Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ. 9: 213–238 (1987)

Sex Determining Mechanism in *Buergeria buergeri* (SCHLEGEL) II. The Effects of Sex Hormones on the Differentiation of Gonads and the Offspring of Sex-reversed Females

By

Shigeru OHTA

Laboratory for Amphibian Biology, Faculty of Science, Hiroshima University, Hiroshima 730, Japan

(With 5 Text-figures)

ABSTRACT

The mechanism of sex determination in *Buergeria buergeri* (SCHLEGEL) was studied by making use of sex-reversed females. There was nearly an equal number of one-nucleolate and two-nucleolate tadpoles in those produced from two pairs of males and females just as in the tadpoles collected from the field. The one-nucleolate and two-nucleolate tadpoles collected from the field as well as those raised in the laboratory were administered estrogen or androgen in order to obtain sex-reversed frogs. Masculinization occurred in a greater or less degree in many of one-nucleolate females administered estrogen or androgen at the tadpole stage. Seven of them administered estrogen or androgen attained sexual maturity. Offspring were obtained from two mature sex-reversed genetic females administered estrogen. Feminization also occurred to a certain degree in many of the juvenile two-nucleolate males administered estrogen at the tadpole stage.

Of the 1067 offspring obtained from the two mature sex-reversed genetic females (1-nu) mated with three normal females (1-nu), 243 (22.8%) had no nucleolus, 525 (49.2%) had one nucleolus, and 299 (28.0%) had two nucleoli, when examined at the neurula, tail-bud and tadpole stages. While all the anucleolate individuals did not live beyond the tail-bud stage, the one- and two-nucleolate individuals mostly survived normally and became females and males, respectively.

It was confirmed that the sex chromosomes are of the ZZ/ZW-type in *Buergeria buergeri* and that the nucleolar organizer is situated in the Z chromosome and inherited as a Mendelian character.

INTRODUCTION

In a previous paper, the present author (1986) has reported that the common bell-ring frog *Buergeria buergeri* is female heterozygous in sex determination and that chromosome pair No. 7 is sex chromosomes of ZZ/ZW-type. One of the homologues of this chromosome pair found in mitoses of bone marrow cells from female frogs has a satellite at the tip of the long arm, while the other homologue has none. In contrast, both homologues of chromosome pair No. 7 from male frogs have a satellite. Moreover, the somatic cell nuclei of male tadpoles have

two nucleoli, while those of female tadpoles have a single nucleolus. The nucleolus seems to have been derived from the nucleolar organizer of the Z chromosome.

Recently, SCHMID, HAAF, GEILE and SIMS (1983) and SCHMID, SIMS, HAAF and MACGREGOR (1986) have analyzed the mitotic and meiotic chromosomes of the marsupial frog *Gastrotheca riobambae* with various banding techniques and have disclosed that this species is male heterogametic and chromosome pair No. 4 is sex chromosomes of the XX/XY-type. The only nucleolus organizer region of the karyotype is located in the short arm of the X chromosome. As found in the cell nuclei of *Buergeria buergeri* tadpoles, SCHMID, SIMS, HAAF and MACGREGOR (1986) have confirmed that the cell nuclei of various somatic tissues from male frogs have one nucleolus, while those from female frogs have two nucleoli, corresponding to the number of X chromosomes in each cell.

ELSDALE, FISCHBERG and SMITH (1958), WALLACE (1960) and GURDON (1977) have observed that all the anucleolate embryos obtained from a cross of two heterozygotes for a mutation reducing the nucleolar number in *Xenopus laevis* die before feeding, although they survive until an abnormal tadpole stage. The present author assumed that one-fourth (WW) of the offspring obtained from mating of a sex-reversed genetic female (ZW) with a normal female (ZW) in *Buergeria buergeri* would be lethal, as they have no nucleoli. Then, he conducted some experiments of sex reversal by administering sex hormones in tadpoles. Fortunately, as two mature sex-reversed genetic females were obtained, matings were made between these sex-reversed females and normal females. The results of these experiments will be reported in the present paper.

MATERIALS AND METHODS

Buergeria buergeri (SCHLEGEL) collected from Sandankyo, Togochi-cho, Hiroshima Prefecture were used as materials. Administration of sex hormones was performed at the tadpole stage. Some of the tadpoles were collected from the field in 1982, while others were obtained from two pairs of mature males and females. These two pairs, female No. WF,82×male No. WM,82 and female No. WF,83× male No. WM,83, laid eggs and produced tadpoles in the laboratory in 1982 and 1983, respectively. Of the 447 tadpoles collected from the field, 234 had one nucleolus, and the other 213 had two nucleoli. Of the 320 tadpoles raised from eggs of a female mated with a male in 1982, 153 had one nucleolus, and the other 167 had two nucleoli. Of the 579 tadpoles raised from eggs of a female mated with a male in 1983, 295 had one nucleolus, and the other 284 had two nucleoli (Table 1).

The tadpoles collected from the field (group I) as well as those raised in the laboratory (groups II and III) were divided into three series. Those of one series were used as controls. Those of another series were administered estrogen after divided into two parts. Each of the tadpoles of one part was injected with 10, 20 or 40 μg of estrogen, while the tadpoles of the other part were immersed in water

TABLE 1
Number of one-nucleolate (1-nu) and two-nucleolate (2-nu) tadpoles obtained from two pairs of *Buergeria buergeri* in 1982 and 1983

Series No.	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 1-nu and 2-nu tadpoles	
	Female	Male						1-nu	2-nu
II	WF, 82	WM, 82	431	431 (100%)	320 (74.2%)	320 (74.2%)	320 (74.2%)	153 (47.8%)	167 (52.2%)
III	WF, 83	WM, 83	637	613 (96.2%)	590 (92.6%)	581 (91.2%)	579 (90.9%)	295 (50.9%)	284 (49.1%)

solution of estrogen which was 125, 250 or 500 μg/l in concentration. The water solution was made by adding 0.2 ml ethanol including 125 μg estrogen and was renewed every other day. Each of the tadpoles of the remaining series was injected with 250 μg of androgen. As estrogen and androgen, Ovahormon benzoat and Enarmon manufactured by Teikoku-Zoki Pharmaceutical Company were used, respectively. The estrogen preparation is standardized to contain 1 mg, 50,000 units, of estradiol benzoate per ml of RINGER's solution, while the androgen preparation is standardized to contain 25 mg, 1,250 units, of testosterone propionate per ml of sesame oil.

Gonads were fixed in NAVASHIN's fluid, together with kidneys, sectioned at 12~15 μm in thickness and stained with HEIDENHAIN's iron hematoxylin. The number of nucleoli which was counted at the tadpole stage was confirmed by observing the cell nuclei of the uriniferous tubules of juvenile or adult frogs.

The embryos and tadpoles were reared at room temperature (22~26°C). A part of the offspring obtained in 1985 from the frogs of the experimental series and the controls were reared outdoors where the maximum water temperature was 34°C.

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

OBSERVATION

I. Effects of sex hormones on sex differentiation in one-nucleolate individuals

1. Controls

Sex differentiation was observed by rearing a series of 167 tadpoles collected from the field and another series of 102 tadpoles raised from eggs in the laboratory. Their gonads were examined at six developmental stages. Although the sex of 20 tadpoles and 56 frogs, 76 in total, of the former series and that of 31 tadpoles and 57 frogs, 88 in total, of the latter series were elucidated by anatomical and histological observations, sex differentiation in the remaining tadpoles and frogs was unknown, owing to postmortem changes or loss (Table 2).

TABLE 2
Number and sex of 1-nu individuals administered sex hormones

Series No.	Dosage (μg)	Tadpoles							Frogs							
		St. IV~XV		St. XIX, XX					Within 2 months after metamorph.					More than 5 months after metamorph.		
		♀u	I	♀n	♀u	I	⚤	♂n	♀n	♀u	I	⚤	♂n	♀n	⚤	♂n
Cont. I	—	10	4	5	1				5	3				48(1)*		
Cont. II	—	2	5		3				6					14		
Cont. III	—	6	5	1	9				14	5				18(6)*		
Total		18	14	6	13				25	8				80(7)*		
Est. I	20	1			2				3					5(1)*		
	40			2	1				3			1		4		
Est. II	10			2	1				1					9(2)*		
	20								3	2		2		1(1)*		
	40			6	3		1		1					2		
Est. III-1	10								3					6(3)*		
	20	2			4		1					1		3(3)*		
	40													6(4)*		
Est. III-2	125**								6					3(1)*		1
	250**	2			5		1		1			7		10(8)*		2
	500**				4				1	1		2		7(3)*	1	2
Total		5		10	20		3		22	3		13		56(26)*	1	5
Test. I	250				2		2			1	1	4		5		
Test. II	250				6	1	1			2		6	1	8		
Test. III	250		2				3	1				5		9(8)*		2
Total			2		8	1	6	1		3	1	15	1	22(8)*		2

*, Number of immature frogs shown in parentheses
**, Concentration (μg/l) of estradiol benzoate
♀n, Female with normal ovaries
♀u, Female with underdeveloped ovaries
♂n, Male with normal testes
⚤, Hermaphrodite with transforming gonads
I, Individual with indifferent gonads

The ovaries began to differentiate at tadpole stages IV~VII. When 24 tadpoles at these stages were examined, they were 20.2~26.2 mm in total length. Their right gonads were 0.8~1.4 mm in length and 0.05~0.1 mm in width, while the left gonads were 0.8~1.5 mm in length and 0.05~0.1 mm in width. Of these tadpoles, 14 had indifferent gonads containing a few germ cells in each cross-section. Some germ cells had one or two yolk granules (Fig. 1a). The gonads of the remaining ten tadpoles were differentiated into ovaries, consisting of the cortical and medullary portions. The cortical portions contained many primary oogonia, while no germ cells were found in the medullary portions.

Eight tadpoles at stages X~XV were 26.0~35.0 mm in total length. Their right gonads were 0.8~1.6 mm in length and 0.1~0.2 mm in width, while their left

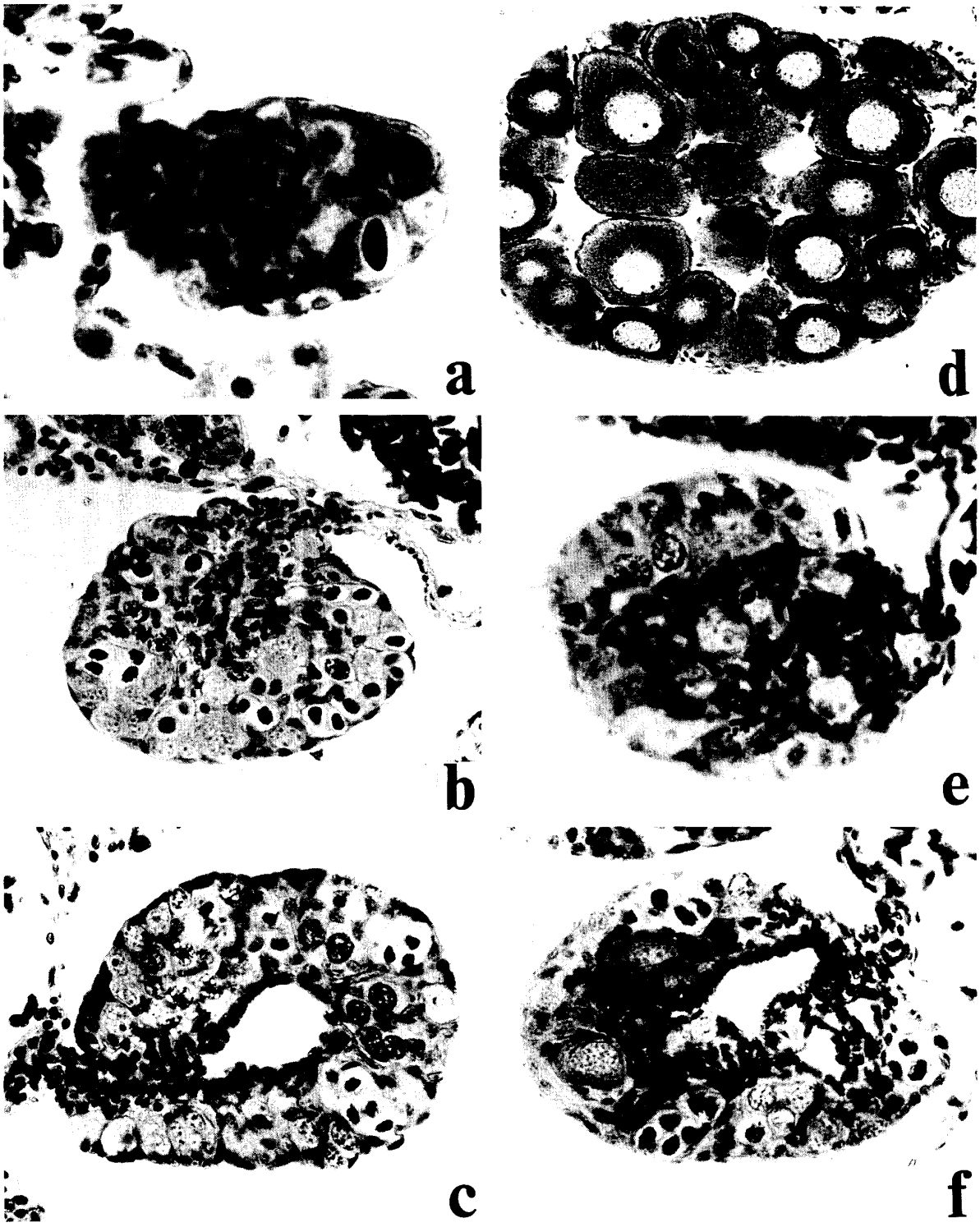


Fig. 1. Cross-sections of the gonads of *Buergeria buergeri* having a single nucleolus in each somatic cell.

a. Indifferent gonad of a control tadpole at stage VI. ×600
b. Normal ovary at the earliest stage of differentiation in a control tadpole at stage XV. ×300
c. Normal ovary of a control tadpole at stage XX. ×300
d. Normal ovary of a control frog at age of two months after metamorphosis. ×150
e. Hermaphroditic gonad of a juvenile frog immediately after completion of metamorphosis reared in estradiol solution of 250 μg/l. ×400
f. Underdeveloped ovary of a two-month-old frog reared in estradiol solution of 250 μg/l. ×320

gonads were 0.7~1.8 mm in length and 0.1~0.2 mm in width. Their gonads were differentiated into ovaries, which were further developed than those of tadpoles at the foregoing stages. These ovaries contained many primary and secondary oogonia and a few first oocytes at the spireme stage. The primary and secondary oogonia were 10~17 μm and about 9 μm in diameter, respectively (Fig. 1b).

Nineteen tadpoles at stages XIX and XX shortly before metamorphosis were 34.2~40.0 mm in total length. Their right gonads were 0.6~1.6 mm in length and 0.1~0.2 mm in width, while their left gonads were 0.7~1.5 mm in length and 0.1~0.2 mm in width. The gonads of six tadpoles were ovaries containing ovarian cavities covered with a thin layer of rete cells. The cortical portions consisted of many small cysts containing primary and secondary oogonia and oocytes at the early stages of meiosis (Fig. 1c). The remaining 13 tadpoles had ovaries which contained a small number of young oocytes in the cortical portions.

The gonads of 23 frogs immediately after completion of metamorphosis, 7.5~16.3 mm in body length, were examined. The right gonads were 0.5~1.3 mm in length and 0.1~0.3 mm in width, while the left gonads were 0.5~1.2 mm in length and 0.1~0.4 mm in width. All these frogs were females. Fifteen of them had ovaries containing ovarian cavities. In cross-sections of the ovaries, several oocytes, being 40~70 μm in diameter, were found. The remaining eight females had ovaries which were at the earliest stage of differentiation and contained a small number of oocytes.

Ten other frogs within 2 months after metamorphosis were 11.5~17.7 mm in body length. All of them were females. Their right ovaries were 0.7~2.4 mm in length and 0.2~0.6 mm in width, while the left ovaries were 0.7~1.9 mm in length and 0.2~0.5 mm in width. The ovaries were filled with many auxocytes, being 70~100 μm in diameter. In the ovaries, there were well-developed ovarian cavities (Fig. 1d).

The gonads of 80 frogs at the ages of more than five months after metamorphosis were examined. These frogs were 32.2~57.2 mm in body length and all of them were females. Their right ovaries were 3.8~29.0 mm in length and 1.8~15.4 mm in width, while the left ovaries were 4.3~27.2 mm in length and 1.6~20.2 mm in width. These ovaries were filled with auxocytes which were 1~3 mm in diameter, except those of seven females. The latter were somewhat immature and contained small auxocytes, being less than 1 mm in diameter.

2. Effects of estrogen on sex differentiation

Forty-one tadpoles collected from the field and 238 tadpoles raised from eggs in the laboratory were administered estrogen. The gonads of six tadpoles and 16 frogs, 22 in total, derived from the former tadpoles, and those of 32 tadpoles and 84 frogs, 116 in total, derived from the latter tadpoles were observed at two tadpole stages and three frog stages. The gonads of the remaining tadpoles and frogs were not observed owing to postmortem changes or loss (Table 2).

a. Tadpoles

Five tadpoles at stages IV~XV were somewhat smaller than the controls, being 18.5~25.6 mm in total length. Although their gonads were inferior to those of the controls in development, they were almost normally differentiating into ovaries.

Thirty-three tadpoles at stages XIX and XX were somewhat smaller than the controls, being 20.2~29.0 mm in total length. Their gonads were also smaller than those of the controls. Three of them were juvenile hermaphrodites whose gonads were transforming from ovaries to testes. Proliferation of rete cells was found in the medullary portions. Some germ cells were surrounded with these cells. The gonads of the remaining 30 tadpoles were underdeveloped or almost normal ovaries in inner structure.

b. Frogs

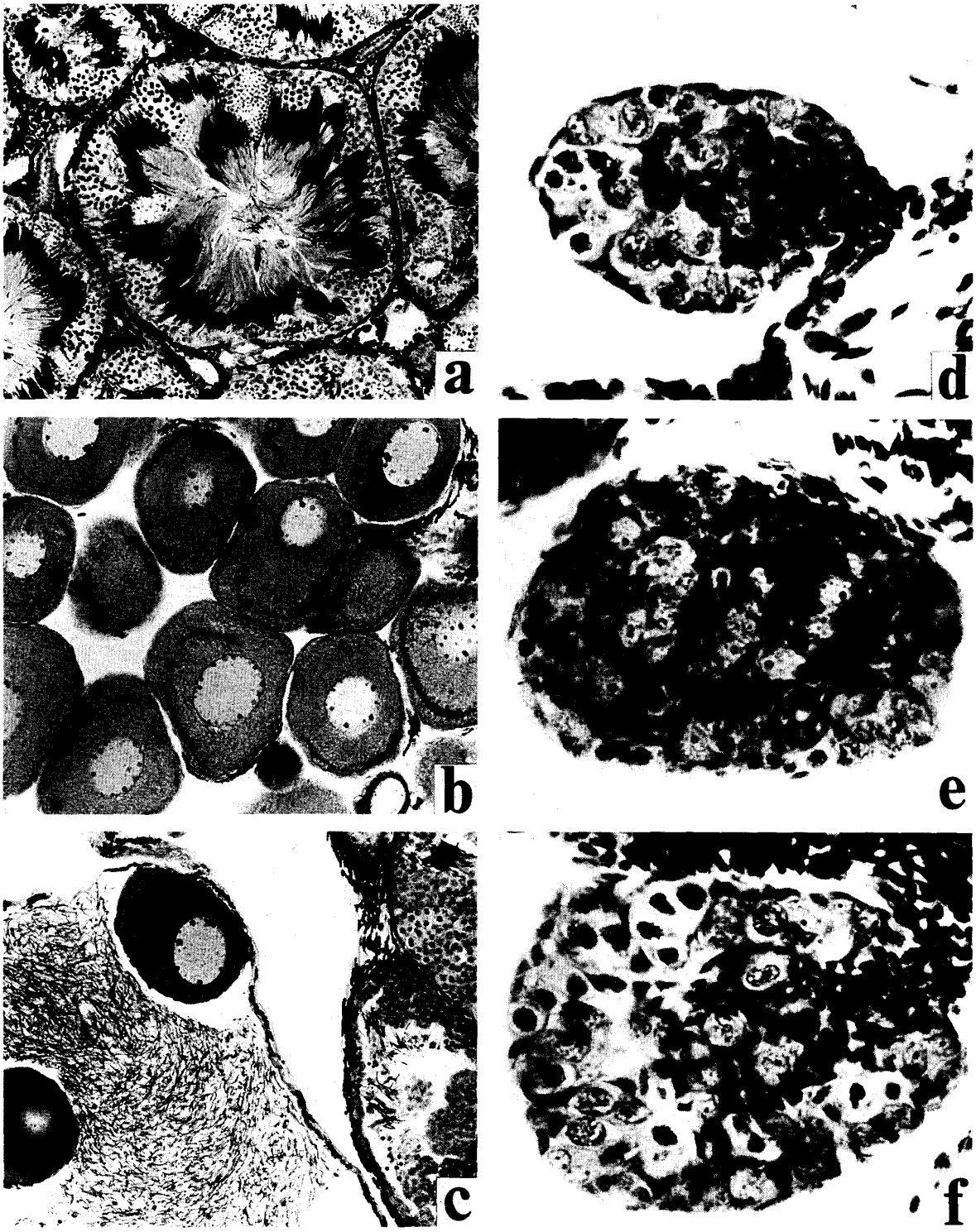
Seventeen juvenile frogs immediately after completion of metamorphosis were nearly the same in body length as well as in gonad size as the controls. Nine of them were juvenile hermaphrodites with gonads transforming from ovaries to testes. Proliferation of rete cells in the medullary portions was observed. A part of these cells migrated into the cortical portions and surrounded some germ cells (Fig. 1e). The remaining eight frogs were females whose ovaries were almost normal or underdeveloped in inner structure.

Twenty-one frogs at the age of about 2 months after metamorphosis did not distinctly differ from the controls in body length as well as in gonad size. Four of them were juvenile hermaphrodites whose gonads were transforming from ovaries to testes. Proliferating rete cells surrounded some germ cells in the cortical portions, although these gonads contained ovarian cavities. The remaining 17 frogs were females whose ovaries were almost normal or underdeveloped (Fig. 1f).

Of the 62 frogs at the ages of more than five months after metamorphosis, five were males reversed from genetic females and had normal testes. They were first identified as males by possessing thumb pads. Of these five males, one, 125E, No.1 (1-nu), two, 250E, Nos.3 and 4 (1-nu), and two, 500E, Nos.1 and 2 (1-nu), were treated with 125, 250 and 500 μg estrogen per liter during their tadpole stage, respectively. In 1985, two of the five male frogs, 125E, No.1 (1-nu) and 500E, No.2 (1-nu), were used in mating experiments. The former male was 38.7 mm in body length. The right testis was 5.9 mm in length and 2.9 mm in width, and the left testis was 5.9 mm in length and 2.8 mm in width. The latter was 38.6 mm in body length. The right testis was 3.9 mm in length and 2.8 mm in width, and the left testis was 4.6 mm in length and 3.6 mm in width. The right testis of each male was used in mating experiments, while the left testis was cut into cross-sections. These cross-sections showed that the testes were normal in inner structure. The central lumens of the seminiferous tubules were filled with many compact bundles of normal spermatozoa (Fig. 2a). The other three of the five males were 35.1~36.3 mm in body length. The right testes were 2.9~5.5 mm in length and 1.9~3.4 mm in width, and the left testes were 2.6~4.1 mm in length and 1.6~2.5 mm in width. These testes were almost the same in inner structure

as those of the foregoing two males.

One of the remaining 57 frogs was a hermaphrodite, 36.1 mm in body length. The right gonad was a normal ovary which was 3.9 mm in length and 1.2 mm in width. This ovary was filled with normal auxocytes which were 130~170 μm in diameter (Fig. 2b). The left gonad consisted of two different parts. The anterior part occupying two-fifths of the gonad was an ovary which was the same in



differentiation as the right ovary, while the posterior part occupying three-fifths of the gonad was a well-developed testis, in which the seminiferous tubules were filled with many spermatocytes and spermatogonia. Besides, numerous spermatozoa were found in the central lumens of seminiferous tubules, and also in the ovarian cavities (Fig. 2c).

The other 56 frogs were nearly the same in body length and gonad size as the controls. They were all normal females, although 26 were somewhat immature and had ovaries which contained small auxocytes, being less than 1 mm in diameter.

These findings seem to show that administration of estrogen disturbs the differentiation of the ovaries in many genetic females having a single nucleolus and gives rise to sex reversal in a few of them.

3. Effects of androgen on sex differentiation

Twenty-six tadpoles collected from the field and 108 tadpoles raised from eggs in the laboratory were injected with androgen. The gonads of four tadpoles and 11 frogs, 15 in total, derived from the former tadpoles, and those of 14 tadpoles and 33 frogs, 47 in total, derived from the latter tadpoles were examined at five developmental stages. The gonads of the other individuals could not be examined owing to postmortem changes or loss (Table 2).

a. Tadpoles

All the tadpoles injected with androgen were somewhat smaller than the controls in total length. Two tadpoles at stages X and XV, being 19.3 mm and 21.1 mm in total length, had indifferent gonads. Sixteen tadpoles at stages XIX and XX, being 28.1~30.2 mm in total length, had gonads which were somewhat smaller than those of the controls. One of them was a male converted from a female and had testes in which the cortical portions were almost degenerated. The medullary portions consisted of many rete cells and included numerous germ cells. Six other tadpoles were juvenile hermaphrodites having gonads transforming from ovaries to testes. In their gonads, rete cells surrounded some germ cells (Fig. 2d). Another tadpole had indifferent gonads. The remaining eight tadpoles had gonads differentiating into ovaries, which were inferior to those of the controls in differentiation. No sign of sex-reversal was found in these gonads.

Fig. 2. Cross-sections of the gonads of *Buergeria buergeri* having a single nucleolus in each somatic cell.

a. Normal testis of a two-year-old frog reared in estradiol solution of 500 μg/l. ×130
b. Normal ovary on the right side of a one-year-old hermaphrodite which was reared in estradiol solution of 500 μg/l. ×130
c. Testicular portion containing a few testis-ova on the left side of the same frog as (b). ×130
d. Hermaphroditic gonad of a tadpole at stage XX injected with 250 μg of testosterone propionate. ×400
e. Testis of a frog at two months after metamorphosis injected with 250 μg of testosterone propionate. ×400
f. Hermaphroditic gonad of a frog at two months after metamorphosis injected with 250 μg of testosterone propionate. ×400

b. Frogs

Nine frogs immediately after completion of metamorphosis, being 6.1~12.5 mm in body length, were smaller than the controls in size. Their gonads were also smaller than those of the controls. Seven of them were juvenile hermaphrodites with gonads transforming from ovaries to testes. While the cortical portions were underdeveloped, the medullary portions were hypertrophied and the rete cells surrounded some germ cells. Another frog had still indifferent gonads. The remaining one had underdeveloped gonads which were somewhat differentiating to ovaries.

The eleven other frogs within 2 months after metamorphosis were 7.3~13.3 mm in body length. They were smaller than the controls in body length. One of them was a male reversed from a female. The cortical portions had almost degenerated, and the seminiferous tubules began to differentiate (Fig. 2e). Eight other frogs were juvenile hermaphrodites whose gonads were transforming from ovaries to testes. In these gonads, proliferation of rete cells was found. Some of these cells surrounded germ cells (Fig. 2f). The remaining two frogs were females, whose ovaries were inferior to those of the controls in differentiation.

Of the 24 frogs at the ages of more than five months after metamorphosis, two were males reversed from genetic females. One of these males was eight months old and 33.5 mm in body length. The right testis was 1.9 mm in length and 0.8 mm in width, while the left testis was 2.2 mm in length and 0.9 mm in width. The other frog was ten months old and 34.4 mm in body length. The right testis was 4.2 mm in length and 1.7 mm in width, while the left testis was 3.5 mm in length and 1.5 mm in width. In the testes of the former male, the seminiferous tubules were filled with numerous spermatocytes and spermatogonia, but they contained no spermatozoa. In the testes of the latter male, the seminiferous tubules were filled with many bundles of normal spermatozoa.

The remaining 22 frogs, being 14.5~41.0 mm in body length, were normal females, and were almost the same in body and ovarian sizes as the controls. The ovaries of 14 of these females were fully developed, although three had no oviducts. The remaining eight females were somewhat immature and had ovaries containing small auxocytes.

These findings seem to show that the administration of androgen suppresses the differentiation of the ovaries of genetic females having a single nucleolus and gives rise to complete sex reversal in a few genetic females.

II. Effects of sex hormones on sex differentiation in two-nucleolate individuals

1. Controls

As controls, a series of 125 tadpoles collected from the field and another series of 105 tadpoles raised from eggs in the laboratory were used. The gonads of 25 tadpoles and 53 frogs, 78 in total, of the former series and 38 tadpoles and 55 frogs,

93 in total, of the latter series were observed at six developmental stages. The sex differentiation of the other tadpoles and frogs was unknown owing to postmortem changes or loss (Table 3).

All the two-nucleolate tadpoles were about the same in total length as the control one-nucleolate tadpoles.

Of the 22 tadpoles which were examined at stages IV~VII and 21.2~27.2 mm in total length, 13 had indifferent gonads which could not be distinguished from those of one-nucleolate tadpoles. The remaining nine tadpoles were juvenile hermaphrodites having gonads differentiating into testes from ovaries. Some

TABLE 3
Number and sex of 2-nu individuals administered sex hormones

Series No.	Dosage (μg)	Tadpoles					Frogs					
		St. IV~XV		St. XIX, XX			Within 2 months after metamorph.					More than 5 months after metamorph.
		I	⚥	⚥	♂u	♂n	♀n	♀u	⚥	♂u	♂n	♂n
Cont. I	—	5	10		7	3					7	46(2)*
Cont. II	—	4			10				1		10	12
Cont. III	—	7	4		13					4	21	7
Total		16	14		30	3			1	4	38	65(2)*
Est. I	20		1	14					1			6
	40			11	2			1	3			3
Est. II	10			1					9			3(1)*
	20						3	1	2			
	40			2			1		3			4
Est. III-1	10									1		2
	20		1	5	1				2			8(3)*
	40				1				1			6(1)*
Est. III-2	125**								2	1		6
	250**		2		3				2	1	1	13(1)*
	500**				4				1	1	1	4
Total			4	33	11		4	2	26	4	2	55(6)*
Test. I	250				4	5				4	4	4(2)*
Test. II	250					3				3	13	21(5)*
Test. III	250	2				4					5	9(6)*
Total		2			4	12				7	22	34(13)*

*, Number of immature frogs shown in parentheses
**, Concentration (μg/l) of estradiol benzoate
♀n, Female with normal ovaries
♀u, Female with underdeveloped ovaries
♂n, Male with normal testes
♂u, Male with underdeveloped testes
⚥, Hermaphrodite with transforming gonads
I, Individual with indifferent gonads

spermatogonia were found in the medullary portions in each cross-section of the gonads, while many oogonia were found in the cortical portions.

Of the eight tadpoles which were examined at stages X~XV and 26.2~32.0 mm in total length, three had indifferent gonads which could not be found in one-nucleolate control tadpoles. The remaining five tadpoles were juvenile hermaphrodites having gonads differentiating into testes. The medullary portions of these gonads were more differentiated than those of the gonads at the foregoing stages, while the cortical portions contained many primary and secondary oogonia and oocytes at the spireme stage (Fig. 3a).

Of the 33 tadpoles which were examined at stages XIX and XX and 32.0~35.9 mm in total length, 30 had gonads differentiating into testes. The medullary portions contained numerous rete cells and many spermatogonia, while the cortical portions almost degenerated. The remaining three were males having testes whose cortical portions had degenerated.

All the two-nucleolate juvenile frogs were nearly the same in body length as the one-nucleolate ones at the same age.

Twenty-five juveniles immediately after completion of metamorphosis, being 9.7~15.7 mm in body length, had gonads which were somewhat smaller than those of one-nucleolate controls. The right gonads were 0.5~1.0 mm in length and 0.1~0.2 mm in width, while the left gonads were 0.6~1.2 mm in length and 0.1~0.2 mm in width. Of these frogs, 21 were males in which the cortical portions of the testes were completely degenerated and the medullary portions contained many primary spermatogonia. The remaining four frogs were males whose testes were underdeveloped.

Of the 18 frogs at the age of about two months, being 10.2~16.2 mm in body length, 17 were males. Their right testes were 0.4~1.4 mm in length and 0.1~0.2 mm in width, and the left testes were 0.3~1.4 mm in length and 0.1~0.2 mm in width. The seminiferous tubules of their testes began to differentiate (Fig. 3b). The remaining one frog was unusual in the differentiation of gonads. The right gonad was 0.6 mm in length and 0.2 mm in width, and the left was 0.7 mm in length and 0.2 mm in width. This frog was a juvenile hermaphrodite. The cortical portions of the gonads were well developed, while the ovarian cavities were surrounded with a thick layer of rete cells which included primary spermatogonia.

Of the 65 frogs examined at the ages of more than five months after metamorphosis, 63 were mature males with thumb pads, and had well-developed testes. The other two frogs were somewhat immature and had small testes. The 65 frogs were 33.0~43.2 mm in body length. The right testes were 1.3~5.7 mm in length and 0.6~3.3 mm in width, and the left testes were 1.4~6.0 mm in length and 0.6~3.5 mm in width. The seminiferous tubules of the testes of the mature frogs were filled with many bundles of normal spermatozoa. There were numerous spermatocytes and spermatogonia in the areas along the walls (Fig. 3c). The seminiferous tubules in the immature frogs contained a small number of spermatozoa.

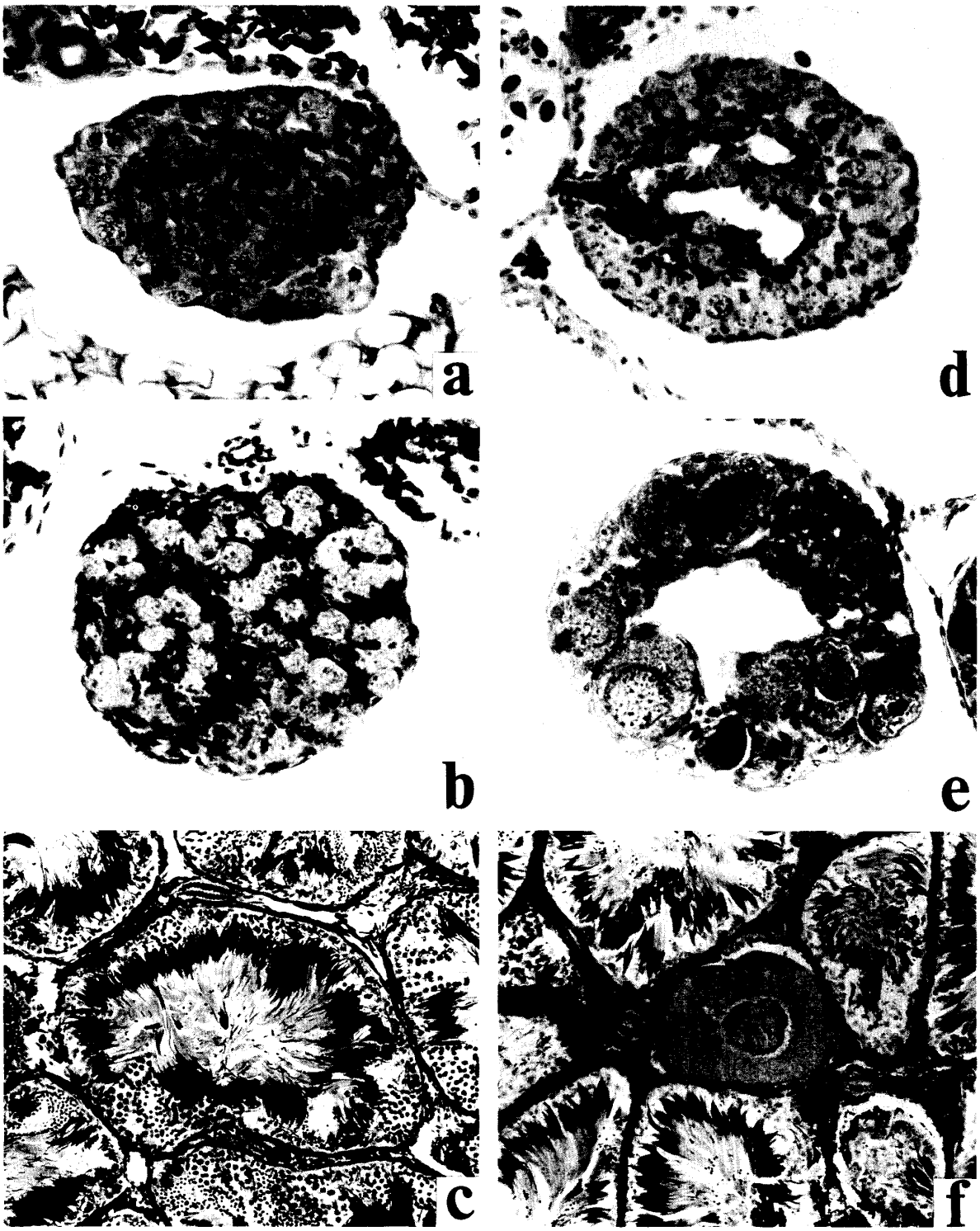


Fig. 3. Cross-sections of *Buergeria buergeri* having two nucleoli in each somatic cell.

a. Testis at the earliest stage of differentiation in a control tadpole at stage XV. ×300
b. Normal testis of a control two-month-old frog. ×300
c. Normal testis of a control two-year-old frog. ×130
d. Hermaphroditic gonad of a tadpole at stage XX injected with 20 μg of estradiol benzoate. ×240
e. Hermaphroditic gonad of a two-month-old frog injected with 10 μg of estradiol benzoate. ×300
f. Testis-ovum contained in the testis of a one-year-old male which was injected with 20 μg of estradiol benzoate. ×130

2. Effects of estrogen on sex differentiation

A series of 59 tadpoles collected from the field and another series of 234 tadpoles raised from eggs in the laboratory were treated with estrogen. The gonads of 28 tadpoles and 14 frogs, 42 in total, of the former series, and those of 20 tadpoles and 79 frogs, 99 in total, of the latter series were examined at five developmental stages. The gonads of the remaining tadpoles and frogs were not observed owing to postmortem changes or loss (Table 3).

a. Tadpoles

Four tadpoles were examined at stages X~XV. They were about the same in size as the controls, being 21.2~25.2 mm in total length. They were juvenile hermaphrodites whose gonads began to differentiate into testes, although the cortical portions were inferior in differentiation to those of the controls.

Forty-four tadpoles at stages XIX and XX were examined. They were smaller than the controls, being 29.0~32.2 mm in total length, while their gonads were larger than those of the controls. Of these tadpoles, 33 were juvenile hermaphrodites whose gonads were transforming from testes to ovaries. These gonads contained ovarian cavities which were covered with numerous rete cells. The cortical portions were superior to those of the controls in differentiation (Fig. 3d). The remaining 11 tadpoles had almost normal gonads which were differentiating as testes.

b. Frogs

The gonads of 11 juvenile frogs immediately after completion of metamorphosis, being 7.7~12.7 mm in body length, and those of 27 frogs at the age of about two months, being 10.9~12.3 mm in body length, were examined. One juvenile and three two-month-old frogs were females. The ovarian cavities of their ovaries were surrounded with rete cells. However, the medullary portions had almost degenerated and contained no germ cells. The cortical portions were superior in differentiation to those of the two-nucleolate controls. One juvenile and one two-month-old frogs had gonads differentiating into ovaries. Although these gonads had no ovarian cavities, no spermatogonia were found in the medullary portions. Six juvenile and 20 two-month-old frogs were hermaphrodites whose gonads were transforming from testes to ovaries. These gonads contained ovarian cavities, although the rete cells were abundant in the medullary portions (Fig. 3e). The remaining three juvenile and three two-month-old frogs had testes or gonads which had almost differentiated into testes. These testes had no evident sign of sex-reversal from ovaries.

Fifty-five frogs at the ages of more than five months after metamorphosis, being 25.8~44.7 mm in body length, were all males. However, one of them had testes containing 21 testis-ova which were 140~220 μm in diameter, and, moreover, it had oviducts which were about 100 μm in diameter at the level of the gonads (Fig. 3f). Six other frogs were immature males.

These findings seem to show that estrogen exerts a feminizing effect on the differentiation of testes to some extent in many individuals having two nucleoli in each cell nucleus, although no complete sex-reversal occurs.

3. Effects of androgen on sex differentiation

A series of 29 tadpoles collected from the field and another series of 112 tadpoles raised from eggs in the laboratory were injected with androgen. The gonads of nine tadpoles and 12 frogs, 21 in total, of the former series, and those of nine tadpoles and 51 frogs, 60 in total, of the latter series were examined at five developmental stages. The gonads of the remaining tadpoles and frogs were not examined owing to postmortem changes or loss (Table 3).

a. Tadpoles

Two tadpoles at stages X and XV, being 19.2 mm and 20.6 mm in total length, respectively, and 16 tadpoles at stages XIX and XX, being 22.5~29.0 mm in total length, were examined. All of them were somewhat smaller than the controls in total length. Their gonads were slightly inferior to those of the controls in differentiation. The two tadpoles at stages X and XV had indifferent gonads. Four of the other 16 tadpoles had underdeveloped testes, while the remaining 12 had normal testes.

b. Frogs

Nine frogs immediately after completion of metamorphosis, being 8.0~11.5 mm in body length, and 20 frogs at the age of about two months, being 8.3~24.4 mm in body length, were examined. They were nearly the same in body length as the controls. They had almost normal testes or those which were slightly inferior in differentiation to those of the controls.

Thirty-four frogs at the ages of more than five months after metamorphosis were examined. One of the males had oviducts which were about 100 μm$\times$130 μm in diameter at the level of the gonads. Thirteen other frogs were immature males and had small testes. The remaining frogs were mature normal males.

These observations indicate that androgen does not suppress differentiation of testes.

III. Offspring of genetic females sex-reversed by administration of sex hormones

By treatment of one-nucleolate tadpoles with sex-hormones, several male frogs were obtained in 1983. In order to confirm the genetic sex of these males, mating experiments were made in 1985 between two of them and three normal one-nucleolate females collected from the field.

1) A normal female, WF, No.1 (1-nu), was mated with a sex-reversed female, 500E, No.2 (1-nu), treated with 500 μg estrogen per liter and a normal male, WM, No.1 (2-nu), collected from the field. 2) A normal female, WF, No.2 (1-nu), was mated with a sex-reversed female, 125E, No.1 (1-nu), treated with 125 μg estrogen

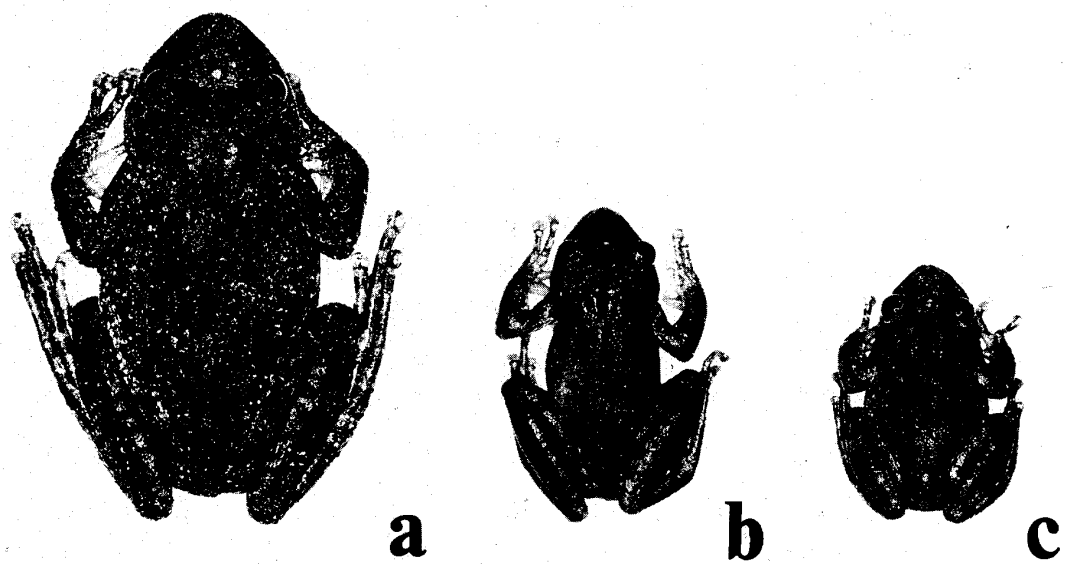


Fig. 4. A female and two males of *Buergeria buergeri* used in the crossing experiments. ×0.7

a. Female, WF, No.1 (1-nu), collected from Sandankyo, Togochi-cho, Hiroshima Prefecture.
b. Male, WM, No.1 (2-nu), collected from the same place as (a).
c. Male, 500E, No.2 (1-nu), having a single nucleolus in each somatic cell. This male was reared in estradiol solution of 500 μg/l.

per liter and a normal male, WM, No.2 (2-nu), collected from the field. 3) A normal female, WF, No.3 (1-nu), was mated with the same two males as used in mating series 2 (Fig. 4).

1. Developmental capacity

The developmental capacity of the offspring of the two sex-reversed genetic females (1-nu) mated with three normal females (1-nu) in the three experimental series (Nos. 1~3) was compared with that of the offspring in the three control series (Nos. 1~3) at the stages from cleavage to completion of metamorphosis. In the three experimental series, of the 387, 445 and 289 eggs of the three females, WF, Nos.1, 2 and 3 (1-nu), inseminated with sperm of the two sex-reversed females, 500E, No.2 (1-nu) and 125E, No.1 (1-nu), 356 (92.0%), 441 (99.1%) and 270 (93.4%) cleaved normally, respectively, and 271 (70.0%), 330 (74.2%) and 195 (67.5%) became normal tail-bud embryos, respectively, while 85 (22.0%), 104 (23.4%) and 64 (22.1%) died at the tail-bud stage, respectively (Fig. 5b). In the three control series, of the 141, 164 and 153 eggs of the three normal females, WF, Nos.1, 2 and 3 (1-nu), inseminated with the two normal males, WM, Nos.1 and 2 (2-nu), 126 (89.4%), 147 (89.6%) and 140 (91.5%) cleaved normally, respectively, and 100 (70.9%), 114 (69.5%) and 129 (84.3%) became normal tail-bud embryos, respectively, while 26 (18.4%), 30 (18.3%) and 8 (5.2%) died at the tail-bud stage, respectively (Fig. 5a). In the three experimental series, 251 (64.9%), 240 (53.9%) and 151 (52.2%) of the inseminated eggs hatched normally, 240 (62.0%),

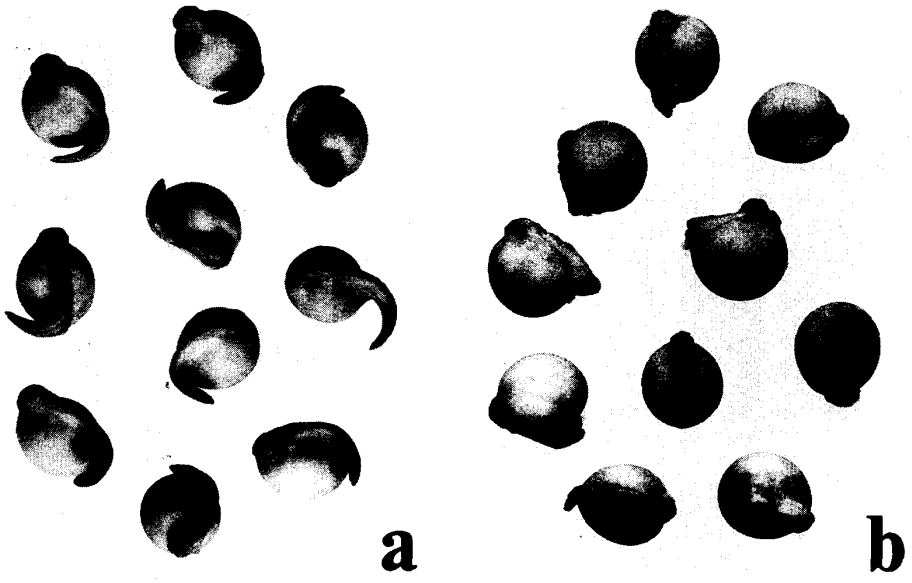


Fig. 5. Embryos having no nucleoli and the controls. ×3

a. Control embryos at the tail-bud stage.

b. Anucleolate embryos which became abnormal at the early tail-bud stage.

240 (53.9%) and 146 (50.5%) became feeding tadpoles, and 237 (61.2%), 230 (51.7%) and 138 (47.8%) metamorphosed normally, respectively. In the three control series, 99 (70.2%), 79 (48.2%) and 91 (59.5%) of the inseminated eggs hatched normally, 87 (61.7%), 79 (48.2%) and 90 (58.8%) became feeding tadpoles and 78 (55.3%), 77 (47.0%) and 83 (54.2%) metamorphosed normally, respectively (Table 4).

These data seem to show that the offspring of normal females mated with the sex-reversed genetic females are approximately the same as those of the same

TABLE 4

Developmental capacity of the offspring of sex-reversed 1-nu females and the controls

Series No.	Parents		No. of eggs	No. of normally cleaved eggs	No. of normal neurulae	No. of normal tail-bud embryos	No. of normally hatched tadpoles	No. of normally feeding tadpoles	No. of metamorphosed frogs
	Female	Male							
1	WF, No. 1	WM, No. 1	141	126 (89.4%)	126 (89.4%)	100 (70.9%)	99 (70.2%)	87 (61.7%)	78 (55.3%)
		500E, No. 2	387	356 (92.0%)	356 (92.0%)	271 (70.0%)	251 (64.9%)	240 (62.0%)	237 (61.2%)
2	WF, No. 2	WM, No. 2	164	147 (89.6%)	144 (87.8%)	114 (69.5%)	79 (48.2%)	79 (48.2%)	77 (47.0%)
		125E, No. 1	445	441 (99.1%)	434 (97.5%)	330 (74.2%)	240 (53.9%)	240 (53.9%)	230 (51.7%)
3	WF, No. 3	WM, No. 2	153	140 (91.5%)	137 (89.5%)	129 (84.3%)	91 (59.5%)	90 (58.8%)	83 (54.2%)
		125E, No. 1	289	270 (93.4%)	259 (89.6%)	195 (67.5%)	151 (52.2%)	146 (50.5%)	138 (47.8%)

females mated with normal males in developmental capacity.

2. Number of nucleoli

In the three control series (Nos. 1, 2 and 3), the number of nucleoli in each somatic cell nucleus was examined in 413 individuals including six abnormal neurulae, 64 abnormal tail-bud embryos, 74 abnormal hatched tadpoles, 13 abnormal feeding tadpoles and 256 normally feeding tadpoles. The results

TABLE 5

Number of 0-nu, 1-nu, 2-nu and 3-nu offspring obtained from sex-reversed 1-nu females and the controls

Series	Parents		Stage	No. of offspring				
	Female	Male		Total	0-nu	1-nu	2-nu	3-nu
Cont.	WF,	WM,	Tail-bud embryo (ab)	26		16	10	
No. 1	No. 1	No. 1	Hatched tadpole (ab)	1		1		
	(1-nu)	(2-nu)	Feeding tadpole (ab)	12		5	7	
			Feeding tadpole (nor)	87		51	36	
Cont.	WF,	WM,	Neurula (ab)	3			3	
No. 2	No. 2	No. 2	Tail-bud embryo (ab)	30		15	15	
	(1-nu)	(2-nu)	Hatched tadpole (ab)	35		16	19	
			Feeding tadpole (nor)	79		42	37	
Cont.	WF,	WM,	Neurula (ab)	3		2	1	
No. 3	No. 3	No. 2	Tail-bud embryo (ab)	8		1	7	
	(1-nu)	(2-nu)	Hatched tadpole (ab)	38		22	16	
			Feeding tadpole (ab)	1				1
			Feeding tadpole (nor)	90		45	45	
Total				413		216 (52.3%)	196 (47.5%)	1 (0.2%)
Exp.	WF,	500E,	Tail-bud embryo (ab)	85	79	5	1	
No. 1	No. 1	No. 2	Hatched tadpole (ab)	20		12	8	
	(1-nu)	(1-nu)	Feeding tadpole (ab)	11		6	5	
			Feeding tadpole (nor)	240		163	77	
Exp.	WF,	125E,	Neurula (ab)	7		3	4	
No. 2	No. 2	No. 1	Tail-bud embryo (ab)	104	98	3	3	
	(1-nu)	(1-nu)	Hatched tadpole (ab)	90		58	32	
			Feeding tadpole (nor)	240		140	100	
Exp.	WF,	125E,	Neurula (ab)	11	7	2	2	
No. 3	No. 3	No. 1	Tail-bud embryo (ab)	64	59	2	3	
	(1-nu)	(1-nu)	Hatched tadpole (ab)	44		29	15	
			Feeding tadpole (ab)	5		2	3	
			Feeding tadpole (nor)	146		100	46	
Total				1067	243 (22.8%)	525 (49.2%)	299 (28.0%)	

ab, abnormal nor, normal

showed that 216 (52.3%) of the 413 individuals had one nucleolus, 196 (47.5%) had two nucleoli and the remaining one (0.2%) had three nucleoli (Table 5). Of the 256 normally feeding tadpoles, 138 (53.9%) had one nucleolus and 118 (46.1%) had two nucleoli. The single three-nucleolate individual was an abnormal feeding tadpole which was assumed to be a triploid, as the nuclei of this tadpole were about 1.5 times larger in diameter than those of the other tadpoles.

In the experimental series (Nos. 1, 2 and 3), the number of nucleoli in each somatic cell nucleus was examined in 1067 individuals including 18 abnormal neurulae, 253 abnormal tail-bud embryos, 154 abnormal hatched tadpoles, 16 abnormal feeding tadpoles and 626 normally feeding tadpoles. The results showed that 243 (22.8%) of the 1067 individuals had no nucleolus, 525 (49.2%) had one nucleolus and the remaining 299 (28.0%) had two nucleoli (Table 5). Thus, the anucleolate, one-nucleolate and two-nucleolate individuals seemed to exist at a ratio of 1:2:1 in the offspring between the normal one-nucleolate females and the sex-reversed genetic one-nucleolate females. As all the individuals having no nucleolus died at the tail-bud stage in the experimental series, the remaining tadpoles had one or two nucleoli. When these two kinds of tadpoles were counted, there were 510 (64.1%) one-nucleolate and 286 (35.9%) two-nucleolate tadpoles among 796 normal and abnormal ones.

3. Sex

In the three control series, the sex of 28, 34 and 34 one-nucleolate frogs or tadpoles, 96 in total, and that of 30, 28 and 25 two-nucleolate frogs, 83 in total, were examined. All of the 96 one-nucleolate individuals were females with ovaries. While 81 of the 83 two-nucleolate individuals were males with testes, the remaining two were females with ovaries (Table 6).

These two females were considered to be triploids, as the cell nuclei of their uriniferous tubules were fairly larger in diameter than those of the 81 two-nucleolate and 96 one-nucleolate individuals. Except for these two two-nucleolate females, the offspring between the three normal females and the two normal males in the control series consisted of 96 (54.2%) females and 81 (45.8%) males.

In the three experimental series, the sex of 127, 66 and 80 one-nucleolate frogs or tadpoles, 273 in total, and the sex of 58, 75 and 41 two-nucleolate frogs or tadpoles, 174 in total, were examined. It was found that all the one-nucleolate individuals were females, while all the two-nucleolate individuals were males. There were 273 (61.1%) females and 174 (38.9%) males in the three experimental series. These numbers of females and males show a ratio of about 1.6: 1, in contrast to the expected ratio of 2: 1 (Table 6).

Concerning the sex of frogs at the age of two or three months after completion of metamorphosis, it was necessary to give consideration to the number of frogs which died or were lost during two or three months after metamorphosis. In experimental series No. 1, 161 of the 163 normally feeding, one-nucleolate tadpoles metamorphosed normally. As 62 (38.5%) of these frogs died or were lost during two or three months after metamorphosis, the gonads of the other 99 (61.5%) were

TABLE 6

Sex of the offspring of sex-reversed 1-nu females and the controls

Series No.	Parents		1-nu							2-nu				
			Total	Tadpoles		Frogs				Total	Tadpoles	Frogs		
				St. XX		Immediately after metamorph.		2~3 months after metamorph.			St. XX	Immediately after metamorph.	2~3 months after metamorph.	
	Female	Male		♀n	♀u ♀d	♀u	♀d	♀n	♀d		♂n ♂d	♂n	♀n	♂n ♂d
1	WF, No. 1 (1-nu)	WM, No. 1 (2-nu)	28	1	3 1			12	11	30				23 7
		500E, No. 2 (1-nu)	127		1	18	11	82	15	58	1 1	1		37 18
2	WF, No. 2 (1-nu)	WM, No. 2 (2-nu)	34					25	9	28			2	17 9
		125E, No. 1 (1-nu)	66		1			60	5	75				63 12
3	WF, No. 3 (1-nu)	WM, No. 2 (2-nu)	34					32	2	25				21 4
		125E, No. 1 (1-nu)	80		1 2			73	4	41				41
Total	Controls		96	1	3 1			69	22	83			2	61 20
	Experiments		273		3 2	18	11	215	24	174	1 1	1		141 30

♀n, Female with normal ovaries

♀u, Female with underdeveloped ovaries

♀d, Female with degenerating ovaries

♂n, Male with normal testes

♂d, Male with degenerating testes

examined, and were found to be ovaries. In the same series, 76 of 77 normally feeding, two-nucleolate tadpoles metamorphosed normally. As 20 (26.3%) of these frogs died or were lost during two or three months after metamorphosis, the gonads of the other 56 (73.7%) were examined and found to be testes. In experimental series No. 2, 133 of the 140 normally feeding, one-nucleolate tadpoles metamorphosed normally. As 68 (51.1%) of these frogs died or were lost during two or three months after metamorphosis, the gonads of the other 65 (48.9%) were examined and found to be ovaries. In the same series, 97 of the 100 normally feeding, two-nucleolate tadpoles metamorphosed normally. As 22 (22.7%) of these frogs died or were lost during two or three months after metamorphosis, the gonads of the other 75 (77.3%) were examined and found to be testes. In experimental series No. 3, 97 of the 100 normally feeding, one-nucleolate tadpoles metamorphosed normally. As 20 (20.6%) of these frogs died or were lost during two or three months after metamorphosis, the gonads of the other 77 (79.4%) were examined and found to be ovaries. In the same series, 41 of the 46 normally feeding, two-nucleolate tadpoles metamorphosed normally. The gonads of these frogs were examined at age of two or three months after metamorphosis and found to be all males.

In the three experimental series, a total of 391 of the 403 normally feeding, one-nucleolate tadpoles metamorphosed normally. Of these frogs, 150 (38.4%) died or were lost during two or three months after metamorphosis, while the gonads of the other 241 (61.6%) were examined and found to be all ovaries. On the other hand, a total of 214 of the 223 normally feeding, two-nucleolate tadpoles metamorphosed normally. Of these frogs, 42 (19.6%) died or were lost during two or three months after metamorphosis, while the gonads of the other 172 (80.4%) were examined and found to be all testes. These data show that the one-nucleolate females are considerably larger than the two-nucleolate males in the number of frogs which died or were lost during the juvenile frog stage. This tendency was most distinct in experimental series No. 2. If all the metamorphosed frogs in the three experimental series were kept alive, the ratio of one-nucleolate frogs (females) to two-nucleolate ones (males) should be evidently nearer the ratio of 2 : 1.

DISCUSSION

The sex determining mechanism in amphibians was reported by WITSCHI (1922) for the first time in *Rana temporaria*. He has presumed that the male is the heterogametic sex on the basis of the results of crossing experiments between the differentiated and undifferentiated races. He has observed the X and Y chromosomes in the meioses of spermatocytes in the males belonging to the differentiated race. These chromosomes seemed to be separated in the second meiotic division. He (1929) has also confirmed the male heterogamety by the results of mating experiments between hermaphrodites and females or males of differentiated or undifferentiated race. The adult hermaphrodites found in *Rana*

temporaria seemed to be genetic females (XX). HARMS (1926) has obtained the offspring from a sex-reversed genetic male whose ovaries were transformed from BIDDER's organs by mating with normal males in *Bufo bufo*. As their sex ratio was 1♀: 2♂, he has considered that the male is heterogametic. According to HARMS, the crossing is XY×XY=1XX:2XY:1YY, and the YY individuals are lethal as found in *Drosophila* and die before gastrulation or soon thereafter. In contrast to HARMS, PONSE (1949, 1950) has reported that the genetic males of *Bufo bufo* converted into females by the same way as adopted by HARMS produced 100% males by mating with normal males. On the basis of this result, PONSE has considered that the male is homozygous (ZZ-type) in sex determination.

KAWAMURA and YOKOTA (1959) have described the male heterogamety (XY-type) in *Rana japonica*, as males converted from females by injection with androgen at the tadpole stage produced only female offspring by mating with normal females. The male heterogamety has also been assumed by KAWAMURA and NISHIOKA (1977) in *Rana nigromaculata* from the results of crossings between sex-reversed genetic females and normal females. In *Hyla arborea japonica*, NISHIOKA and UEDA (1977) have observed that three male albinos collected from two separated stations produced only males by mating with normal females. These results seem to show that these male albinos are YY in genotype and at the same time that the male of *Hyla arborea japonica* is heterogametic (KAWAMURA and NISHIOKA, 1977), as the conversion of males into females by administration with estrogen has been reported by TAKAHASHI (1958, 1959) and KAWAMURA and NISHIOKA (1977). Three-fourths of the offspring obtained from a sex-reversed male (XY) by mating with a normal male (XY) were males (KAWAMURA and NISHIOKA, 1977). Thus, it seems evident that the YY individuals can survive in *Hyla arborea japonica* in contrast to the case of *Bufo bufo* observed by HARMS.

It has been reported that the female of *Xenopus laevis* is heterozygous with respect to a sex determining factor (GALLIEN, 1953, 1955; CHANG and WITSCHI, 1955, 1956; MIKAMO and WITSCHI, 1963a, b, 1964). According to MIKAMO and WITSCHI (1963a), ZW females converted into males by testicular grafts produced offspring having a ratio of 1 male: 3 females by mating with normal ZW females. They (1964) have described that all the three kinds of individuals, 1 ZZ male: 2 ZW females: 1 WW female, can be raised until sexual maturity and are fertile.

In urodeles, HUMPHREY (1942a) has reported that the females of *Ambystoma mexicanum* and *A. tigrinum* are heterozygous in sex determination. He induced sex-reversal from genetic females to males by orthotopic implantation of testis preprimordia. The grafted testes with possibility of function were removed long before matings. The offspring of a sex-reversed genetic female axolotl mated with a normal female included approximately 25% males and 75% females (1942b). Thus, it has been assumed that the normal female is heterozygous (ZW-type) in sex determination and the WW individual becomes a viable female. GALLIEN (1951, 1954) has also assumed that the male *Pleurodeles waltl* is homozygous (ZZ), as the genetic male converted into a female by administration with estrogen produced 100% males by mating with a normal male.

It is rather strange that heteromorphic sex chromosomes in amphibians have never been discovered during more than thirty years after the time of WITSCHI (1922, 1929), in spite of a large accumulation of reports on karyology in numerous kinds of anurans and urodeles. In 1957, YOSIDA has observed heteromorphic sex chromosomes in *Hyla arborea japonica*. Since 1965, a great number of investigators have reported the XX/XY-type or ZZ/ZW-type chromosomes in various anuran and urodelan species, owing to recent improvement of cytological techniques.

The present author (1986) has reported that the common bell-ring frog, *Buergeria buergeri,* is probably female heterozygous and that chromosome pair No. 7 is the sex chromosomes of the ZZ/ZW-type. Both homologues of the sex chromosome pair have a satellite at the tip of the long arm in the mitoses of the male, while only one homologue of the sex chromosome pair has a satellite in the mitoses of the female. It is interesting that the male tadpoles have two nucleoli in the nuclei of somatic cells, while the female tadpoles have a single nucleolus. The nucleolus seemed to have been derived from the nucleolar organizer of the Z chromosome.

In the present study, masculinization of one-nucleolate frogs was induced by administration with androgen or estrogen at the tadpole stage. Two mature sex-reversed, one-nucleolate genetic females administered estrogen were mated with three normal one-nucleolate females. Of the 1067 offspring at the embryonic and tadpole stages obtained from these matings, about one-fourth, one-half and one-fourth had no, one and two nucleoli, respectively. While all the anucleolate individuals did not live beyond the tail-bud stage, the one- and two-nucleolate ones survived normally and became females and males, respectively.

A mutation reducing the number of nucleoli in the nucleus has been discovered by ELSDALE, FISCHBERG and SMITH (1958) in *Xenopus laevis* and also by JOTTERAND and FISCHBERG (1974) in *Xenopus borealis*. The Mendelian ratio, 25% with two-nucleolate cells, 50% with one-nucleolate cells and 25% with anucleolate cells, has been obtained by them among the offspring from a cross of two heterozygotes. All the homozygous mutants die at the young tadpole stage, showing a constant syndrome of abnormalities. Such a lethality of homozygous mutants in *Xenopus laevis* has been confirmed by WALLACE (1960) and GURDON (1977). KAHN (1962) has observed that the mutation reducing nucleolar number in interphase nuclei also reduces the number of secondary constrictions which are the nucleolar-organizing regions. SCHMID (1983) has reported that the female of the South African bull frog *Pyxicephalus adspersus* is heterogametic and that chromosomes No. 8 among the 13 pairs in total are sex chromosomes of the ZW-type. On the other hand, chromosomes No. 6 have the nucleolus organizer region which is specifically demonstrable by silver staining in the short arm. In contrast, SCHMID, HAAF, GEILE and SIMS (1983) and SCHMID, SIMS, HAAF and MACGREGOR (1986) have reported that the marsupial frog *Gastrotheca riobambae* is male heterozygous and chromosomes No. 4 of the 13 pairs in total are sex chromosomes of the XY-type. The Y chromosome is considerably larger than the X chromosome and almost completely heterochromatic. The only nucleolus

organizer region of the karyotype is localized in the short arm of the X chromosome. Thus, the number of the nucleolus organizer regions causes a sex-specific difference. While the female has two in diploid cells, the male has one. SCHMID, SIMS, HAAF and MACGREGOR (1986) have explained that this constitutes an extremely rare situation in the karyotype of vertebrates.

Buergeria buergeri studied by the present author is somewhat similar to *Gastrotheca riobambae* in that the nucleolus organizer region is located in a sex chromosome. However, the case of *Buergeria buergeri* differs from that of *Gastrotheca riobambae* in that the former species is of the ZW-type and the nucleolus organizer region is borne by the Z chromosome. It has been confirmed that the WW (0-nu) individuals in *Buergeria buergeri* are lethal, and the offspring of heterozygous parents (ZW) consist of ZZ males (2-nu) and ZW females (1-nu) at the rate of nearly 1 : 2. It is supposed that the YY (0-nu) individuals produced from matings between heterozygous parents (XY) in *Gastrotheca riobambae* may be lethal just as the WW individuals in *Buergeria buergeri* are.

In *Xenopus laevis*, it has been reported that anucleolate individuals are lethal (WALLACE, 1960, 1962; GURDON, 1977), being independent of the sex ratio (MIKAMO and WITSCHI, 1963a, b, 1964). This seems to be attributable to the fact that the nucleolus organizer regions are not situated in sex chromosomes.

ACKNOWLEDGMENTS

The author wishes to express his heartfelt gratitude to Professor Emeritus Toshijiro KAWAMURA and Professor Midori NISHIOKA, Hiroshima University, for their warm encouragement and guidance during the course of this study and their critical review of the original manuscript.

LITERATURE

CHANG, C. Y. and E. WITSCHI 1955. Breeding of sex-reversed males of *Xenopus laevis* DAUDIN. Proc. Soc. exp. Biol. and Med., **89**: 150–152.

———— 1956. Genic control and hormonal reversal of sex differentiation in *Xenopus*. Proc. Soc. exp. Biol. and Med., **93**: 140–144.

ELSDALE, T. R., M. FISCHBERG and S. SMITH 1958. A mutation that reduces nucleolar number in *Xenopus laevis*. Exp. Cell Res., **14**: 642–643.

GALLIEN, L. 1951. Sur la descendance unisexuée d'une femelle de *Pleurodeles waltlii* MICHAH. ayant subi, pendant sa phase larvaire, l'action gynogène du benzoate d'oestradiol. C. R. Acad. Sc. Paris, Sér. D, **233**: 828–830.

———— 1953. Inversion totale du sexe chez *Xenopus laevis* DAUD. à la suite d'un traitement gynogène par le benzoate d'oestradiol, administré pendant la vie larvaire. C. R. Acad. Sc. Paris, Sér. D, **237**: 1565–1566.

———— 1954. Démonstration de l'homogamétie du sexe mâle chez le Triton *Pleurodeles waltlii* MICHAH. par l'étude de la descendance d'animaux à sexe physiologique inversé, après un traitement hormonal gynogène (benzoate d'oestradiol). C. R. Acad. Sc. Paris, Sér. D, **238**: 402–404.

———— 1955. Descendance unisexuée d'une femelle de *Xenopus laevis* DAUD. ayant subi, pendant sa phase larvaire, l'action gynogène du benzoate d'oestradiol. C. R. Acad. Sc. Paris, Sér. D, **240**: 913–915.

GURDON, J. B. 1977. The croonian lecture, 1976. Egg cytoplasm and gene control in development. Proc. R. Soc. Lond. B, **198**: 211–247.

HARMS, J. W. 1926. Beobachtungen über Geschlechtsumwandlungen reifer Tiere und deren F_1 Generation. Zool. Anz., **67**: 67–79.

HUMPHREY, R. R. 1942a. Studies on sex reversal in *Amblystoma*: XII. Sterility after reversal of ovary to testis in the axolotl. Growth, **6**: 185–201.

———— 1942b. Sex reversal and the genetics of sex determination in the axolotl (*Amblystoma mexicanum*). Anat. Rec., **84**: 465.

JOTTERAND, M. and M. FISCHBERG 1974. A chromosome mutation affecting the number of nucleoli in *Xenopus borealis* PARKER. Experientia, **30**: 1003–1005.

KAHN, J. 1962. The nucleolar organizer in the mitotic chromosome complement of *Xenopus laevis*. Quart. J. micr. Sci., **103**: 407–409.

KAWAMURA, T. and M. NISHIOKA 1977. Aspects of the reproductive biology of Japanese anurans. The Reproductive Biology of Amphibians, edited by D. H. TAYLOR and S. I. GUTTMAN, pp. 103–139. Plenum Press, New York and London.

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.

MIKAMO, K. and E. WITSCHI 1963a. Functional sex-reversal in genetic females of *Xenopus laevis*, induced by implanted testes. Genetics, **48**: 1411–1421.

———— 1963b. Zuchtversuche mit geschlechtskonvertierten Krallenfröschen (*Xenopus laevis*). Experientia, **19**: 536–537.

———— 1964. Masculinization and breeding of the WW *Xenopus*. Experientia, **20**: 622–623.

NISHIOKA, M. and H. UEDA 1977. Genetic and morphologic studies on ten albino stocks in *Hyla arborea japonica*. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **2**: 103–163.

OHTA, S. 1986. Sex determining mechanism in *Buergeria buergeri* (SCHLEGEL). I. Heterozygosity of chromosome pair No. 7 in the female. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ., **8**: 29–43.

PONSE, K. 1949. La différenciation du sexe et l'intersexualité chez les vertébrés. F. Rouge & Cie, S. A. Librairie de l'Université Lausanne.

———— 1950. La génétique du sexe chez les batraciens avec un aperçu des travaux de R. R. HUMPHREY. Arch. d'Anat. micr. et Morph. exp., **39**: 183–214.

SCHMID, M. 1983. Evolution of sex chromosomes and heterogametic systems in Amphibia. Differentiation, **23**: 13–22.

SCHMID, M., T. HAAF, B. GEILE and S. SIMS 1983. Chromosome banding in Amphibia. VIII. An unusual XY/XX-sex chromosome system in *Gastrotheca riobambae* (Anura, Hylidae). Chromosoma (Berl.), **88**: 69–82.

SCHMID, M., S. H. SIMS, T. HAAF and H. C. MACGREGOR 1986. Chromosome banding in Amphibia. X. 18S and 28S ribosomal RNA genes, nucleolus organizers and nucleoli in *Gastrotheca riobambae*. Chromosoma (Berl.), **94**: 139–145.

TAKAHASHI, H. 1958. Gonadal reaction in the tree-frog larvae (*Hyla arborea japonica* GUENTHER) to the androgen. J. Fac. Sci. Hokkaido Univ., Ser. VI, Zool., **14**: 92–99.

———— 1959. Partial feminization of larval gonads of *Hyla arborea japonica* GUENTHER induced by treatment with estradiol. J. Fac. Sci. Hokkaido Univ., Ser. VI, Zool., **14**: 210–221.

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

WALLACE, H. 1960. The development of anucleolate embryos of *Xenopus laevis*. J. Embryol. exp. Morph., **8**: 405–413.

———— 1962. Cytological and biochemical studies of anucleolate *Xenopus* larvae. Quart. J. micr. Sci., **103**: 25–35.

WITSCHI, E. 1922. Vererbung und Zytologie des Geschlechts nach Untersuchungen an Fröschen. Zeitschrift f. ind. Abstammungs- u. Vererbungslehre, **29**: 31–68.

———— 1929. Studies on sex differentiation and sex determination in amphibians. III. Rudimentary hermaphroditism and Y chromosome in *Rana temporaria*. J. Exp. Zool., **54**: 157–223.

YOSIDA, T. H. 1957. Sex chromosomes of the tree frog, *Hyla arborea japonica*. J. Fac. Sci. Hokkaido Univ., Ser. VI, Zool., **13**: 352–358.