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Offspring of Sex-reversed Males in *Bufo viridis* Laur.

By

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ABSTRACT

In order to determine the sex-determining mechanism in *Bufo viridis*, males were sex-reversed into females by castration and subsequent administration with estrogen, and mated with normal males to obtain the offspring. Of the 25 castrated and estrogen-injected males, 17 lived for more than 15 months. While seven of these toads had regenerated testes and normal Bidder's organs, the other ten had well-developed ovaries transformed from their Bidder's organs, although the oviducts were defective in five of them. By pituitary injection, four of the remaining five sex-reversed males laid 473, 62, 5368 and 7316 eggs. By insemination with sperm of regenerated testes, 822 eggs attained completion of metamorphosis. Among 784 offspring, there were 197 females and 587 (74.9%) males. These numbers seem to show that the male in *Bufo viridis* is heterogametic (XY) and YY toads produced from matings between sex-reversed genetic males (XY) and normal males (XY) can survive and become males.

INTRODUCTION

Bufo viridis is a beautiful green toad species which is distributed in Europe, Asia and northern Africa (Nikol'skii, 1918; Ballasina, 1984). Heteromorphic sex-chromosomes have never been observed in this species (Galgano, 1933; Morescalchi, 1963; Bogart, 1972). Kawamura, Nishioka and Ueda (1980) have reported that *B. viridis* from Turkey is probably of the XX/XY-type in sex-determining mechanism. In their hybridization experiments, the hybrids between a male *B. viridis* and females of four other *Bufo* species in addition to a female *B. viridis* were all males. Thus, they have assumed that the exceptional male *B. viridis* was YY in sex-chromosome constitution.

In order to confirm that the male is heterogametic in *Bufo viridis*, the present author attempted to change males into functional females by castration and subsequent administration with estrogen and to examine the sex of their offspring. A preliminary report of this study was made a few years ago (Ueda, 1985).

MATERIALS AND METHODS

One hundred and twenty-nine offspring obtained from a pair of *Bufo viridis*

LAUR. from Turkey in 1979 were all males (KAWAMURA, NISHIOKA and UEDA, 1980). On 29 October 1980, 25 of these males were castrated at the age of 17 months to make their BIDDER's organs develop into functional ovaries. They were 38~58 mm, 51.6 mm on the average, in body length and had testes which were 2.0~8.0 mm in length and 1.0~3.5 mm in width. Their BIDDER's organs were 2.5 mm×2.0 mm~5.5 mm×5.5 mm, 4.5 mm×4.1 mm on the average, in size (Table 1). As the BIDDER's organ covered the anterior part of the testis like a cap, the testis was excised together with a portion of the BIDDER's organ (Fig. 1a). The Müllerian ducts were very slender and their posterior parts were usually undeveloped at the time of castration, in contrast to those of many other *Bufo* species in which they are relatively distinct in adult males (WITSCHI, 1933). For the purpose of making the undeveloped Müllerian ducts active, each toad was injected with 0.05 mg estradiol-benzoate (Teikoku-zoki Inc.) on 27 November and 12 December 1980 and on 16 January and 16 February 1981.

As four castrated males spawned three years after castration by injecting with suspension of pituitaries obtained from *Rana catesbeiana,* the eggs of the sex-reversed males were fertilized with sperm of normal males. The tadpoles obtained were fed on boiled spinach. The metamorphosed toads were fed on nymphs at various developmental stages of the two-spotted cricket, *Gryllus bimaculatus* DE GEER, and thereafter on adults. They were reared from two months to the mature stage after metamorphosis.

Each castrated toad was named as Cast. 1, Cast. 2 and so on, according to the order of the performed operation.

OBSERVATION

I. Results of castration

After castration, five toads, Cast. 3, Cast. 6, Cast. 12, Cast. 22 and Cast. 25, died within a month, and three others, Cast. 1, Cast. 2 and Cast. 17 died within four months (Table 1). Their BIDDER's organs were almost unchanged. Cast. 13, Cast. 15 and Cast. 20, which died 21.1, 16.5 and 15.2 months after operation, respectively, had somewhat small regenerated testes and normal BIDDER's organs. Cast. 7 died 22.9 months after operation. The BIDDER's organs of this toad were 33.0 mm×21.0 mm×18.0 mm and 35.0 mm×18.0 mm×17.0 mm in size. They were filled with mature eggs. There was no left Müllerian duct, while there was a slender anterior four-fifths of the right Müllerian duct. Cast. 11, which died 25.0 months after the operation, had the ovaries transformed from the BIDDER's organs. The right and left ovaries were 38.0 mm×23.5 mm×12.0 mm and 32.0 mm×20.0 mm×12.0 mm in size, respectively. A pair of complete oviducts were observed which were somewhat slender and weakly waving and their posterior ends opened into the cloaca (Fig. 1c).

Twelve castrated toads were alive in May 1983. Seven of them, Cast. 5, Cast. 8, Cast. 9, Cast. 14, Cast. 16, Cast. 19, and Cast. 24, showed a female appearance,

TABLE 1
Effects of castration in male *Bufo viridis*

No.	Measurements at the time of castration					Period from castration to killing (month)	Condition of BIDDER's organs at the time of killing	Remarks
	Body length (mm)	Size of testes		Size of BIDDER's organ				
		Right (mm)	Left (mm)	Right (mm)	Left (mm)			
Cast. 1	41.5	2.5×1.0	2.5×1.0	4.0×4.0	3.5×4.0	3.8	Unchanged	Regenerated testes
Cast. 2	44.0	3.0×1.5	2.5×1.5	4.0×3.0	4.0×3.0	3.7	Early stage of development	
Cast. 3	41.0	2.5×1.5	2.0×1.0	3.0×2.5	3.0×2.5	0.9	Unchanged	
Cast. 4	52.0	4.0×1.5	4.0×1.5	5.0×4.0	4.5×4.5	56.0	Well-developed ovaries	Small testis and defective oviducts
Cast. 5	52.5	6.5×2.5	6.5×2.5	5.0×3.5	4.5×4.0	41.2	Well-developed ovaries	Defective oviducts
Cast. 6*	57.5	8.2×3.0	8.0×3.0	5.0×4.5	5.0×3.5	0.9	Unchanged	
Cast. 7	56.5	8.2×3.5	9.0×3.5	5.0×4.0	5.5×4.0	22.9	Well-developed ovaries	Defective oviducts
Cast. 8*	57.0	8.0×4.0	8.5×3.5	5.0×5.0	5.0×4.5	32.1	Well-developed ovaries	473 eggs laid
Cast. 9	40.5	6.5×3.5	6.5×4.0	2.5×2.0	3.0×2.0	33.1	Well-developed ovaries	62 eggs laid
Cast. 10	38.0	2.5×1.0	3.0×1.0	3.0×3.0	3.5×4.0	56.0	Unchanged	Normal testes
Cast. 11	40.0	3.0×1.0	3.5×1.0	3.5×3.5	3.5×2.5	25.0	Well-developed ovaries	Complete oviducts
Cast. 12	50.0	5.5×3.0	6.0×2.5	5.0×4.5	5.0×4.0	0.7	Unchanged	
Cast. 13	45.0	2.0×1.0	4.5×2.0	4.5×4.0	4.0×3.5	21.1	Unchanged	Normal testes
Cast. 14	51.0	6.0×3.0	7.0×3.0	5.0×2.5	5.0×4.0	41.2	Well-developed ovaries	Defective oviducts
Cast. 15	51.5	5.5×2.5	5.0×2.0	5.5×5.5	5.0×5.0	16.5	Unchanged	Regenerated testes
Cast. 16	54.0	5.0×2.0	6.0×2.0	5.5×5.0	5.0×5.0	30.4	Well-developed ovaries	5368 eggs laid
Cast. 17	54.0	5.5×2.5	6.0×3.0	5.0×4.5	5.0×4.5	1.5	Unchanged	
Cast. 18	55.0	6.0×3.0	6.0×3.0	5.0×5.0	4.5×5.0	56.0	Unchanged	Normal testes
Cast. 19	53.5	5.0×2.5	6.0×2.5	5.0×4.5	5.0×4.0	41.2	Well-developed ovaries	7316 eggs laid
Cast. 20*	58.0	6.5×3.0	8.0×3.0	5.0×5.0	5.0×4.5	15.2	Unchanged	Normal testes
Cast. 21*	55.0	7.0×3.0	7.5×3.0	5.0×4.5	5.0×4.5	32.0	Unchanged	Normal testes
Cast. 22*	55.0	7.0×3.5	7.0×3.5	5.0×5.0	4.0×5.5	0.5	Unchanged	
Cast. 23*	57.0	6.5×3.0	7.0×3.0	4.5×5.0	4.0×4.0	56.0	Unchanged	Normal testes
Cast. 24*	66.2	7.0×3.0	7.0×3.0	5.0×4.0	5.0×4.5	41.2	Well-developed ovaries	Defective oviducts
Cast. 25*	63.5	5.0×3.5	8.5×3.5	5.0×4.0	5.0×4.5	0.3	Unchanged	

*Nuptial-pads were observed at the time of castration.

while the other five, Cast. 4, Cast. 10, Cast. 18, Cast. 21 and Cast. 23, had the male secondary sexual characters. The former seven were injected with pituitary suspension of *Rana catesbeiana* on 14 May 1983. The results showed that four of them, Cast. 8, Cast. 9, Cast. 16 and Cast. 19, laid 473, 62, 5368 and 7316 eggs, respectively, on the following day, while the other three laid no eggs. Although

TABLE 2
Ovaries and oviducts of sex-reversed males which lived for more than 30 months after castration

No.	Body length (mm)	Size of ovaries		No. of urogenital arteries	Oviducts			Occurrence of ovulation	No. of eggs laid
		Right (mm)	Left (mm)		Width		Type		
					Right (mm)	Left (mm)			
Cast. 4*	80.3	37×36×30	35×34×28	1 pair	1.2	1.5	Cul-de-sac	——	——
Cast. 5	81.5	36×30×27	34×32×20	1 pair	1.0	1.2	Cul-de-sac	No	——
Cast. 8	84.0	35×25×16	35×24×20	1 pair	2.0	2.0	Complete	Yes	473
Cast. 9	85.0	41×30×22	42×31×25	1 pair	2.0	2.0	Right, Complete Left, Cul-de-sac	Yes, but slightly	62
Cast. 14	86.0	51×35×20	48×35×16	1 pair	1.5	1.5	Cul-de-sac	No	——
Cast. 16	89.5	25×23×12**	20×15×10**	1 pair	2.5	2.5	Complete	Yes	5368
Cast. 19	93.5	50×35×21	48×32×23	1 pair	2.5	2.5	Complete	Yes	7316
Cast. 24	88.0	41×40×19	41×36×20	1 pair	2.0	2.5	Cul-de-sac	Yes, but slightly	——
Normal female 1	85.0	50×33×22	52×33×25	5 pairs	3.5	3.5	Complete	Yes	15652
Normal female 2	86.0	51×34×20	52×32×23	5 pairs	3.5	3.5	Complete	Yes	12658

*Hermaphrodite having a regenerated small testis

**Measured just after spawning

Normal females 1 and 2 are the offspring between a sex-reversed male, Cast. 16, and a normal male, Cast. 21.

the latter three toads were injected again with pituitary suspension in the following season, they laid no eggs. Subsequently, they were preserved to observe their urogenital organs. It was found that their oviducts were culs-de-sac in spite of the existence of well-developed ovaries (Table 2). No eggs were found in the abdominal cavities nor in the anterior parts of the oviducts of both Cast. 5 and Cast. 14, while only a small number of eggs were found in the normal anterior parts of the oviducts of Cast. 24, after these females were injected with pituitary suspension which was sufficient for making normal females fully spawn (Fig. 2c, d).

The four females which spawned were killed within 12 months after spawning to observe the appearance of their urogenital organs. Three females, Cast. 8, Cast. 16 and Cast. 19, had complete oviducts although these were slender and weakly waving (Fig. 1d, Fig. 2a). The remaining Cast. 9 had a complete oviduct on the right side, while the left oviduct was defective and had no posterior half. The ovaries of these four sex-reversed males were nearly the same in size as those of normal females (Table 2). The ovaries derived from the BIDDER's organs were connected with the anterior parts of the kidneys, and they were somewhat roundish, as were the original BIDDER's organs. A pair of the urogenital arteries

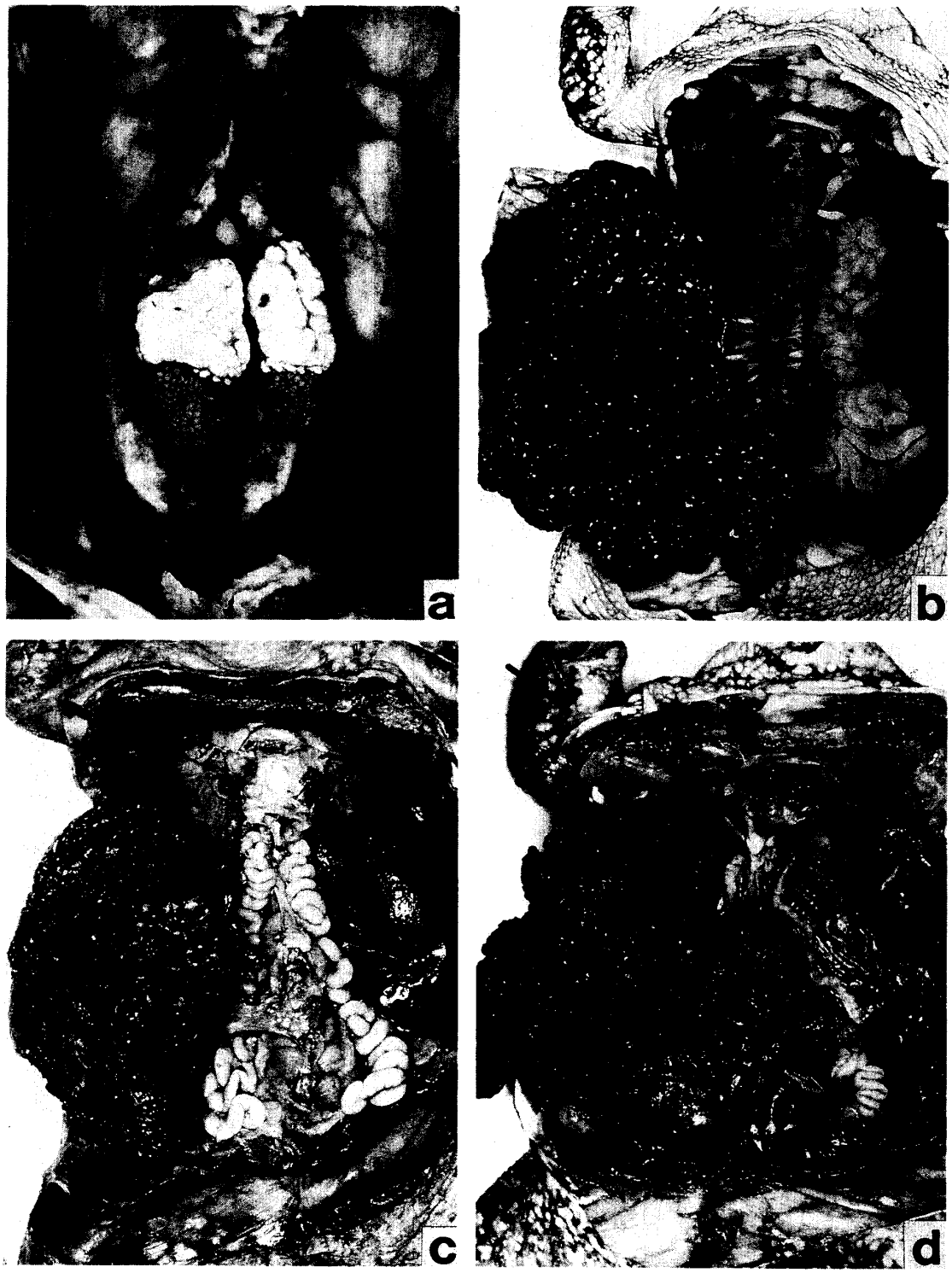

Fig. 1. Urogenital organs of normal male and female and two sex-reversed males in *Bufo viridis*. The left ovary of each female is removed.

a. A normal male, 17 months old. The BIDDER's organs covered the anterior parts of the testes before castration. ×4

b. A normal two-year-old female produced from a sex-reversed male, Cast. 16. ×1.1

c. A sex-reversed male, Cast. 11, with well-developed ovaries and somewhat slender oviducts. ×1.1

d. A sex-reversed male, Cast. 8, with well-developed ovaries, very slender oviducts and expanded uteri (arrow) containing eggs. ×1.1

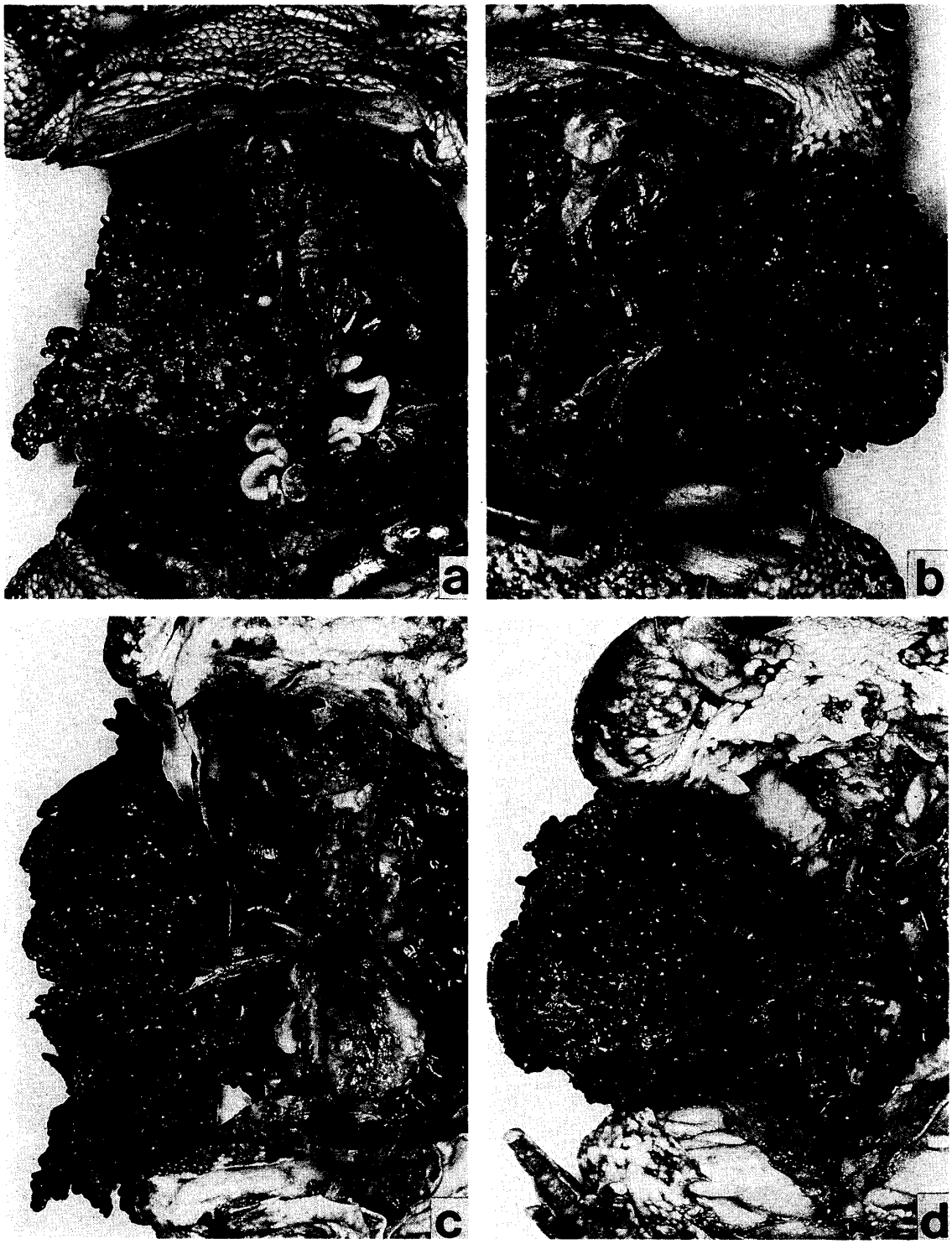

Fig. 2. Urogenital organs of four castrated males in *Bufo viridis*. Left or right ovary of each toad is removed. ×1.2

a. A sex-reversed male, Cast. 16, with ovaries after ovulation, somewhat slender oviducts and expanded uteri (arrow) containing a large number of eggs.

b. A hermaphrodite, Cast. 4, with well-developed ovaries, defective oviducts and a testis (arrow).

c. A castrated male, Cast. 14, with well-developed ovaries and defective oviducts.

d. A castrated male, Cast. 24, with well-developed ovaries and defective oviducts of which anterior parts contain a small number of eggs (arrow).

was distributed in these ovaries, in contrast to those of normal females in which five pairs of urogenital arteries were generally distributed. On 15 May 1983, two castrated males, Cast. 18 and Cast. 21, were examined by laparotomy on the urogenital organs. They had completely regenerated testes and normal male BIDDER's organs. Their testes were used in matings with sex-reversed males, because they should be normal in sex-chromosome constitution. Three castrated males, Cast. 4, Cast. 10 and Cast. 23, were killed to observe their urogenital organs in 1985. Two of them, Cast. 10 and Cast. 23, had completely normal male urogenital organs, but Cast. 4 was a typical hermaphrodite, which had not only well-developed ovaries derived from the BIDDER's organs but also a small testis. This testis was 6.0 mm×6.0 mm×3.5 mm in size and was connected to the central part of the right kidney (Fig. 2b). The histological preparations of the testis showed evidence of normal spermatogenesis. The oviducts of this hermaphrodite were culs-de-sac.

II. Offspring of the sex-reversed males

On 15 May 1983, the unfertilized eggs obtained from two sex-reversed, castrated males, Cast. 8 and Cast. 9, were inseminated with sperm of the regenerated testes of a male, Cast. 18, while the eggs obtained from the other two sex-reversed

TABLE 3
Results of mating experiments between sex-reversed males and normal males

Parents		No. of eggs	No. of normal cleavages	No. of normal neurulae	No. of normally hatched tadpoles	No. of normally feeding tadpoles	No. of metamorphosed toads
Sex-reversed male	Normal male						
Cast. 8	Cast. 18	473	301(63.6%)	284(60.0%)	268(56.7%)	197(41.6%)	126(26.6%)
Cast. 9	Cast. 18	62	59(95.2%)	55(88.7%)	51(82.3%)	43(69.4%)	39(62.9%)
Cast. 16	Cast. 21	5368	3912(72.9%)	2658(49.5%)	1782(33.2%)	1235(23.0%)	657(12.2%)
Cast. 19	Cast. 21	7316	0				

TABLE 4
Sex of the offspring of sex-reversed males mated with normal males in *Bufo viridis* from Turkey

Parents		No. of metamorphosed toads	Young toads			Mature toads			Total			
Sex-reversed male	Normal male		No.	♀	♂	No.	♀	♂	No.	♀	♂	(%)
Cast. 8	Cast. 18	126	18	5	13	93	22	71	111	27	84	(75.7%)
Cast. 9	Cast. 18	39	16	6	10	17	2	15	33	8	25	(75.8%)
Cast. 16	Cast. 21	657	363	95	268	277	67	210	640	162	478	(74.7%)
Total		822	397	106	291	387	91	296	784	197	587	(74.9%)

Young toads preserved from two to 12 months after metamorphosis.

castrated males, Cast. 16 and Cast. 19, were inseminated with sperm of the regenerated testes of a male, Cast. 21. It was found that 301 (63.6%) out of 473, 59 (95.2%) out of 62 and 3912 (72.9%) out of 5368 eggs obtained from Cast. 8, Cast. 9 and Cast. 16, respectively, cleaved normally, while all of the 7316 eggs obtained from Cast. 19 did not cleave, probably owing to overripeness. Thereafter, most of the normally cleaved eggs died, and eventually, 126 (26.6%), 39 (62.9%) and 657 (12.2%) completed metamorphosis, respectively (Table 3).

About half the offspring of each sex-reversed, castrated male were killed two months after metamorphosis, while the remaining were left to survive. Afterwards, the sex of all the offspring which had been raised for more than two months after the metamorphosis were examined. The results are shown in Table 4. There were five females and 13 males among 18 young toads, and 22 females and 71 males among 93 mature ones in the offspring of Cast. 8. Twenty-seven were females and 84 (75.7%) were males in total. There were six females and 10 males among 16 young toads, and two females and 15 males among 17 mature ones in the offspring of Cast. 9. In total, eight were females and 25 (75.8%) were males. There were 95 females and 268 males among 363 young toads and 67 females and 210 males among 277 mature ones in the offspring of Cast. 16. In total, 162 were females and 478 (74.7%) were males. In the offspring of the three sex-reversed, castrated males mated with none-sex-reversed males, there were 197 females and 587 (74.9%) males.

DISCUSSION

According to WITSCHI (1933), BIDDER's organ is specific in *Bufo* and is a region of the gonad which consists entirely of the cortex whose development is kept at a standstill throughout life. It has been shown in *Bufo bufo* that complete elimination of the gonad proper enables the BIDDER's organs of both sexes to develop into functional ovaries (HARMS, 1921, 1926; PONSE, 1925, 1942, 1949, 1950). HARMS (1926) crossed a sex-reversed male with a normal male in *B. vulgaris* (=*B. bufo*) from Germany. It was found that 1828 of the 2078 deposited eggs began to develop. However, mortality was very high, and eventually 57 females and 104 males were produced. As the ratio of female to male was about 1 : 2, he assumed that the male was heterogametic (XY), and the YY animals were lethal. PONSE (1925), who performed the same experiment as HARMS did by using Italian *B. vulgaris* (=*B. bufo*). She obtained 247 eggs by the first mating between a sex-reversed genetic male and a normal male. Of these eggs only 11 could reach the tadpole-stage. Afterwards, by repeating the same kind of experiments, she examined the sex of 1080 offspring of seven sex-reversed males obtained by the same kind of experiments, and confirmed that all of them were males. On the basis of this result, she concluded that the female is heterogametic (ZW) and the male is homogametic (ZZ) in this species (PONSE, 1942, 1949, 1950). Although nobody has reexamined these contradictory results, PONSE's opinion seems to be generally accepted (ENGEL and SCHMID, 1981; SCHMID, 1983; BEÇAK, 1983).

ENGEL and SCHMID (1981) have supported the female heterogamety by the H–Y antigen studies in *Bufo bufo*. WEN, LU and XIANG (1983) and SHANG and DENG (1983) have reported that chromosomal pair No. 10 in female *B. bufo gargarizans* (*B. japonicus gargarizans*) is heteromorphic in BrdU-Hoechst-Giemsa replication pattern.

In the present study, three matings between three sex-reversed genetic males and two normal males were performed in *B. viridis*, and the sex of their offspring was examined. The results showed that the number of females and males in three matings were 27 and 84 (75.7%), 8 and 25 (75.7%) and 162 and 478 (74.7%) respectively. There were 197 females and 587 (74.9%) males in total. The ratio of females to males was nearly 1 to 3. If *B. viridis* is XY type in sex-determining mechanism and the sex-reversed male (XY) is mated with normal male (XY), three kinds of offsprings, XX, XY and YY should be produced at the ratio of 1 : 2 : 1. If the YY toads become males as KAWAMURA, NISHIOKA and UEDA (1980) have suggested, the sex ratio of females to males should be 1 : 3. Thus, it seems to be evident that the male in *B. viridis* is heterogametic (XY) and YY individuals of this species can survive and become males. The result obtained by the present author in *B. viridis* is in contrast to that obtained by PONSE in *B. bufo*, where the female is considered to be heterogametic. While the male in *B. bufo* is assumed to be heterogametic by HARMS, he considered that YY individuals are lethal. It seems probable that the sex-determining mechanism in genus *Bufo* is not always the same and may differ with species and populations.

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