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The Sex of Triploids and Gynogenetic Diploids in *Bombina orientalis*

By

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INTRODUCTION

According to WITSCHI (1929), the males of *Rana* are rudimentary hermaphrodites. While the differentiated race of *Rana temporaria* is a gonochorist, the males of the semidifferentiated and the undifferentiated races have ovaries at first, which are then transformed into testes after a short or long period of time. Thus, transitory hermaphrodites are found before or after metamorphosis in these races. On the other hand, adult hermaphrodites have been frequently found in this species (CREW, 1921). WITSCHI (1929) has assumed that the male of *Rana temporaria* is heterogametic on the basis of the sex of the offspring of adult hermaphrodites as well as the hereditary males of different races.

Japanese true frog species like *Rana japonica* and *R. nigromaculata* are of the semidifferentiated type in sex differentiation of males. The sex of these species is so unstable that the genetic females can be reversed into functional males by administration of androgen. By making use of such sex-reversed genetic females in crosses with normal females, it has been assumed that the male of these species is heterogametic (KAWAMURA and YOKOTA, 1959; KAWAMURA and NISHIOKA, 1977).

In contrast with *Rana*, the oriental fire-bellied toad, *Bombina orientalis* (BOULENGER), an archaic species belonging to Discoglossidae is a typical gonochorist. In a preliminary report, the present author (1967) has described that the differentiation of indifferent gonads into testes and ovaries begins at the stage of tadpoles having 2.3～3.0 mm long hind legs. Moreover, the sex of this species cannot be reversed by injection of sex hormones or transplantation of testes or ovaries removed from juveniles into tadpoles, or by rearing tadpoles under high temperature. Such stability of sex differentiation in *Bombina orientalis* makes it difficult to utilize sex-reversed individuals for the purpose of determining the heterogametic sex. However, the assumption of heterogametic sex can be made by examining the sex of both triploids and gynogenetic diploids especially in the case of species with stable sex differentiating mechanisms.

In order to determine the heterogametic sex of *Bombina orientalis*, the present author produced many triploids and gynogenetic diploids and examined their

sex. The results will be reported in this paper.

MATERIALS AND METHODS

Bombina orientalis (BOULENGER) were collected from Korea. Fourteen females and 25 males that were originated from one pair collected from suburbs of Seoul and four females collected from Kyongiu were used in this study. The methods for obtaining oviducal eggs and rearing tadpoles and metamorphosed toads were the same as those described by KAWAMURA, NISHIOKA and UEDA (1972). Triploids were produced from fertilized eggs by suppressing extrusion of the second polar body by refrigeration.

Gynogenetic diploids were obtained by refrigerating eggs in order to suppress extrusion of the second polar body after insemination of the eggs with UV-irradiated sperm. The UV-lamp of Toshiba GUL-5-J type was utilized in irradiating the sperm. This lamp was operated at 75 V, 125 mA where the spectrum of 2537 Å was the highest in intensity.

Chromosomes were observed in epidermal cells of the tail-tips of tadpoles by the squash method with water pretreatment (MAKINO and NISHIMURA, 1952). More than half of individuals in each experimental series and the control were preserved in NAVASHIN's fluid. The others were continuously reared until sexual maturity. The gonads were sectioned at 15 μ and stained with HEIDENHAIN's hematoxylin.

OBSERVATION

I. Production of triploids and gynogenetic diploids

1. Triploids

In order to establish the best method of refrigeration in producing triploids, eggs were kept at 0~1°C for 15, 30 or 60 minutes, or at 2~3°C for 15, 30, 60 or 90 minutes, 23 minutes after fertilization (Figs. 1 and 2). The results showed that refrigeration at 0~1°C for 30 or 60 minutes and at 2~3°C for 90 minutes gave rise to high mortality, although the tadpoles raised from refrigerated eggs were all triploids. Refrigeration at 2~3°C for 60 minutes did not bring about a high mortality, but it produced a high rate of triploids. Thus, this procedure was exclusively utilized in the experiments performed in 1968 and 1971.

In 1968, as a result of refrigeration of 112 eggs obtained from three females, 98 (87.5%) cleaved normally, 74 (66.1%) hatched normally and 68 (60.7%) became feeding tadpoles. Of these tadpoles, 58 (85.3%) were triploids, while three, five and two of the remaining were diploids, diploid-triploid mosaics and individuals whose ploidy could not be determined, respectively. A total of 54 triploid tadpoles completed metamorphosis.

In 1971, 153 eggs obtained from four females were refrigerated after insemination (Table 1). As a result, 124 (81.0%) cleaved normally and 57 (37.3%)

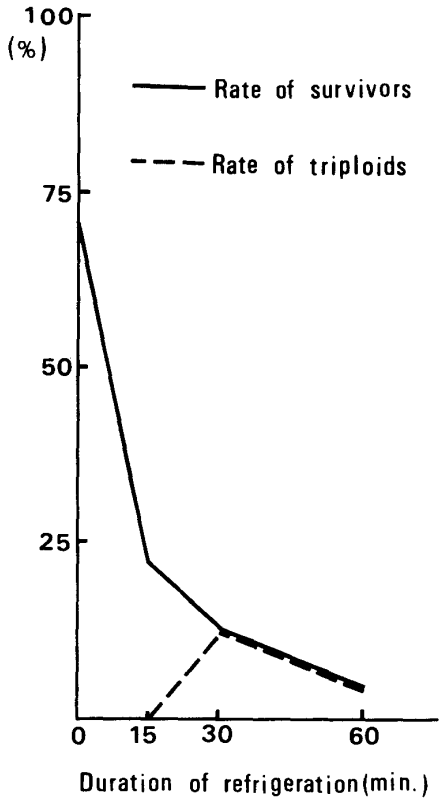

Fig. 1. Relation between survivors and triploids at the feeding stage when the eggs were refrigerated at 0~1°C.

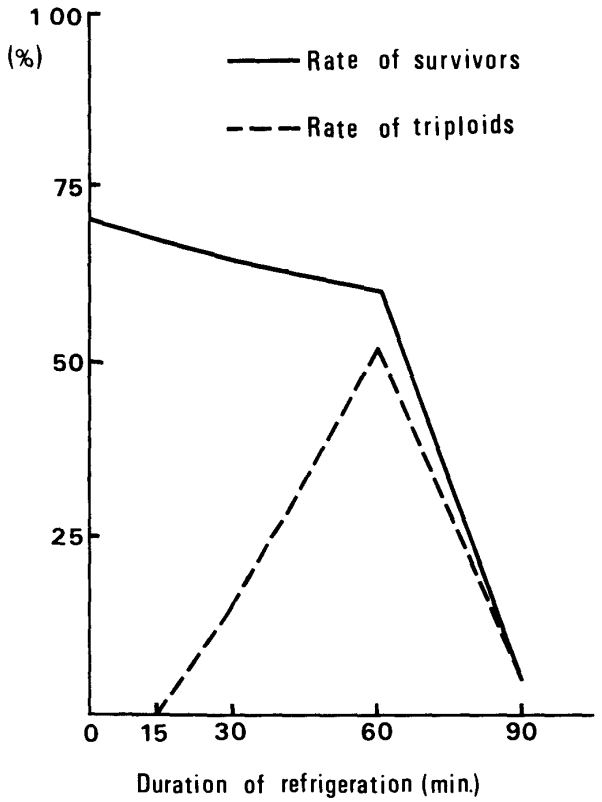

Fig. 2. Relation between survivors and triploids at the feeding stage when the eggs were refrigerated at 2~3°C.

hatched normally, while 558 (87.9%) out of 635 control eggs cleaved normally and 480 (75.6%) hatched normally. At the feeding stage there were 53 (34.6%) and 452 (71.2%) tadpoles in the experimental and the control series, respectively. While 428 (67.4%) control tadpoles completed metamorphosis, only 46 (30.1%) raised from refrigerated eggs did so.

Shortly after tadpoles began to eat, the chromosomes of the tadpoles raised from refrigerated eggs were examined in epidermal cells of removed tail-tips. It was found that 46 (86.8%) of the above 53 tadpoles were triploids. Of the remaining seven tadpoles, three were diploids, three were mosaics consisting of diploid and triploid cells and one could not be determined. The triploid tadpoles completed metamorphosis nearly at the same time as the controls did.

2. Gynogenetic diploids

Sperm suspension was exposed to UV-rays at a distance of 17 cm for 30, 60, 120, 180 or 240 seconds in order to determine the optimum time for inactivation of sperm nuclei. In these five conditions, the total energies were 1080, 2160, 4320, 6480 and 8640 erg/mm^2, respectively. Eggs of normal females were inseminated with the UV-irradiated sperm. The relationship between survivors and haploids at the tail-bud stage when eggs were inseminated with sperm irradiated with UV-rays for different durations is shown in Fig. 3. The rate of viable

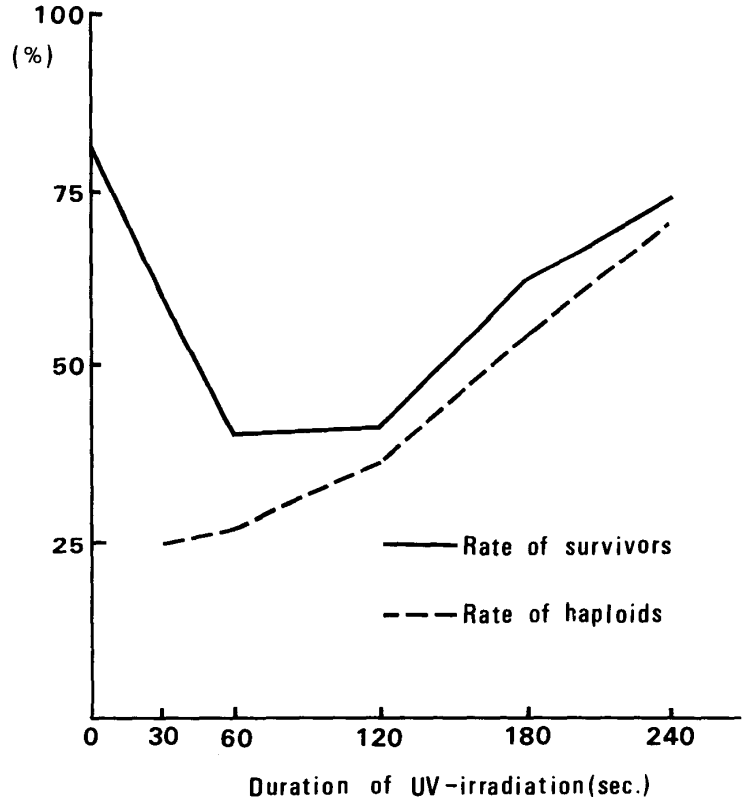

Fig. 3. Relation between survivors and haploids at the tail-bud stage when eggs were inseminated with UV-irradiated sperm.

embryos was the lowest by irradiation for 60 and 120 seconds. When 140 eggs were inseminated with sperm irradiated for 240 seconds, 99 (70.1%) became haploid embryos and two others were haploid-diploid mosaics. Although the remaining three embryos showed haploid syndrome, their chromosome number was not determined. On the basis of the results obtained from this preliminary examination as well as from the above described experiments in suppressing extrusion of the second polar body, gynogenetic diploids were produced by the following procedure.

In 1968, 1970 and 1971, eggs were removed from 2, 6 and 15 normal females,

TABLE 1

Developmental capacity of eggs in three kinds of experimental series and the controls

Series	No. of eggs	No. of cleaved eggs		No. of tail-bud embryos		No. of hatched tadpoles		No. of feeding tadpoles		Ploidy			No. of normal metamorphosed toads
		Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	2n	3n	Others	
Control	635	558 (87.9%)	36 (5.7%)	505 (79.5%)	23 (3.6%)	480 (75.6%)	31 (4.9%)	452 (71.2%)	23 (3.6%)	—	—	—	428 (67.4%)
3n	153	124 (81.0%)	7 (4.6%)	71 (46.4%)	33 (21.6%)	57 (37.3%)	23 (15.0%)	53 (34.6%)	16 (10.5%)	3	46	3	46 (30.1%)
GD	1118	632 (56.5%)	316 (28.3%)	352 (31.5%)	96 (8.6%)	239 (21.4%)	140 (12.5%)	166 (14.8%)	56 (5.0%)	149	4	9	130 (11.6%)
H	430	360 (83.7%)	61 (14.2%)	4 (0.9%)	318 (74.0%)	4 (0.9%)	303 (70.5%)	4 (0.9%)	0 (0.0%)	4	0	0	0 (0.0%)

3n, producing triploids GD, producing gynogenetic diploids H, producing haploids

respectively, and inseminated with sperm exposed to UV-rays for 240 seconds. About 23 minutes later, these eggs were refrigerated at 2~3°C for 60 minutes. It was found that metamorphosed toads were raised from among eggs of 14 females, while the eggs obtained from the other toads all died at the embryonic or early tadpole stage. The developmental capacity of the eggs obtained from the 14 females is presented in Table 1.

Of a total of 1118 eggs, 632 (56.5%) cleaved normally, 352 (31.5%) became normal tail-bud embryos and 239 (21.4%) hatched normally. At the hatching stage, 140 (12.5%) were abnormal and 42 of them revealed haploid syndrome. Of the normally hatched tadpoles, 166 became normal feeding tadpoles. The results of chromosome examination showed that 149 of them were diploids (Fig. 4b), four were triploids (Fig. 4c), five were diploid-triploid mosaics, two were hyperdiploids (2n+1) and two were diploid-tetraploid mosaics. The chromosome number of the remaining four tadpoles could not be examined. At the feeding stage, there were 56 abnormal tadpoles; 38 were edematous and 16 were of a thin and dark body. Eventually, 130 (11.6%) diploids completed metamorphosis. Among these gynogenetic diploids, there were a few color mutants.

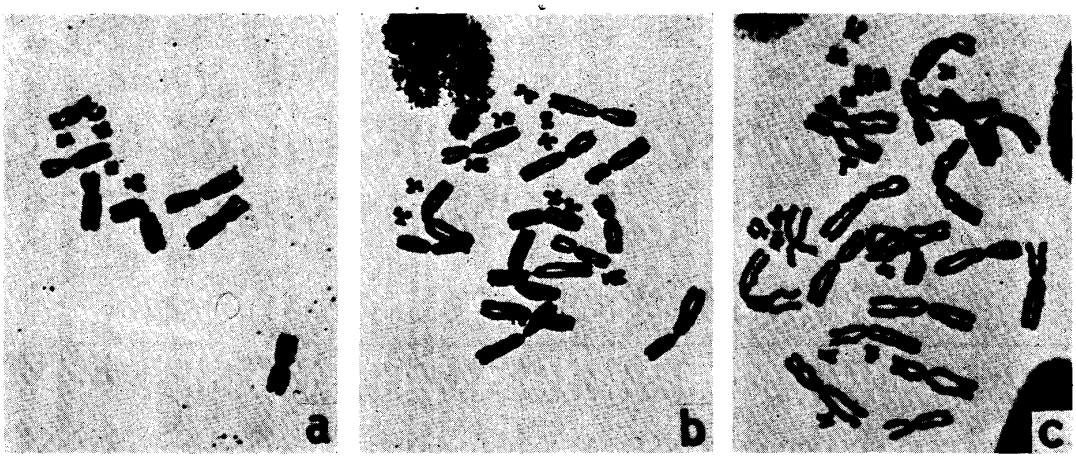

Fig. 4. Metaphase spreads from the tail-tips of haploid, diploid and triploid individuals. ×950
a. A haploid at the hatching stage b. A gynogenetic diploid at the early tadpole stage
c. A triploid at the early tadpole stage

Of 430 eggs inseminated with UV-irradiated sperm, 360 (83.7%) cleaved normally and 322 (74.9%) attained the late tail-bud stage. Of these embryos, 318 were haploids (Fig. 4a), having a typical haploid syndrome such as ascites, microcephaly and ill-development of external gill rudiments. The other four were diploids and normal in appearance.

II. Sex of triploid toads

1. Differentiation of gonads

There were 48 females and 52 males among 100 juvenile and adult triploids, while there were 59 females and 58 males among 117 controls (Table 2). Of

TABLE 2
Sex of triploid toads and the controls

Exp. no.	Triploids					Controls			
	Juveniles		Mature toads			Juveniles		Mature toads	
	Normal ovaries	Normal testes	Abnormal ovaries	Degenerated ovaries	Abnormal testes	Normal ovaries	Normal testes	Normal ovaries	Normal testes
68(3n)	12	13	9	4	16	3	6	8	9
71GD5(3n)	7	4	0	0	0	3	2	8	11
71GD10(3n)	1	2	2	1	3	4	3	10	8
71GD16(3n)	8	10	0	0	0	8	7	5	6
71GD19(3n)	3	3	1	0	1	3	3	6	4
Total	31	32	12	5	20	21	21	37	38

the triploid toads, 54 and 46 were those which had been produced in 1968 and 1971, respectively. Twenty-five of the former and 38 of the latter were killed immediately after metamorphosis to examine their sex. There were 31 females and 32 males among the toads produced in both years. Of 42 controls, 21 were females and 21 were males. The ovaries of the triploid females as well as the testes of the triploid males were apparently the same as those of the controls in structure. The remaining 29 and 8 triploids produced in 1968 and 1971, respectively, were continuously reared. Eight of them died at the age of 7～9 months, while 17 others were killed at the age of one year. The other 12 triploids lived two years or more; four of them lived eight years or more. Among the 37 triploids in total, there were 17 females and 20 males. The age, body length, gonad size and some other characters of these females and males are presented

TABLE 3
Ovaries of adult triploid females

Individual no.	Age (months)	Body length (mm)	Hind-limb length (mm)	Size of gonads		Type of ovaries
				Right (mm)	Left (mm)	
No. 68(3n)2	7	30	33	4.0×2.5	5.0×2.0	2
No. 68(3n)3	7	32	36	3.0×0.5	3.0×0.5	3
No. 68(3n)4	7	30	35	4.5×2.0	5.0×2.0	2
No. 68(3n)7	9	38	41	5.0×2.0	5.5×2.0	2
No. 68(3n)16	12	36	43	8.0×2.5	8.0×2.0	2*
No. 68(3n)17	12	38	42	2.0×0.5	2.0×0.5	3
No. 68(3n)18	12	42	45	8.0×2.5	9.0×2.0	2*
No. 68(3n)19	12	40	43	7.0×2.5	7.5×2.5	2
No. 68(3n)20	12	41	44	2.5×0.5	—	3
No. 68(3n)21	12	39	42	2.0×0.5	2.0×0.5	3
No. 71GD10(3n)2	12	40	44	10.0×2.5	7.5×3.0	2*
No. 68(3n)22	24	47	55	12.0×3.0	8.0×3.0	2*
No. 71GD19(3n)2	25	48	52	12.0×3.0	9.0×3.0	1
No. 71GD10(3n)4	56	51	55	2.5×0.5	2.5×0.5	3
No. 68(3n)27	96	53	55	10.0×4.0	9.5×5.0	1
No. 68(3n)29	132	55	60	Living		
No. 71GD10(3n)6	96	45	52	Living		

* Containing abnormal spermatozoa

TABLE 4
Testes of adult triploid males

Individual no.	Age (months)	Body length (mm)	Hind-limb length (mm)	Size of gonads	
				Right (mm)	Left (mm)
No. 68(3n)1	7	31	36	2.5×2.0	2.5×2.0
No. 68(3n)5	7	32	36	2.5×2.0	2.5×2.0
No. 68(3n)6	8	30	33	2.5×1.5	2.5×1.5
No. 71GD10(3n)1	9	35	41	3.5×2.0	3.5×2.5
No. 68(3n)8	12	38	44	4.0×2.5	4.0×2.5
No. 68(3n)9	12	38	46	4.0×3.0	4.0×3.0
No. 68(3n)10	12	43	48	4.5×3.0	4.5×3.0
No. 68(3n)11	12	42	47	4.0×2.5	4.5×2.5
No. 68(3n)12	12	45	50	5.0×3.0	5.0×3.0
No. 68(3n)13	13	45	51	4.5×3.0	4.5×3.0
No. 68(3n)14	13	41	45	4.0×2.0	4.0×2.0
No. 68(3n)15	13	41	46	4.0×2.0	4.0×2.0
No. 71GD10(3n)3	13	43	48	4.0×3.0	5.0×3.0
No. 71GD19(3n)1	13	45	50	4.5×3.0	4.5×3.0
No. 68(3n)23	26	51	56	5.0×3.0	5.0×3.0
No. 68(3n)24	32	53	60	5.0×3.5	5.0×3.5
No. 68(3n)25	46	42	49	4.0×3.0	4.5×3.0
No. 68(3n)26	59	55	62	5.5×3.0	5.5×3.0
No. 71GD10(3n)5	72	43	49	4.0×2.0	4.0×2.0
No. 68(3n)28	132	53	60	Living	

in Tables 3 and 4. Seven females at the age of one year were 36～42 mm, 39.4 mm on the average, in body length, while ten males at the age of about one year were 38～45 mm, 42.1 mm on the average, in body length. Although both males and females thereafter became slightly larger, they were 55 mm in maximum body length. These triploid males and females were nearly the same in body length as the controls.

The ovaries of adult triploid females were very small and abnormal, as compared with those of the controls (Fig. 5a, b). They were 2.0～12.0 mm in length and 0.5～5.0 mm in width, which were not more than those of juvenile controls that were about 10 days after metamorphosis in differentiation of germ cells. The ovaries of these triploid females were classified into the following three types. The ovaries of type 1 consisted of numerous small cysts containing primary and secondary oogonia and oocytes at the early stage (Fig. 6a). Each ovary had narrow ovarian cavities which were surrounded with a thin layer of flattened cells. There were a few auxocytes which were as large as about 50 μ in diameter. Besides, there were many follicles containing a degenerating auxocyte or a large vacuole in place of the former auxocyte. The ovaries of type 2 consisted of large spherical or polyhedral cysts which were not so numerous as the cysts of type 1 ovaries. When there were a few cysts in each ovary, the ovarian cavities were wide and each cyst was spherical (Fig. 6c). In contrast, when the cysts were comparatively numerous, they were polyhedral and surrounded by slit-like ovarian cavities. In both cases, each cyst was filled with germ cells which mostly appeared as if they were spermatocytes. As a matter

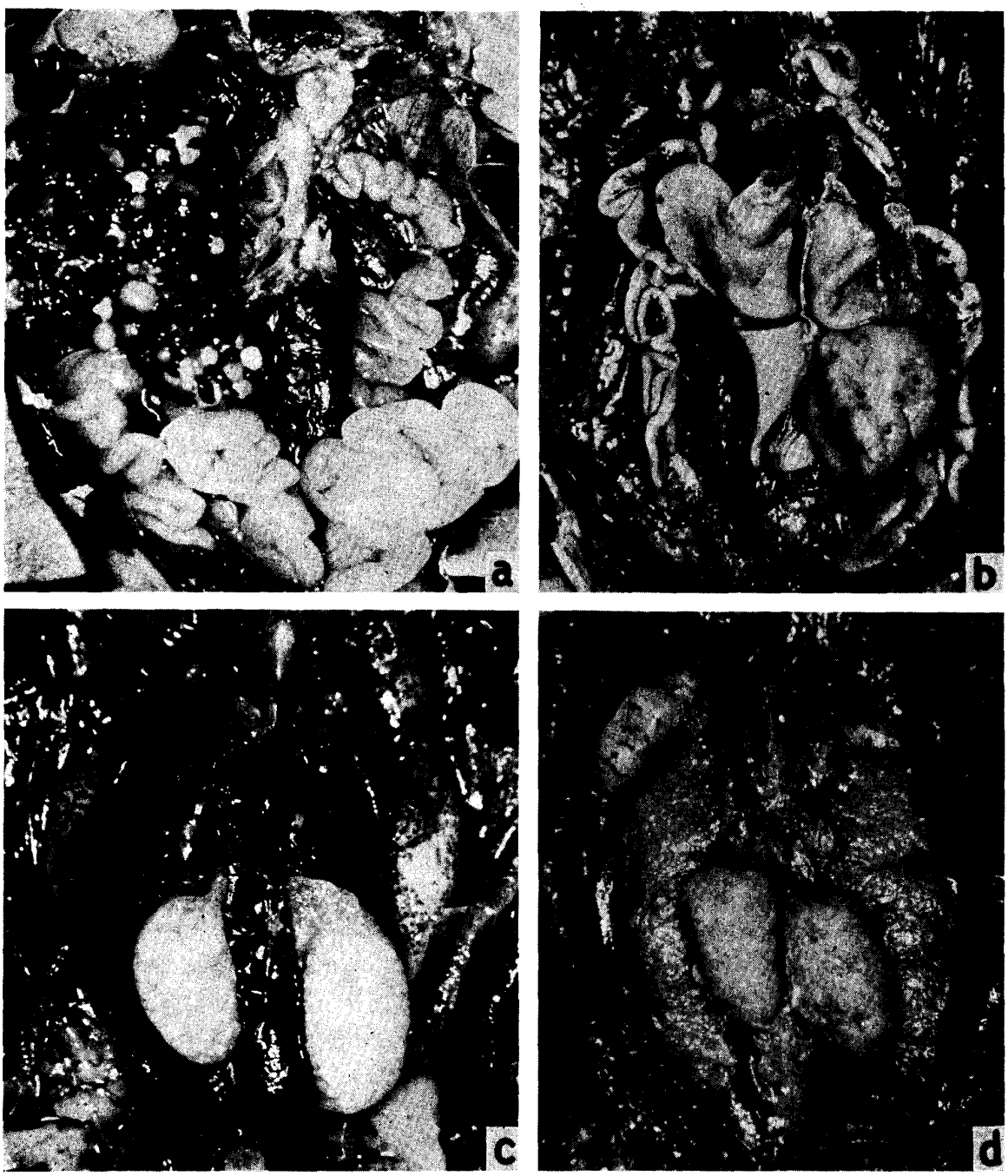

Fig. 5. Gonads of mature triploid toads.

a. Control female, No. 71GD19cont.1, 12 months old ×3.5
b. Triploid female, No. 71GD19(3n)2, 25 months old ×3.5
c. Control male, No. 68cont.2, 24 months old ×4
d. Triploid male, No. 68(3n)24, 32 months old ×4

of fact, a few abnormal spermatozoa were found in some ovaries (Fig. 6d). It was evident that these abnormal spermatozoa were derived from oocytes as a kind of intermediate product of degeneration, since there were various stages of transformation from oocytes to abnormal spermatozoa. In addition to these germ cells, there were a few oogonia and many degenerating germ cells. A few

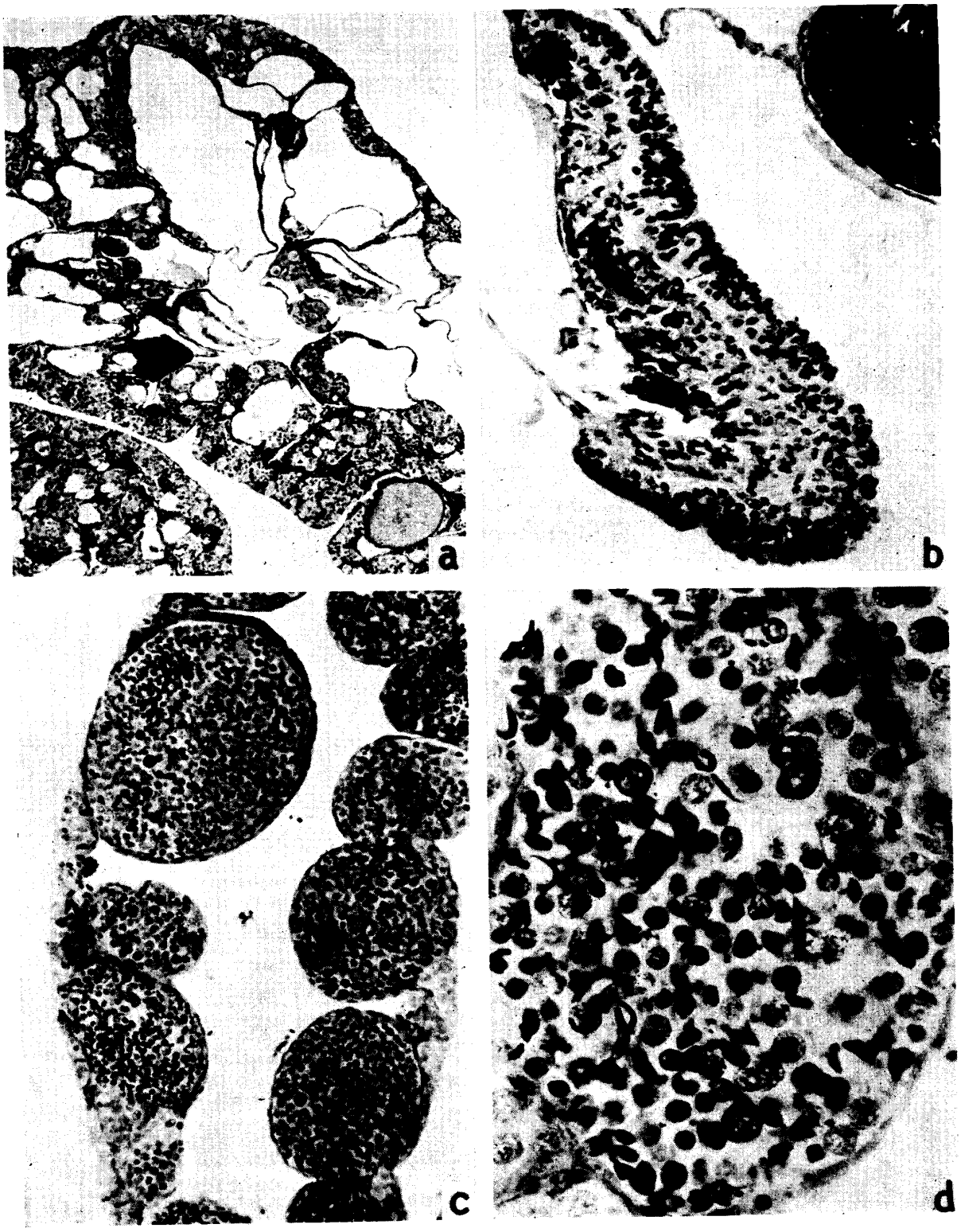

Fig. 6. Cross-sections of the ovaries of mature triploid females.

a. Ovary (type 1) of a triploid female, No. 71GD19(3n)2 ×40
b. Ovary (type 3) of a triploid female, No. 68(3n)21 ×190
c. Ovary (type 2) of a triploid female, No. 68(3n)18 ×95
d. The same as (c) ×380

mitotic figures of germ cells were also found. The ovaries of type 3 were extremely small, that is, 2.0~3.0 mm in length and 0.5 mm in width, and very degenerative (Fig. 6b). Each ovary consisted of shrunk cortical portion and the walls of slit-like ovarian cavities. There was no sign of hypertrophy in the medullary portion. In one female, No. 68(3n)20, the left gonad was changed into a part of the fat body.

The types of ovaries of 15 triploid females whose gonads were examined are presented in Table 3. The ovaries of two, eight and five females were of type 1, type 2 and type 3, respectively. In spite of differences in ovarian type, all these females had well-developed Müllerian ducts. Of the eight females having ovaries of type 2, four had a few abnormal spermatozoa. Two triploid females are now living at the age of 8 and 11 years, respectively.

The testes of adult triploid males were nearly normal in size and shape (Table 4, Fig. 5c, d). Those of four males preserved at the age of 7~9 months were 2.5~3.5 mm in length and 1.5~2.5 mm in width. Ten other males at the age of 12 or 13 months had testes which were 4.0~5.0 mm in length and 2.0~3.0 mm in width. Five males preserved at the age of 2~6 years had testes which were 4.0~5.5 mm in length and 2.0~3.5 mm in width. In addition to these 19 males in total, there was a living triploid male which was 11 years old.

Although the testes of the triploid males were nearly the same as the controls in appearance, their seminiferous tubules were remarkably larger in cross section

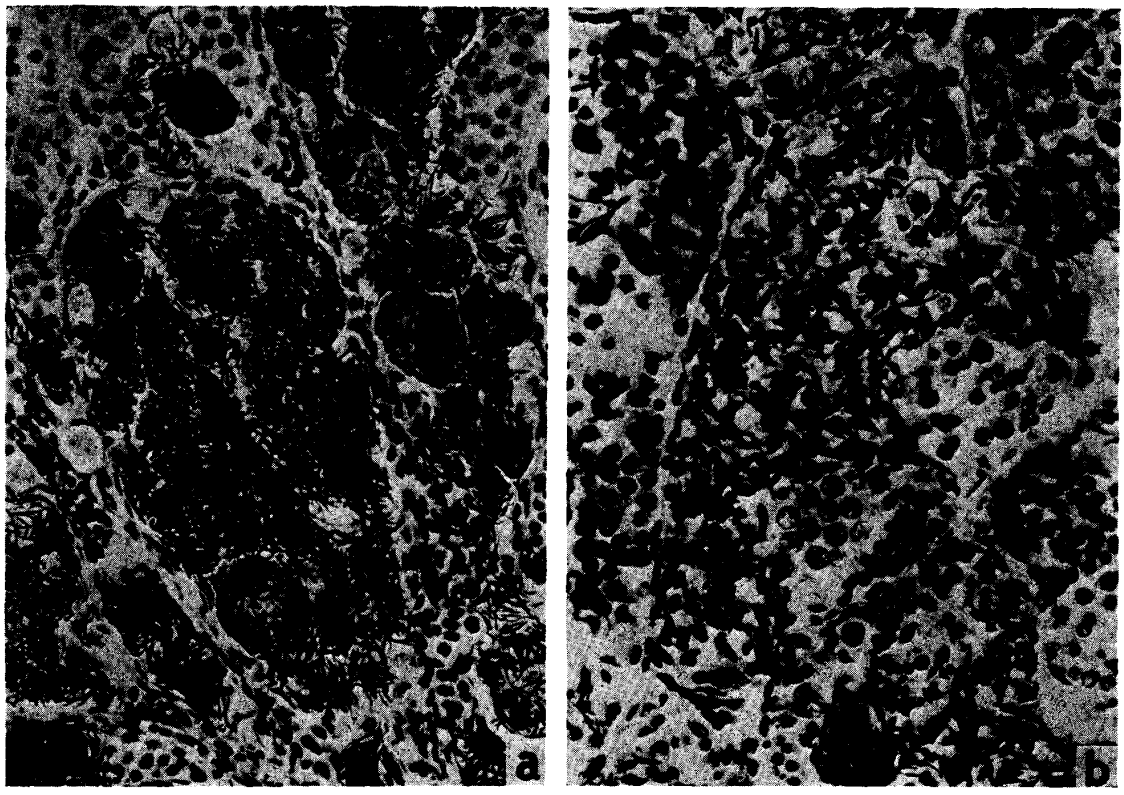

Fig. 7. Cross-sections of testes of a mature triploid and the control. × 190

a. Control male, No. 68cont.1 b. Triploid male, No. 68(3n)9

than those of the controls. There were no individual differences in inner structure of the testes among the triploid males. Spermatogenesis was almost normal from spermatogonia through first spermatocytes. Thereafter, meiotic division was very abnormal and produced large and small spermatids. As a result of abnormal divisions, many germ cells degenerated, while the remaining spermatids were transformed into spermatozoa. These spermatozoa were not uniform in size and shape. They were remarkably fewer and most of them were distinctly larger than those of the control males (Fig. 7).

2. Reproductive capacity

All the triploid males produced in 1968 were 38~45 mm in body length and revealed their secondary sexual characters at the age of one year. Seven of them were divided into two groups of four and three animals. On June 30, 1969, the four triploid males belonging the first group and a diploid male were mated with two normal females by artificial fertilization. As a result, 45~64% of the respective number of eggs cleaved normally by sperm of the triploid males, while 89% of eggs did so by the diploid male (Table 5). Thereafter, in the experimental series derived from sperm of triploid males, 8~39% became normal tail-bud embryos and 0~14% hatched normally. All or most of the embryos produced by each triploid male died or became abnormal before the hatching stage. Although 19 out of 381 eggs in total became normal tadpoles, most of them could not eat and died in a short time. Only five tadpoles began to eat and grew slowly. They were all hyperdiploids; four were trisomics and the other had two additional chromosomes. All of them were feeble and died before attaining the metamorphosis stage. In contrast, 82% of eggs inseminated with sperm of the diploid male became normal tail-bud embryos, 79% hatched nor-

TABLE 5

Results of crosses between diploid females and triploid males

Date of experiments	Parents		No. of eggs	No. of cleaved eggs		No. of tail-bud embryos		No. of hatched tadpoles		No. of feeding tadpoles	
	Female	Male		Normal (%)	Abnormal (%)	Normal (%)	Abnormal (%)	Normal (%)	Abnormal (%)	Normal (%)	Abnormal (%)
June 30, 1969	S64F$_2$f2, 4	68(2n)1	103	92 (89.3)	0 (0)	84 (81.6)	2 (1.9)	81 (78.6)	1 (1.0)	75 (72.8)	1 (1.0)
		68(3n)9	78	50 (64.1)	19 (24.4)	6 (7.7)	21 (26.9)	0 (0)	0 (0)	0 (0)	0 (0)
		68(3n)10	79	46 (58.2)	17 (21.5)	31 (39.2)	12 (15.2)	5 (6.3)	25 (31.6)	2 (2.5)	0 (0)
		68(3n)11	83	46 (55.4)	23 (27.7)	27 (32.5)	17 (20.5)	12 (14.5)	23 (27.7)	3 (3.6)	0 (0)
		68(3n)12	141	64 (45.4)	25 (17.7)	17 (12.1)	22 (15.6)	2 (1.4)	21 (14.9)	0 (0)	0 (0)
July 29, 1969	S64F$_3$f5,	68(2n)2	75	70 (93.3)	5 (6.7)	60 (80.0)	7 (9.3)	51 (68.0)	10 (13.3)	51 (68.0)	0 (0)
	S65F$_2$f2	68(3n)13	54	1 (1.9)	8 (14.8)	1 (1.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		68(3n)14	121	2 (1.7)	15 (12.4)	2 (1.7)	1 (0.8)	1 (0.8)	1 (0.8)	0 (0)	0 (0)
		68(3n)15	96	15 (15.6)	5 (5.2)	8 (8.3)	9 (9.4)	3 (3.1)	5 (5.2)	1 (1.0)	0 (0)

mally, and 73% became feeding tadpoles.

The three triploid males belonging to the second group and a diploid male were mated with two normal diploid females on July 29, 1969. Of 75 eggs inseminated with sperm of the diploid male, 93% cleaved normally, 80% became normal tail-bud embryos and 68% hatched and grew normally. In contrast with this, only 2~16% of the respective number of eggs cleaved normally and 2~8% became normal tail-bud embryos by sperm of triploid males (Table 5). Although four (1.5%) out of 271 eggs in total hatched normally, only one tadpole began to eat. This tadpole died of ill-development before the chromosomes were examined.

III. Sex of gynogenetic diploids

The sex of 90 juvenile and 39 mature gynogenetic diploids was examined (Table 6). It was found that all of them were females except one juvenile and one mature toad. In contrast with this, there was nearly an equal number of males and females in the control series; of 266 juvenile, 133 were females and 133 were males. At the mature stage, there were 75 females and 86 males. The ovaries of the female gynogenetic diploids were nearly the same in size, shape and structure as those of control females, except one female which had degenerated ovaries. The two exceptional males found among gynogenetic diploids had normal testes in which there was no indication that they came from ovaries by sex reversal. Their Müllerian ducts were also the same in thickness as those of the control males. Thus, the two males seemed to have been raised from eggs which were fertilized with uninjured spermatozoa and escaped from the effect of refrigeration.

TABLE 6

Sex of gynogenetic diploids and the controls

Exp. no.	Gynogenetic diploids				Controls			
	Juvenile		Mature		Juvenile		Mature	
	♀	♂	♀	♂	♀	♂	♀	♂
68GD1	14	1	—	—	27	25	—	—
68GD2	6	0	—	—	9	6	—	—
70GD1	3	0	—	—	17	23	—	—
70GD2	3	0	—	—	12	16	—	—
70GD3	10	0	9	—	14	11	8	12
70GD5	3	0	—	—	5	7	—	—
71GD5	10	0	4*	0	3	2	8	11
71GD6	5	0	—	—	2	3	23	29
71GD8	5	0	—	—	8	7	10	10
71GD10	8	0	12	1	4	3	10	8
71GD11	1	0	4	0	4	6	5	6
71GD14	6	0	—	—	17	14	—	—
71GD16	10	0	6	0	8	7	5	6
71GD19	5	0	3	0	3	3	6	4
Total	89	1	38	1	133	133	75	86

* One of them had degenerated ovaries.

DISCUSSION

The assumption that the male of the genus *Rana* is heterogametic was first made by WITSCHI (1914) who reexamined the results obtained by R. HERTWIG (1912) from crossing experiments between different sex races of *Rana temporaria* and *R. esculenta,* and was subsequently confirmed by his own studies on crosses of hermaphrodites with normal males and females of different sex races and crosses between different sex races in *Rana temporaria* (1922, 1923a, b, 1929). PARMENTER (1925) and KAWAMURA (1939) supported the male heterogamety from their studies on the sex of parthenogenetically produced frogs or tadpoles in *Rana pipiens* and *Rana nigromaculata,* respectively. Recently, KAWAMURA and NISHIOKA (1977) made the same support on the basis of the sex of gynogenetic diploids in *Rana nigromaculata, R. brevipoda, R. japonica, R. tsushimensis* and *R. rugosa.*

On the other hand, examination of the sex of the offspring of sex-reversed frogs seemed to be the most useful means in determining the heterogametic sex. The offspring of a male *Rana japonica* produced parthenogenetically were all females (MORIWAKI 1959). In the same species, the offspring of phenotypic males which had been reversed from genetic females by administration of androgen were almost exclusively females (KAWAMURA and YOKOTA, 1959). The same matter was also confirmed in *Rana nigromaculata* (KAWAMURA and NISHIOKA, 1977). These findings could not be explained by other than male heterogamety.

SATO (1938) has reported that the chromosomes of *Bombina orientalis* are 24 in diploid number, and that there are no particular chromosomes corresponding to sex chromosomes. However, the present author (1967) has confirmed that this species is a typical gonochorist and the sex is so stable as this cannot be reversed by injection of sex hormones or by transplantation of a gonad removed from a young toad of the other sex into the body cavity of a tadpole, or by rearing tadpoles under high temperature until the completion of metamorphosis. Thus, it is impossible to determine the heterogametic sex of *Bombina orientalis* by making use of sex-reversed individuals.

The present study has definitely demonstrated the following two facts; that gynogenetic diploids are all females and that there is nearly an equal number of males and females among triploids raised from fertilized eggs by suppressing the extrusion of the second polar body. If sex chromosomes make a prereductional separation, the fact that all the gynogenetic diploids are females seems to indicate the male heterogamety of *Bombina orientalis.* If sex chromosomes make postreductional separation, as observed by WITSCHI (1922, 1924, 1929) in *Rana temporaria,* it is difficult to determine the heterogametic sex, because the gynogenetically produced females may be XX in the case of male heterogamety or may be ZW in the case of female heterogamety. On the other hand, the fact that there is nearly an equal number of males and females among triploid toads cannot be explained by female heterogamety, because all the triploids should be ZZW, that is, females in the latter case. It seems to be evident that the male is hetero-

gametic sex in *Bombina orientalis*, that the sex chromosomes make prereductional separation, and that male and female triploid toads are XXY and XXX in sex chromosome condition.

There was another interesting finding in the present study. This is the production of abnormal spermatozoa in the degenerative ovaries of some triploid females. Although no hypertrophy of the medullary portion was found in these ovaries, the spherical or polyhedral cysts were somewhat similar to seminiferous tubules in the point that they were filled with many small germ cells of similar size and shape. These germ cells mostly appeared to be oocytes which could not make a start of growth. Abnormal spermatozoa seemed to be a kind of intermediate products derived from oocytes on the way of degeneration. It is probable that the cysts of the degenerative ovaries had a physiological condition somewhat similar to the seminiferous tubules of normal testes.

SUMMARY

1. Both triploids and gynogenetic diploids were produced and their sex was examined in order to determine the heterogametic sex in *Bombina orientalis*.

2. Thirty percent of fertilized eggs refrigerated after insemination became metamorphosed triploids, while 67 percent of the control eggs attained the completion of metamorphosis. Among the triploids there was nearly an equal number of males and females.

Mature triploid females had very small, abnormal ovaries which were classified into three types. Some triploids produced a few abnormal spermatozoa in their ovaries. Mature triploid males produced some spermatozoa that were mostly abnormal. Eggs of normal diploid females inseminated with sperm of triploid males developed into abnormal embryos and tadpoles.

3. Twelve percent of eggs refrigerated after insemination with UV-irradiated sperm became gynogenetic diploid toads. All the gynogenetic diploids were females.

4. On the basis of the sex of both triploids and gynogenetic diploids, it is assumed that the male is heterogametic in *Bombina orientalis*.

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