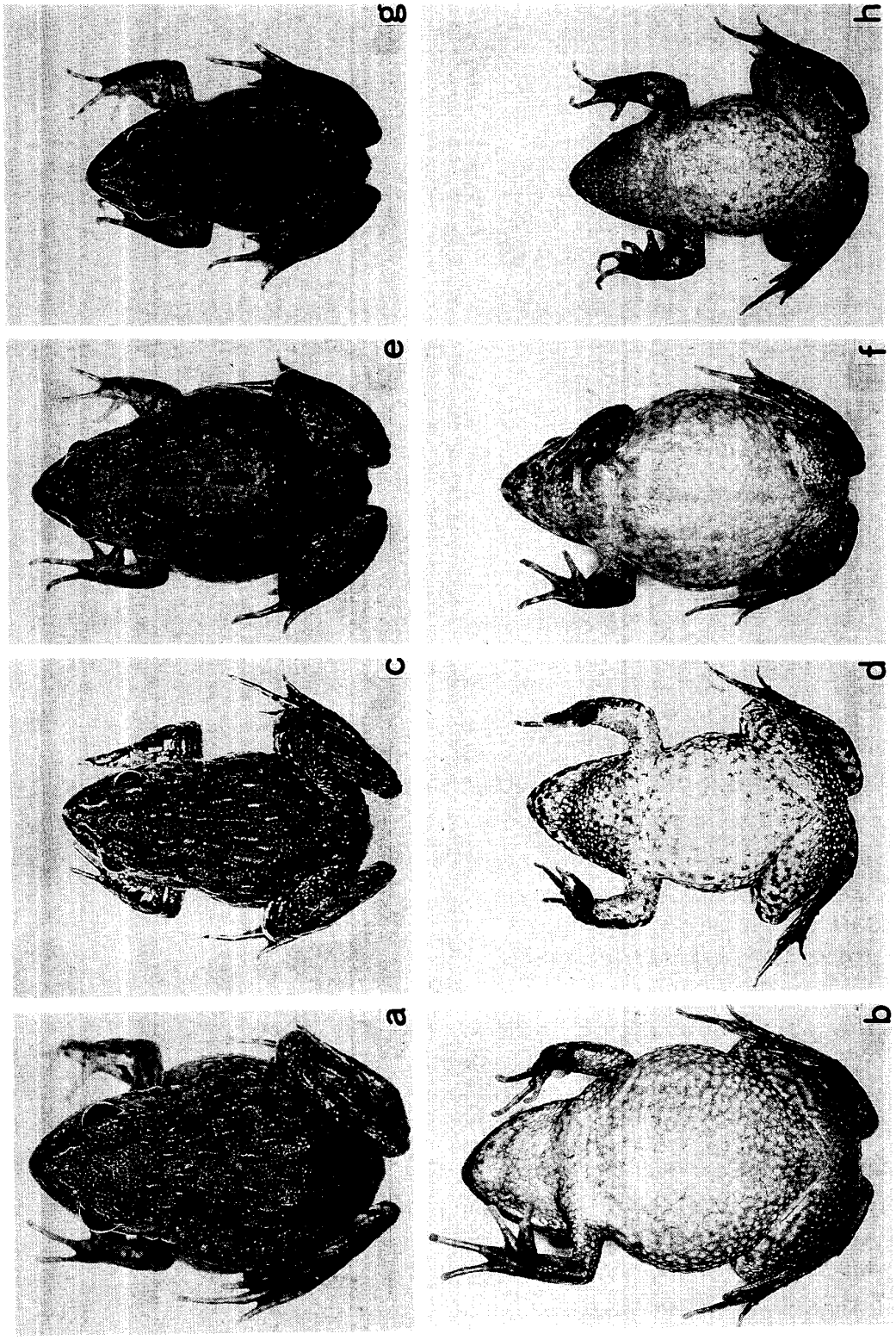

Fig. 4. Mature gynogenetic diploid frogs and normal diploid frogs used in breeding experiments. ×1

a, b. Dorsal and ventral views of a normal diploid female frog, collected in field
c, d. Dorsal and ventral views of a normal diploid male frog, collected in field
e, f. Dorsal and ventral views of a gynogenetic diploid female frog
g, h. Dorsal and ventral views of a gynogenetic diploid male frog

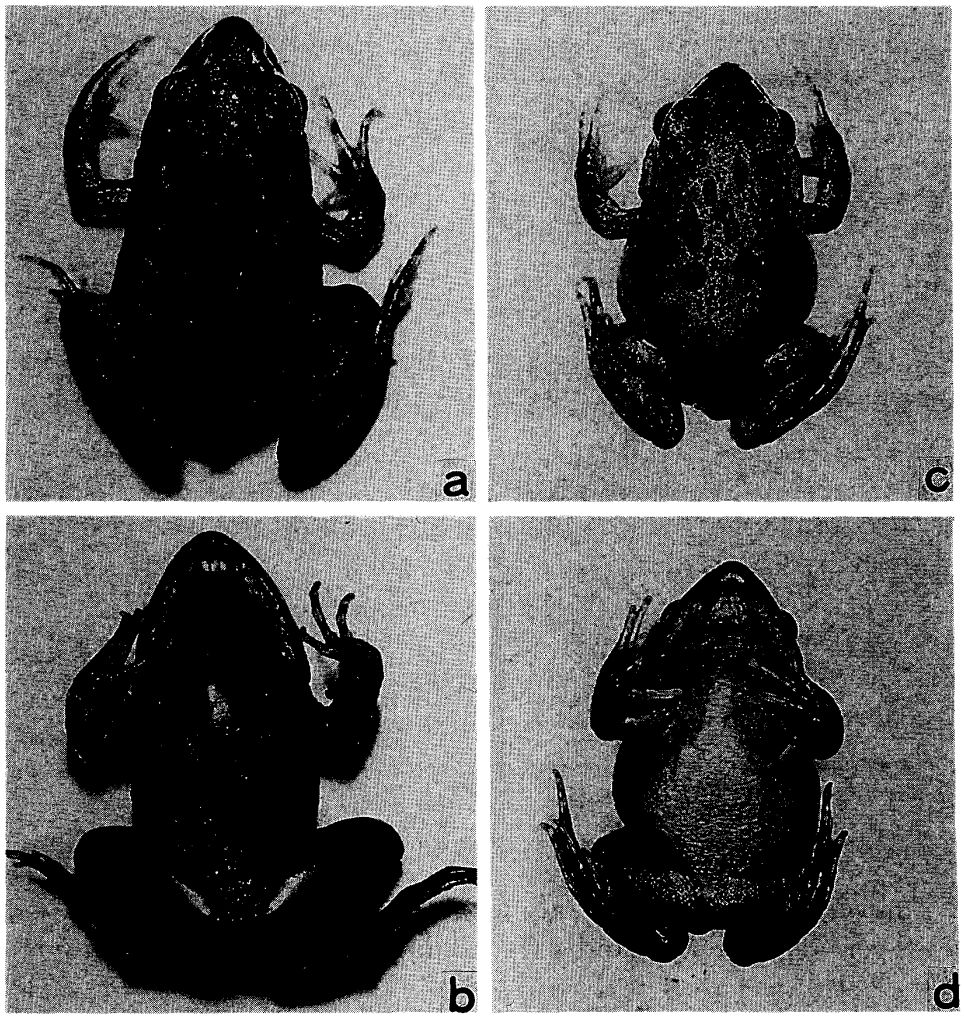

Fig. 5. Mature color mutants found in gynogenetic diploid frogs. ×1.4
a, b. Black-eyed mutant (♀), GD10, No. 3
c, d. Gray-eyed mutant (♀), GD8, No. 2

and mature gynogenetic diploid frogs.

3. Structure of gonads in mature frogs

a. Males

Six one-year-old gynogenetic diploid males and 10 control males attained sexual maturity at the age of one year. The reproductive capacity was examined in these gynogenetic diploids and three of the controls (Table 4). In addition, two normal field-caught males (W♂, Nos. 1 and 2) were used in mating experiments. After measurement of the body size, histological observations and mating experiments were performed by making use of the left and right testes, respectively. The testes were divided into the following two types by their inner structures, as described by KAWAMURA and NISHIOKA (1972).

Type 1. The testis is quite normal in inner structure. Seminiferous tubules are filled with close bundles of normal spermatozoa. A small number of pycnotic nuclei are found.

TABLE 4
Testes of one-year-old gynogenetic diploid males and the control

Kind	Individual no.	Body length (mm)	Size of testes		Type
			Right (mm)	Left (mm)	
Control	C7, No. 1	35.2	3.9×2.8	3.8×2.6	1
Gynogenetic diploid	GD7, No. 1	36.8	2.0×1.5	2.2×1.8	1
	GD7, No. 2	35.0	3.2×2.2	3.0×2.2	1
	GD7, No. 3	29.8	2.5×1.8	2.5×1.5	1
Control	C10, No. 2	35.0	3.9×2.0	3.7×2.1	1
	C10, No. 3	34.0	3.9×2.5	4.0×2.4	1
Gynogenetic diploid	GD10, No. 4	35.2	3.2×2.2	3.2×2.0	2
	GD10, No. 5	35.3	2.9×2.4	2.9×2.5	1
	GD10, No. 6	34.9	2.2×1.8	2.0×1.5	2

Type 2. The testis is almost normal in inner structure. When compared with the testis of type 1, this type is retarded in development. Somewhat small and coarse bundles of normal spermatozoa are found in some of the seminiferous tubules. In the seminiferous tubules which contain no bundles of normal spermatozoa, there are abundantly normal first and second spermatocytes and spermatids.

The three control males (C7♂, No. 1 and C10♂, Nos. 2 and 3) were 34.0~35.2 mm, 34.7 mm on the average, in body length. Their testes were 3.7~4.0 mm, 3.9 mm on the average, in length and 2.0~2.8 mm, 2.4 mm on the average, in width. They were all of type 1 in inner structure (Fig. 6a). The six gynogenetic diploid males (GD7♂, Nos. 1~3 and GD10♂, Nos. 4~6) were 29.8~36.8 mm, 34.5 mm

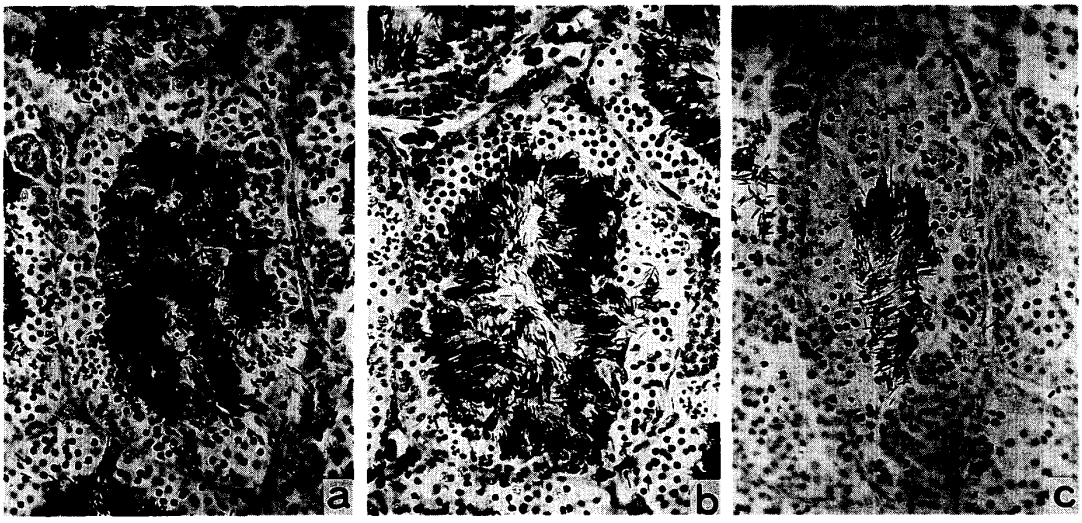

Fig. 6. Cross-sections of the testes of mature gynogenetic diploid frogs and the control at the age of one year. ×170

a. Testis of type 1 of the control male frog, C10, No. 2
b. Testis of type 1 of a gynogenetic diploid male frog, GD7, No. 1
c. Testis of type 2 of a gynogenetic diploid male frog, GD10, No. 4

on the average, in body length. The testes of these males were much inferior to those of the controls in size. They were 2.0~3.2 mm, 2.7 mm on the average, in length, and 1.5~2.5 mm, 2.0 mm on the average, in width. Of the six males, four (GD7♂, Nos. 1~3 and GD10♂, No. 5) were of type 1 (Fig. 6b), while the other two (GD10♂, Nos. 4 and 6) were of type 2 (Fig. 6c).

b. Females

Of the 29 one-year-old mature gynogenetic diploid females, 17 wild-type females, including 10 of GD7♀ and seven of GD10♀, five gray-eyed females, including five of GD8♀, and seven black-eyed females, including seven of GD10♀, were injected with suspension of bullfrog pituitaries to accelerate ovulation. The results showed that four wild-type females (GD7♀, Nos. 1 and 5 and GD10♀, Nos. 2 and 6) and two black-eyed females (GD10♀, Nos. 3 and 4) laid eggs normally. On the other hand, five of the one-year-old control females (C7♀, Nos. 1 and 2 and C10♀, Nos. 1~3) were injected with suspension of bullfrog pituitaries. It was found that two of them (C7♀, No. 1 and C10♀, No. 2) laid eggs normally. The number and size of their eggs are presented in Table 5. The two controls (C7♀, No. 1 and C10♀, No. 2) were 43.8 mm and 42.5 mm, 43.2 mm on the average, in body length. After pituitary injection, they deposited 653 and 602 eggs, which were 1.3±0.01 mm and 1.4±0.02 mm in diameter, respectively. The six gynogenetic diploid females were 32.3~44.7 mm, 40.1 mm on the average, in body length. They deposited 215~674 eggs, 476 eggs on the average, which were 1.1±0.01~1.4±0.01 mm in diameter. Besides them, four field-caught normal females (W♀, Nos. 1~4) were used in mating experiments.

TABLE 5
Eggs of one-year-old gynogenetic diploid females and the control

Kind	Individual no.	Body length (mm)	No. of eggs	Mean diameter of 20 eggs (mm)
Control	C7, No. 1	43.8	653	1.3±0.01
	C10, No. 2	42.5	602	1.4±0.02
Gynogenetic diploid	GD7, No. 1	44.2	674	1.2±0.01
	GD10, No. 2	44.7	631	1.3±0.02
	GD10, No. 3*	36.0	327	1.1±0.01
	GD10, No. 4*	32.3	215	1.1±0.01
	GD7, No. 5	41.3	471	1.4±0.01
	GD10, No. 6	42.1	536	1.4±0.01

* Black-eyed mutant

III. Reproductive capacity

1. Gynogenetic diploid males

In eight control matings between four field-caught females (W♀, Nos. 1~4) and

two control (C7♂, No. 1 and C10♂, No. 2) and two field-caught (W♂, Nos. 1 and 2) males, 74.7~91.3%, 83.0% on the average, of the 53~146 eggs cleaved normally. Of the normally cleaved eggs, 14, 31 and 19 became abnormal and died at the gastrula, tail-bud and hatching stages, respectively, while 56.2~85.1%, 72.4% on the average, hatched normally. Thereafter, 28 of them died without taking food, and 69 died by metamorphosis. Eventually, 42.5~67.6%, 56.7% on the average, became normally metamorphosed frogs (Table 6).

In six matings between the same four field-caught females as those used in the control matings and three gynogenetic diploid males (GD7♂, Nos. 2 and 3 and GD10♂, No. 5), 59.1~96.9%, 82.0% on average, of the 112~320 eggs cleaved normally. Eleven, 60 and 48 of the normally cleaved eggs became abnormal and died at the gastrula, tail-bud and hatching stages, respectively, while 43.1~90.2%, 73.1% on the average, hatched normally. Of these tadpoles, 22 died without taking food and 206 were ill-developed and died by metamorphosis, while 39.3~68.3%, 57.1% on the average, of the normally cleaved eggs attained the completion of metamorphosis. However, the reproductive capacities of another gynogenetic diploid male (GD7♂, No. 1) in the following two matings considerably differed from each other. In one mating with a field-caught female (W♀, No. 1), 32.3% of the 223 eggs cleaved normally. One of the normally cleaved eggs became abnormal and died at the tail-bud stage, while 23.3% hatched normally. Thereafter, one of the normally hatched tadpoles died without taking food and nine died by metamorphosis, while 18.8% attained the completion of metamorphosis. In the other mating using a field-caught female (W♀, No. 2), 72.8% of the 232 eggs cleaved normally. Of the normally cleaved eggs, one became abnormal and died at the tail-bud stage, while 72.4% hatched normally. Of the normally hatched tadpoles, one died without taking food and 57 died by metamorphosis. Eventually, 47.4% became normally metamorphosed frogs.

In four matings between two field-caught females (W♀, Nos. 3 and 4) and the remaining two gynogenetic diploid males (GD10♂, Nos. 4 and 6), 18.2~83.1%, 57.9% on the average, of the 110~241 eggs cleaved normally. While eight, 60 and 19 became abnormal and died at the gastrula, tail-bud and hatching stages, respectively, 13.6~59.5%, 44.5% on the average, hatched normally. Of these tadpoles, 20 died without taking food and 81 died by metamorphosis, while 4.5~37.5%, 29.2% on the average, attained the completion of metamorphosis.

2. Gynogenetic diploid females

In four control matings between two control females (C7♀, No. 1 and C10♀, No. 2) and two control males (C7♂, No. 1 and C10♂, No. 3) and two field-caught males (W♂, Nos. 1 and 2), 77.8~89.1%, 84.7% on the average, of the 88~101 eggs cleaved normally. Four, 15 and 14 of the normally cleaved eggs died of various kinds of abnormalities at the gastrula, tail-bud and hatching stages, respectively, and 70.0~82.8%, 75.8% on the average, hatched normally. Of the normally hatched tadpoles, 19 died without taking food and 21 died by metamorphosis. Eventually, 61.4~70.0%, 65.1% on the average, attained the comple-

TABLE 6

Reproductive capacity of gynogenetic diploid males and the control

Parents		No. of eggs	No. of normal cleavages	No. of normal tail-bud embryos	No. of normally hatched tadpoles	No. of normally feeding tadpoles	No. of metamorphosed frogs
Female	Male						
W, No. 1	W, No. 1	53	45 (84.9%)	41 (77.4%)	38 (71.7%)	38 (71.7%)	35 (66.0%)
	C7, No. 1	146	109 (74.7%)	85 (58.2%)	82 (56.2%)	81 (55.5%)	62 (42.5%)
W, No. 2	W, No. 1	61	52 (85.2%)	46 (75.4%)	43 (70.5%)	38 (62.3%)	31 (50.8%)
	C7, No. 1	69	63 (91.3%)	60 (87.0%)	56 (81.2%)	52 (75.4%)	41 (59.4%)
W, No. 3	W, No. 2	60	45 (75.0%)	43 (71.7%)	42 (70.0%)	40 (66.7%)	38 (63.3%)
	C10, No. 2	61	52 (85.2%)	50 (82.0%)	48 (78.7%)	48 (78.7%)	41 (67.2%)
W, No. 4	W, No. 2	68	57 (83.8%)	54 (79.4%)	53 (77.9%)	53 (77.9%)	46 (67.6%)
	C10, No. 2	101	91 (90.1%)	88 (87.1%)	86 (85.1%)	70 (69.3%)	57 (56.4%)
W, No. 1	GD7, No. 1	223	72 (32.3%)	53 (23.8%)	52 (23.3%)	51 (22.9%)	42 (18.8%)
	GD7, No. 2	168	131 (78.0%)	117 (69.6%)	107 (63.7%)	105 (62.5%)	76 (45.2%)
	GD7, No. 3	274	162 (59.1%)	127 (46.4%)	118 (43.1%)	115 (42.0%)	109 (39.8%)
W, No. 2	GD7, No. 1	232	169 (72.8%)	168 (72.4%)	168 (72.4%)	167 (72.0%)	110 (47.4%)
	GD7, No. 2	286	277 (96.9%)	270 (94.4%)	258 (90.2%)	255 (89.2%)	187 (65.4%)
	GD7, No. 3	265	231 (87.2%)	226 (85.3%)	226 (85.3%)	216 (81.5%)	181 (68.3%)
W, No. 3	GD10, No. 4	241	139 (57.7%)	106 (44.0%)	103 (42.7%)	95 (39.4%)	83 (34.4%)
	GD10, No. 5	320	286 (89.4%)	273 (85.3%)	260 (81.3%)	256 (80.0%)	217 (67.8%)
	GD10, No. 6	195	162 (83.1%)	131 (67.2%)	116 (59.5%)	107 (54.9%)	62 (31.8%)
W, No. 4	GD10, No. 4	110	20 (18.2%)	16 (14.5%)	15 (13.6%)	12 (10.9%)	5 (4.5%)
	GD10, No. 5	112	81 (72.3%)	77 (68.8%)	73 (65.2%)	73 (65.2%)	44 (39.3%)
	GD10, No. 6	112	60 (53.6%)	59 (52.7%)	59 (52.7%)	59 (52.7%)	42 (37.5%)

W, Field-caught
C, Control
GD, Gynogenetic diploid

tion of metamorphosis (Table 7).

Seven matings were made between four gynogenetic diploid females which laid eggs normally, including two wild-type (GD7♀, No. 1 and GD10♀, No. 2) and two black-eyed (GD10♀, Nos. 3 and 4) females, and five males, including two field-caught (W♂, Nos. 1 and 2) and three control (C7♂, No. 1 and C10♂, Nos. 2 and 3) males. In three matings between two wild-type gynogenetic diploid females (GD7♀, No. 1 and GD10♀, No. 2) and a control male (C7♂, No. 1) and two field-caught males (W♂, Nos. 1 and 2), 13.9~41.8%, 25.3% on the average, of the 110~165 eggs cleaved normally. Of the normally cleaved eggs, two, 31 and six became abnormal and died at the gastrula, tail-bud and hatching stages, respectively, while 12.7~23.6%, 16.4% on the average, hatched normally. Thereafter, nine of them died without taking food and 13 died by metamorphosis. Eventually, 8.5~15.5%, 11.4% on the average, became normally metamorphosed frogs (Table 7). On the other hand, in four matings between the two black-eyed gynogenetic diploid females (GD10♀, Nos. 3 and 4) and two control males

TABLE 7

Reproductive capacity of gynogenetic diploid females and the control

Parents		No. of eggs	No. of normal cleavages	No. of normal tail-bud embryos	No. of normally hatched tadpoles	No. of normally feeding tadpoles	No. of metamorphosed frogs
Female	Male						
C7, No. 1	W, No. 1	88	74 (84.1%)	66 (75.0%)	62 (70.5%)	61 (69.3%)	56 (63.6%)
	C7, No. 1	101	90 (89.1%)	86 (85.1%)	80 (79.2%)	70 (69.3%)	62 (61.4%)
C10, No. 2	W, No. 2	90	70 (77.8%)	65 (72.2%)	63 (70.0%)	61 (67.8%)	59 (65.6%)
	C10, No. 3	93	81 (87.1%)	79 (84.9%)	77 (82.8%)	71 (76.3%)	65 (70.0%)
GD7, No. 1	W, No. 1	163	42 (25.8%)	26 (16.0%)	25 (15.3%)	24 (14.7%)	19 (11.7%)
	C7, No. 1	110	46 (41.8%)	31 (28.2%)	26 (23.6%)	25 (22.7%)	17 (15.5%)
GD10, No. 2	W, No. 2	165	23 (13.9%)	21 (12.7%)	21 (12.7%)	14 (8.5%)	14 (8.5%)
GD10, No. 3*	C10, No. 2	181	139 (76.8%)	129 (71.3%)	120 (66.3%)	116 (64.1%)	90 (49.7%)
	C10, No. 3	146	112 (76.7%)	99 (67.8%)	71 (48.6%)	69 (47.3%)	53 (36.3%)
GD10, No. 4*	C10, No. 2	101	77 (76.2%)	62 (61.4%)	55 (54.5%)	51 (50.5%)	31 (30.7%)
	C10, No. 3	114	74 (64.9%)	61 (53.5%)	61 (53.5%)	61 (53.5%)	45 (39.5%)

W, Field-caught
C, Control
GD, Gynogenetic diploid
*, Black-eyed mutant

(C10♂, Nos. 2 and 3), 64.9~76.8%, 74.2% on the average, of the 101~181 eggs cleaved normally. While 51 and 44 of the normally cleaved eggs became abnormal and died at the tail-bud and hatching stages, respectively, 48.6~66.3%, 56.6% on the average, hatched normally. Of the normally hatched tadpoles, 10 died without taking food and 78 were ill-developed and died by metamorphosis, while 30.7~49.7%, 40.4% on the average, became normally metamorphosed frogs.

IV. Sex of the offspring of gynogenetic diploids

The sex of the offspring of gynogenetic diploid males and females and the controls was examined within one month after metamorphosis. The results are presented in Tables 8 and 9.

TABLE 8
Sex of the offspring of gynogenetic diploid males and the control

Series	Parents		Sex of frogs			
	Female	Male	No. of frogs	♀	⚥	♂ (%)
Control	W, No. 1	W, No. 1	35	18	0	17 (48.6)
		C7, No. 1	15	10	0	5 (33.3)
	W, No. 2	W, No. 1	31	11	0	20 (64.5)
		C7, No. 1	36	18	0	18 (50.0)
	W, No. 3	W, No. 2	28	15	0	13 (46.4)
		C10, No. 2	21	9	0	12 (57.1)
	W, No. 4	W, No. 2	32	17	0	15 (46.9)
		C10, No. 2	41	20	0	21 (51.2)
	Total		239	118	0	121 (50.6)
Experimental	W, No. 1	GD7, No. 1	40	40	0	0 (0)
		GD7, No. 2	71	71	0	0 (0)
		GD7, No. 3	81	81	0	0 (0)
	W, No. 2	GD7, No. 1	108	108	0	0 (0)
		GD7, No. 2	162	162	0	0 (0)
		GD7, No. 3	123	123	0	0 (0)
	W, No. 3	GD10, No. 4	78	64	0	14 (17.9)
		GD10, No. 5	157	152	0	5 (3.2)
		GD10, No. 6	48	48	0	0 (0)
	W, No. 4	GD10, No. 4	4	2	1	1 (25.0)
		GD10, No. 5	44	41	1	2 (4.5)
		GD10, No. 6	40	39	0	1 (2.5)
	Total		956	931	2	23 (2.4)

W, Field-caught
C, Control
GD, Gynogenetic diploid
⚥, Hermaphrodite

TABLE 9
Sex of the offspring of gynogenetic diploid females and the control

Series	Parents		Sex of frogs		
	Female	Male	No. of frogs	♀	♂ (%)
Control	C7, No. 1	W, No. 1	54	25	29 (53.7)
		C7, No. 1	38	23	15 (39.5)
	C10, No. 2	W, No. 2	50	27	23 (46.0)
		C10, No. 3	65	30	35 (53.8)
	Total		207	105	102 (49.3)
Experimental	GD7, No. 1	W, No. 1	19	8	11 (57.9)
		C7, No. 1	13	7	6 (46.2)
	GD10, No. 2	W, No. 2	14	10	4 (28.6)
	GD10, No. 3*	C10, No. 2	80	37	43 (53.8)
		C10, No. 3	50	28	22 (44.0)
	GD10, No. 4*	C10, No. 2	30	14	16 (53.3)
		C10, No. 3	42	20	22 (52.4)
	Total		248	124	124 (50.0)

W, Field-caught
C, Control
GD, Gynogenetic diploid
*, Black-eyed mutant

a. Offspring of gynogenetic diploid males

In eight control matings between four field-caught females (W♀, Nos. 1~4) and two control (C7♂, No. 1 and C10♂, No. 2) and two field-caught (W♂, Nos. 1 and 2) males, 118 of the 239 frogs were females and 121 (50.6%) were males (Table 8). In the experimental series, consisting of 12 matings between the same four field-caught females as those used in the control series and four gynogenetic diploids (GD7♂, Nos. 1~3 and GD10♂, Nos. 4~6), 931 of the 956 frogs were females, two (0.2%) were hermaphrodites and 23 (2.4%) were males (Table 8).

b. Offspring of gynogenetic diploid females

In the control series consisting of four matings between two control females (C7♀, No. 1 and C10♀, No. 2) and two control (C7♂, No. 1 and C10♂, No. 3) and two field-caught (W♂, Nos. 1 and 2) males, 105 of the 207 frogs were females and the other 102 (49.3%) were males (Table 9). In the experimental series, consisting of seven matings between two wild-type gynogenetic diploid (GD7♀, No. 1 and GD10♀, No. 2) and two black-eyed gynogenetic diploid (GD10♀, Nos. 3 and 4) females and the same four males as those used in the control series and another control male (C10♂, No. 2), 124 of the 248 offspring were females and 124 (50.0%) were males.

DISCUSSION

1. Production of gynogenetic diploid frogs

The purpose of the production of gynogenetic diploids is to obtain genetically pure strains. The normal viability of these individuals is probably disturbed by recessive lethal genes which vary in number among species. VOLPE and DASGUPTA (1962) produced gynogenetic diploids by inseminating *Rana pipiens* eggs with *Scaphiopus holbrooki* sperm followed by administration of shock. Of the normally cleaved eggs, only 2.1% developed into metamorphosed frogs. Abundant diploids were gynogenetically obtained from *Rana nigromaculata* eggs by refrigeration after inseminating with UV-irradiated sperm of either *Rana pipiens* or *Rana brevipoda* (KAWAMURA and NISHIOKA, 1977; NISHIOKA, 1977). In their experiments, 30.0~45.6% of the eggs became metamorphosed frogs, in contrast to 85.7% of the control eggs which did so. Of these gynogenetic diploid frogs, 59% survived for one year or more, while 88% survived in the control series. RICHARDS and NACE (1978) produced many gynogenetic diploids in *Rana pipiens* by heat shock after inseminating with UV-irradiated *Rana clamitans* sperm. KAWAMURA and NISHIOKA (1981) also produced gynogenetic diploids in *Rana japonica* and *R. tsushimensis*. While hybrids between these two species failed to metamorphose, 36.6% and 37.4% of the normally cleaved eggs of *Rana japonica* became metamorphosed frogs, if the eggs were heat-shocked after inseminating with UV-irradiated sperm of *R. japonica* and *R. tsushimensis*, respectively. When the eggs of *R. tsushimensis* were heat-shocked after inseminating with UV-irradiated sperm of *R. tsushimensis* and *R. japonica*, 43.6% and 41.3% of the normally cleaved eggs became metamorphosed frogs, respectively, while 86.6% of the normally cleaved eggs developed into frogs in the control series.

In the present study, gynogenetic diploids were produced from eggs of *Rana rugosa* by refrigeration after inseminating with UV-irradiated sperm of the same species. In the experimental series, 73.1% of the eggs cleaved normally, and 20.9% of the normally cleaved eggs became metamorphosed frogs, while 88.0% of the eggs in the control series cleaved normally and 70.3% of the normally cleaved eggs became metamorphosed frogs. These results show that the percentage of eggs which become gynogenetic diploid frogs in *Rana rugosa* by suppression of the second polar body after inseminating with UV-irradiated sperm was remarkably smaller than those in *Rana nigromaculata*, *R. japonica* and *R. tsushimensis*. It can not be believed that the lower percentages of gynogenetic diploid frogs in *Rana rugosa* than those in *R. nigromaculata*, *R. japonica* and *R. tsushimensis* are due to presence of abundant lethal genes, as the gynogenetic haploids of *R. rugosa* seem to be exceptionally viable as compared with those of the other anuran species (KASHIWAGI, 1980). Thus, the lower percentages of gynogenetic diploid frogs in this species seem to be attributable to weak resistance of the eggs against refrigeration.

2. Sex of gynogenetic diploid frogs

KAWAMURA and NISHIOKA (1977) have reported that there were 1387 females and 75 (5.1%) males, including 11 hermaphrodites, among the 1462 gynogenetic diploid *Rana nigromaculata*. Of the 1798 diploid frogs produced gynogenetically from *Rana brevipoda* eggs, 1773 were females and 25 (1.4%) were males, including 11 hermaphrodites. In the control series of the above two studies, approximately an equal number of males and females were obtained. The 1269 gynogenetic diploids produced by RICHARDS and NACE (1978) in *Rana pipiens* consisted of 967 females and 302 (23.8%) males including 35 hermaphrodites, while there was approximately an equal number of males and females among the 467 control frogs. According to KAWAMURA and NISHIOKA (1981), of the 984 frogs produced gynogenetically from *Rana japonica* eggs by insemination with UV-irradiated sperm of the same species, 865 were females and 119 (12.1%) were males including 23 hermaphrodites, and of the 1339 frogs produced gynogenetically from *R. japonica* eggs by insemination with UV-irradiated sperm of *R. tsushimensis*, 1182 were females and 157 (11.7%) were males including 25 hermaphrodites. The control *R. japonica* consisted of 573 females and 561 (49.5%) males including two hermaphrodites. In *R. tsushimensis*, the 401 gynogenetically produced frogs by inseminating with UV-irradiated sperm of the same species consisted of 358 females and 43 (10.7%) males including 10 hermaphrodites. The 469 gynogenetic frogs produced from *R. tsushimensis* eggs by insemination with UV-irradiated sperm of *R. japonica* consisted of 422 females and 47 (10.0%) males including 15 hermaphrodites. Among the 271 control frogs of *R. tsushimensis* there were 132 females and 139 (51.3%) males. On the basis of the finding that an overwhelming majority of gynogenetic diploids were females, KAWAMURA and NISHIOKA assumed that the male is heterogametic in *R. japonica* and *R. tsushimensis*, and sex reversal seems to occur in gynogenetic diploids as a by-product of some developmental abnormality.

Of the 457 diploid *Rana rugosa* produced gynogenetically in the present study, 418 were females and 39 (8.5%) were males including 12 hermaphrodites, while the 509 control frogs consisted of 266 females and 243 (47.7%) males. When the gynogenetic males were mated with normal females, the offspring were predominantly females; 931 of the 956 offspring were females and 25 (2.6%) were males including two hermaphrodites, while the 239 control frogs consisted of 118 females and 121 (50.6%) males. These results seem to show that the gynogenetic males were sex-reversed genetic females with an XX chromosome constitution. An assumption that *R. rugosa* is XX-XY type in sex determining mechanism has also been made by NISHIOKA, MIURA and SAITOH (1993) by using specimens collected from Hiroshima Prefecture. While half of the males, which had been raised from tadpoles injected with testosterone propionate, produced only females by mating with normal females, the other males produced males and females in nearly an equal number by mating with normal females. Thus, it was believed that *R. rugosa* distributed in Hiroshima Prefecture at least is XX-XY type.

3. Color mutants in gynogenetic diploids

RICHARDS, TARTOF and NACE (1969) found melanoid mutants among individuals gynogenetically produced from two female *Rana pipiens* and indicated that these mutants are due to a recessive gene. NISHIOKA (1977) exposed spermatozoa from three males and unfertilized eggs from three females to X-rays or neutrons in order to produce color mutants in *Rana nigromaculata*. When 75 females raised from irradiated gametes were examined by the method of diploid gynogenesis in order to detect induced mutation, it was found that 24 produced nine kinds of color mutants. Two kinds of black-eyed mutants of *Hyla japonica* were discovered by NISHIOKA and UEDA (1985b). One of them appeared in diploids produced gynogenetically from eggs of a female collected from Hesaka, Hiroshima City.

In the present study of producing gynogenetic diploids in *Rana rugosa*, 26 black-eyed and 23 gray-eyed mutants were found in tadpoles produced from two females collected from Fuchu-cho near Hiroshima City. The black-eyed mutants were derived from a female (W♀, No. 10), while the gray-eyed mutants from the other female (W♀, No. 8). Of these tadpoles, 16 black-eyed and 13 gray-eyed mutants became metamorphosed frogs, and seven black-eyed and five gray-eyed mutants attained sexual maturity. The fact that black-eyed females mated with normal males produced only normal offspring indicates that the black-eyed mutants at least are genetically recessive. From the results of the present investigation, it can be assumed that of the 10 females collected from the field, one (10%) had a black-eyed gene, one (10%) had a gray-eyed gene and eight (80%) had normal genes.

4. Reproductive capacity

The present study showed that female gynogenetic diploids of *Rana rugosa* were somewhat inferior to the controls in reproductive capacity; 8.5~49.7% of the eggs of each female became normally metamorphosed frogs, in contrast to 61.4~70.0% of the eggs laid by the control females which did so. Male gynogenetic diploids were also inferior to the controls in reproductive capacity; 4.5~68.3% of the eggs of normal females mated with male gynogenetic diploids became normally metamorphosed frogs, in contrast to 42.5~67.6% of the eggs of normal females mated with normal males which did so. This defect in reproductive capacity of gynogenetically produced male and female diploids can be genetically explained by the homozygosity of a gene or genes unfavorable to this capacity. As the gynogenetic diploid frogs derived from eggs whose pronucleus fused with the retained second polar body nucleus, they are considered to be homozygous for the majority of gene pairs. In contrast, MORIWAKI (1957, 1960) examined the reproductive capacity of parthenogenetic diploids of *Rana japonica* and confirmed that one of the six females was fertile, another four were partially fertile and the remaining one was sterile. While one of the four males was fertile, the other three were quite sterile. The superiority of the gynogenetically produced diploids to the parthenogenetically produced ones in reproductive capacity may be attributable to

the presence of a certain degree of heterozygosity necessary for normal reproductive capacity in them.

According to NACE, RICHARDS and ASHER (1970), the repetition of diploid gynogenesis for three generations is sufficient to obtain highly homozygous strains of *Rana pipiens*. In the absence of selection, complete homozygosity is attained in less than 10 generations (ASHER, 1970). In fact, NISHIOKA (1971) repeated the experiments of producing gynogenetic diploids by refrigeration of eggs fertilized with UV-irradiated sperm for three generations in *Rana nigromaculata* and *R. brevipoda*. In the first generation, most of the gynogenetic diploids died by the completion of metamorphosis, and nearly all of the frogs which remained alive and became matured were females. The viability of the gynogenetic diploids of the second generation obtained from these females by the same method were considerably improved as compared with those of the first generation. Almost all of the mature frogs of the second generation were also females. Gynogenetic diploids of the third generation were obtained from them by the same method. It was found that in this generation their viability was distinctly improved, and especially the gynogenetic diploids of *R. nigromaculata* did not differ much in viability from the control frogs. As *Rana rugosa* attains without fail sexual maturity in one year under laboratory conditions, this species is believed to be one of the excellent animals for producing genetically pure strains by repeating the method of diploid gynogenesis for several generations.

ACKNOWLEDGMENTS

The author wishes to express her 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.

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