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Reproduction of the Oriental Fire-bellied Toad, *Bombina orientalis*, with Special Reference to the Superiority of this Species as a Laboratory Animal

By

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INTRODUCTION

The oriental fire-bellied toad is a beautiful species of Discoglossidae. This is distributed in the temperate region of eastern Asia, that is, Korea, northeastern China and the most southeastern districts of Siberia (OKADA, 1931; KOBA, 1937; LIU and HU, 1961; NIKOL'SKII, 1962). The upperside of the body and extremities is usually light green or brownish grey in ground color and had many black spots. When it is brownish grey, there are often a few green speckles on the back. The underside is red or orange with black speckles.

Although OKADA (1931, '66) has listed Tsushima, a Japanese solitary group of islands lying between Korea and Kyushu of Japan as an area of distribution, it is open to doubt (GORIS, 1965). In spite of our frequent trips for collecting amphibians, nobody has confirmed the existence of this species in Tsushima. In Japan, the oriental fire-bellied toad is called bell frog, as the voice of both sexes is somewhat like the tinkling of a small bell.

The present writers have raised a lot of oriental fire-bellied toads collected from Korea in their laboratory. In the meanwhile, they were aware of the superiority of this species as a laboratory animal, judging from their many years' experiences in rearing various kinds of anurans. It is their belief that *Bombina orientalis* will be raised abundantly and utilized as good material for biological researches in many laboratories in the near future.

MATERIAL AND METHODS

Three males and two females of Korean *Bombina orientalis* (BOULENGER) were first collected from the vicinity of Seoul by the courtesy of Professor G. Goo, Seoul Municipal Agricultural College, and Professor C. M. KIM, Korea University, in summer of 1963. Since then, these oriental fire-bellied toads have produced four generations of offspring in our laboratory. Besides, many males and females of this species were collected from the vicinity of Kyongiu, North Kyonsang Province, the southeastern part of Korea by Mr. FUJIKAWA in 1969 and from the vicinity of Taejon, South Chungchong Province, the southwestern part of Korea, by Professor G. W. NACE, Department of Zoology, the University

of Michigan, in 1970. In 1971 several males and females were also collected by the courtesy of Professor H. K. KIM, Ewha Women's University, from the vicinity of Seoul. These toads were added to the above colony in our laboratory.

Matured males of this species can easily be distinguished from females by the following sexual characters.

1) The fore and hind legs of the male are comparatively longer than those of the female.

2) The male in the breeding season has the forelegs which are remarkably thick and have a distinct brown black oval swelling on the inner side of the first finger. Besides, the inner metatarsal tubercle is hypertrophied, and the tarsus and toes of the hind leg are distinctly thick.

3) The dorsal skin of the male is rough, as there are numerous spinous protuberances.

All these sexual characters appear in company with the sexual maturity. Accordingly, the sex can not be identified from appearances in young toads which are less than 3 cm. in body length. The sexual characters of males do not disappear all the year round, if they are reared at 25°C or so.

In the present paper the individual number or identification sign of each toad is shown with a combination of some letters and figures which are arranged like No. S64F_1f2. S is the capital letter of the name of the city, Seoul, near where the specimen was collected. The next 64 represents 1964, when the toad was developed. F_1 is the offspring in the first generation and f2 means a female No. 2.

The developmental stages of tadpoles are indicated by those of *Rana pipiens* in TAYLOR and KOLLROS' table.

RESULTS

I. Ovulation and amplexus

Ovulation in the female and coupling behavior of the male are reliably accelerated by gonadotropic hormones of frog pituitaries (NAKAMURA, 1938). Accordingly, amplexus, oviposition and fertilization easily occur after injection of pituitary hormones. For artificial fertilization it is unnecessary to inject hormones into males.

The donors of pituitaries were usually *Rana nigromaculata* HALLOWELL or *Rana brevipoda* ITO in the experiments performed by the present writers. One dose was prepared from two or three pituitaries in the months April to October, or from four in the months November to March. These pituitaries were mashed in 0.2 m*l*. RINGER's fluid and injected into the abdominal cavity of a matured toad. Then the latter was left at the temperature of about 25°C. By this way ovulation or amplexus occurred at any time throughout the year. The same result was obtained by injecting the pituitary hormones of *Rana catesbeiana* SHAW. In this case half the number of pituitaries described above was enough for the ovulation and amplexus.

II. Natural and artificial fertilization

Natural fertilization of eggs always occurred in an aquarium which was kept dark and at the temperature of about 25°C. The aquarium contained dechlorinated tap water, which was about 5~6 cm. in depth. Two or three stones were put in the aquarium so that their tops on the water became resting places of the toads. A male and a female were transferred to the aquarium after their skins turned dark by the pituitary injection. When artificial fertilization was scheduled, the female was transferred to a small container which was kept dark and at the temperature of about 25°C. For making the inside of the container damp, some sponges full of water were put on the bottom.

Five or six hours in general, ten hours at the latest, after the pituitary injection the male showed sexual behavior in the way of inguinal amplexus. About five hours later the female began to lay eggs, and the oviposition continued for eight to ten hours. During the period of these hours numerous eggs were intermittently spawned, laying a small mass of 1~23 eggs at a time.

The female of this species could deposit a comparatively small number of eggs in the uterian parts of the oviducts. These eggs were laid without amplexus or easily pushed out by pressure put on the lower abdomen. Accordingly, for the purpose of obtaining as many uterian eggs as possible, it was necessary to take them out as soon as the natural oviposition began to take place. After these eggs were taken out of the uterian parts of the oviducts many other eggs were found in the coelomic cavity and the other parts of the oviducts. Accordingly, the second and third acquisitions of unfertilized eggs were possible after intervals of three or four hours, although the most numerous eggs were obtained at the first.

The unfertilized eggs taken out of the oviducts were placed in rows on a slide-glass and covered with a small amount of sperm-containing water. After the slide-glass had been left in air for about ten minutes, it was put in rearing water. The sperm-containing water was made by crushing a testis in 2 m*l*. dechlorinated tap water. The best fertilization rate was shown by the eggs obtained at the first acquisition after a pituitary injection. The later the eggs were taken out, the worse the fertilization became.

III. Rearing

1. Embryos

Embryos were reared in dechlorinated tap water contained in PETRI dishes, 15 or 18 cm. in diameter. It was appropriate to keep less than 40 eggs in a 15 cm. dish and less than 80 in a 18 cm. An egg mass was cut into as many pieces as possible, in order to make the gas exchange of eggs easier. The water in PETRI dish was usually renewed once a day. The number of days from the beginning of development to the hatch of embryos at 15.6~25.0°C are given in Table 1.

When the temperature was comparatively high, the outer envelopes of embryos were apt to become opaque and shrink by infection. As a result, the embryos

TABLE 1
Relation between the velocity of development and the rearing temperature

Female no.	Date of fertilization	Water temperature during embryonal stages	Period of time during embryonal stage (days)	Water temperature during larval stages	Period of time from fertilization to protrusion of the forelegs (days)
S63Pf1	Apr. 22, '66	25°C	3	25°C	25～ 30
	Jun. 1, '67	21.2～22.0°C	3	20.5～24.0°C	28～ 36
	Jun. 3, '68	21.0～21.5°C	3	21.0～24.2°C	28～ 35
$S64F_1f1$	May 8, '66	18.1～18.5°C	3	18.1～21.5°C	27～ 38
	Mar. 30, '67	15.6～16.2°C	5	15.6～20.0°C	35～ 43
$S64F_1f2$	May 28, '66	20.2～21.2°C	3	18.2～22.7°C	31～ 45
	Jun. 28, '66	22.0～22.9°C	3	22.0～27.5°C	23～ 27
	Jun. 28, '66	22.0～22.9°C	3	32.0～34.0°C	26～ 74
	Jun. 24, '70	25°C	3	25°C	23～ 29
$S65F_2f3$	Nov. 1, '71	16～17°C	5	12～17°C	50～ 89
S71Pf1	Nov. 1, '71	16～17°C	5	12～17°C	45～ 95
S71Pf2	Nov. 1, '71	16～17°C	5	12～17°C	51～123

were occasionally delayed in development and eventually died. Such infection, however, was prevented by making use of a kind of sulphanilamide dosage, Herus tabloids (Haseherus Pharmaceutical Company). When embryos were reared from the outset in water containing this dosage in a definite ratio, no infection did occur. Even when outer envelopes became opaque by infection, most embryos could develop normally, if the embryos were kept in water containing the above dosage after the opaque outer envelopes had been removed. The spread of the infection could be prevented to some degree by frequent renewal of rearing water, too.

2. Tadpoles

For rearing tadpoles indoors, enamel or plastic pans, about 33 cm. × 23 cm. × 6 cm. in dimensions, were used. These pans were easy to handle, owing to their lightness. More than 20 tadpoles were safely kept in each pan. Dechlorinated water in the pan was usually renewed once a day, and the inner surfaces were always kept clean. Boiled soft vegetables like Japanese spinach were given once a day immediately after renewal of rearing water. The vegetables could occasionally be replaced with vegetable gelatin containing compressed food for goldfish or fish meal.

The appropriate density of tadpoles in each pan was different with the size of tadpoles. When tadpoles were about 15 mm. in total length, nearly 100 were kept in each pan. At the stage V, about 23 mm. in total length, 40～60 tadpoles were reared, while 20～35 were at the stages XV～XX. The maximum number of tadpoles which could grow without stragglers was about 50.

The number of days from the fertilization to the protrusion of one or both forelegs of each tadpole is given in Table 1, together with the water temperatures. At 25°C eggs required 23~30 days for attaining to that stage. Generally speaking, the higher the rearing temperature became, the more rapidly the development of individuals proceeded. However, when tadpoles were raised at an extremely high temperature such as 33±1°C after the age of 13 days, their development was rather distinctly retarded, although only half of them did not require more than 26~39 days in order to attain to the stage of protrusion of the forelegs from the fertilization. The other half could barely reach this stage 40~74 days after the beginning of development. When tadpoles were reared at 12~17°C, they required 45~123 days for attaining to the stage of protrusion of the forelegs from the fertilization.

Besides rearing indoors, some tadpoles were raised in large cement tanks, which were 83 cm. × 54 cm. × 17 cm. in dimensions and left outdoors. The water was about 14 cm. deep. About 100 tadpoles were kept in each tank until the completion of their metamorphosis. In this case nearly all the tadpoles could develop normally, even if the water had never been renewed. The outdoor rearing, however, was restricted to the warm seasons of the year.

An infectious disease of tadpoles occurred once for all in these ten years. Some tadpoles produced from a female collected from the southeastern part of Korea in 1969 suffered from this disease. Blood extravasations appeared on the skins of the abdomen, hind legs, tails and other parts of tadpoles, which eventually died of ascites. In order to prevent the spread of this disease, it was necessary to isolate an infected tadpole from the others on sight, and to put the latter in 0.6% salt or 2.5 mg./*l.* oxytetracycline solution for about 24 hours. A slight disease was cured by the same treatment, too.

3. Toads

Toads were reared in enamel or plastic pans which were 33 cm. × 23 cm. × 6 cm. or 46 cm. × 31 cm. × 11 cm. in dimensions and covered with wire gauzes. The water in each pan was about 5 mm. in depth. Pieces of a flowerpot served the toads in the pan as hiding or resting places. The water was usually renewed once a day and the inner surfaces of the pan were always kept clean.

Fire-bellied toads were usually reared with worms of Tubificidae, such as *Limnodrilus* or *Tubifex*. Although they ate mosquitos, *Chironomus*, *Drosophila* and aphides during their youngest stage, and later flies, bagworms and various other insects of such a size, it was remarkable that they grew into matured adults by feeding on worms of Tubificidae only. The latter were easily and sufficiently obtained from shops of pet fish all the year round.

Food was given immediately after renewal of water. The wet weight of worms given at a time was about 8 g. for each small pan and about 15 g. for each large one, as shown in Table 2. The period of time from fertilization to sexual maturity differed in accordance with rearing conditions, such as the density of toads and water temperatures, as presented in the same table. In the best con-

TABLE 2
Relation between the density of toads and the period of time from fertilization to sexual maturity

Dimensions of a rearing pan and weight of food given at a time	Number of toads			Period of time from fertilization to sexual maturity (years)	
	Immediately after metamorphosis	About 2 cm. in body length	About 3 cm. in body length	At ca. 25°C during the cold season	At indoor or outdoor temperatures
33×23×6 cm³	40	15	5	1	2
8 g.	40	20	10	2	2
46×31×11 cm³	100	30	10	1	2
15 g.	100	50	40	2	3

dition nearly all the toads in each pan attained their sexual maturity just one year after the fertilization. When toads had been reared in comparatively low density and at 25°C or somewhat higher temperatures all the year round, most of them became larger than 40 mm. in body length at the age of one year.

If there were no accidents, the toads seldom died when the water in the pans was kept clean; they could healthily stand against severe temperatures and infectious diseases. The most cause of death was putrefaction of water, owing to the death of food worms. The water in pans was occasionally putrefied in such a case as that water temperature was too high, the excrements of toads were accumulated too much, the inner surfaces of the pan became very dirty, or the worms were given too much.

The putrefaction of water was prevented by the use of running water. In a large tank, 83 cm.×54 cm.×17 cm. in dimensions, into which running water was poured to the depth of about 5 mm, the toads always grew healthily, by making clean the inner surfaces once in three or four weeks.

IV. Reproduction

1. Toads collected from the field

The most active breeding season of *Bombina orientalis* in Korea seems to be May and June. The two females Nos. S63Pf1 and S63Pf2 and two of the three males, Nos. S63Pm1 and S63Pm2, which had been collected from Korea in 1963, produced many offspring after matings induced by pituitary injection in June, 1964 and January, February and April, 1965. The results of these matings are presented in Table 3. In the year 1964, the two females and two males were injected with pituitary hormones on the nineth of June and mated. The ovulation began to occur about 12 hours after the injection at 20°C, and the females spawned on the next day. The eggs were put in the water of PETRI dishes and left at the room temperature. After hatching the tadpoles were raised in large cement tanks placed outdoors.

All the 215 eggs obtained from one female were normally fertilized and became normal embryos. Moreover, all these embryos hatched and grew into

TABLE 3
Viability of the offspring of *Bombina orientalis*

Date of fertilization	Female no.	Male no.	No. of total eggs	No. of normal blastulae (%)	No. of normal neurulae (%)	No. of normal, tail-bud embryos (%)	No. of normal, hatched tadpoles (%)	No. of metamorphosed toads (%)
June 10, 1964	S63Pf1	S63Pm1	215	215(100)	215(100)	215(100)	215(100)	213(99.1)
〃	S63Pf2	S63Pm2	230	230(100)	229(99.6)	229(99.6)	229(99.6)	225(97.8)
Jan. 6, 1965	S63Pf1	S63Pm1	118	115(97.5)	110(93.2)	109(92.4)	105(89.0)	94(79.7)
Feb. 10, 1965	S63Pf2	S63Pm2	125	103(82.4)	95(76.0)	91(72.8)	86(68.8)	70(56.0)
April 30, 1965	S63Pf1	S63Pm1	260	249(95.8)	249(95.8)	248(95.4)	245(94.2)	241(92.7)
〃	S63Pf2	S63Pm2	310	301(97.1)	299(96.5)	293(94.5)	290(93.5)	282(91.0)
April 22, 1966	S63Pf1	S63Pm1	308	293(95.1)	281(91.2)	270(87.7)	270(87.7)	[1]
June 30, 1966	S63Pf1	S63Pm1	301	290(96.3)	276(91.7)	271(90.0)	271(90.0)	267(88.7)
June 1, 1967	S63Pf1	S63Pm1	678	661(97.5)	548(80.8)	548(80.8)	543(80.1)	512(75.5)
June 24, 1970	K69Pf2	K69Pm3	452	422(93.4)	420(92.9)	411(90.9)	411(90.9)	362(80.1)
Sept. 3, 1970	〃	〃	292	279(95.5)	263(90.1)	253(86.6)	251(86.0)	[2]
Aug. 4, 1970	T70Pf2	T70Pm	73	70(95.9)	69(94.5)	69(94.5)	69(94.5)	[2]
Sept. 3, 1970	〃	〃	133	131(98.5)	129(97.0)	129(97.0)	127(95.5)	36(27.1) [3]
Nov. 30, 1970	〃	〃	160	142(88.8)	140(87.5)	140(87.5)	139(86.9)	135(84.4)
Nov. 1, 1971	S71Pf1	S71Pm1	328	305(93.0)	281(85.7)	262(79.9)	258(78.7)	250(76.2)
Nov. 1, 1971	S71Pf2	S71Pm2	520	495(95.2)	487(93.7)	466(89.6)	399(76.7)	370(71.2)

[1] Used for another experiment at the tadpole stage
[2] Died of an infectious disease at the tadpole stage
[3] Mostly died of an infectious disease at the tadpole stage

normal tadpoles. All these tadpoles, but two, completed their metamorphosis at the ages of 20～25 days. Similarly, all the 230 eggs of the other female were fertilized and became normal blastulae. Although one of them became abnormal at the neurula stage, the other 229 hatched and grew into normal tadpoles; 225 completed their metamorphosis at the ages of 20～25 days.

On January 5th of the following year, 1965, a female and a male were injected with pituitary hormones. On the next day 118 eggs were obtained from this pair. With one exception these eggs cleaved normally and 115 became normal blastulae. Five, one and four embryos became abnormal and died at the neurula, tail-bud and hatching stage, respectively, and 105 became normal tadpoles. Ninety-four of the latter developed normally and completed their metamorphosis. From another female 125 eggs were obtained on February 10th of the same year by mating with a male after pituitary injection. Out of these eggs 108 cleaved and 103 became normal blastulae. Eight, four and five embryos became abnormal and died at the neurula, tail-bud and hatching stage, respectively, and 86 hatched normally. Seventy of the latter grew normally and became metamorphosed toads. The offspring of the above two females were raised in enamel pans at the temperature 25°C during the time from the beginning of development to the completion of metamorphosis. The protrusion of the forelegs of each tadpole occurred at the ages between 24 and 28 days.

The same two females and two males were injected again with pituitary hor-

mones and mated on April 29th of the same year. The females spawned 260 and 310 eggs on the next day. Out of these eggs 249 and 301 cleaved normally and became blastulae. Only four of the former and eleven of the latter became abnormal by the hatching stage and died; 245 and 290 embryos hatched normally and grew into swimming tadpoles. Nearly all of these tadpoles, that is, 241 and 282, completed their metamorphosis and became normal young toads. These offspring of the two pairs were raised from the beginning of the development in large cement tanks placed outdoors. In this case the forelegs of each tadpole protruded at the ages between 28 and 36 days.

In the year 1966 one of the two females collected in 1963 spawned 308 eggs on April 22nd and 301 eggs on June 30th after pituitary injections and mating with a male. Out of these two groups of eggs 293 and 290, respectively, cleaved normally and 270(87.7%) and 271(90.0%), respectively, became swimming tadpoles.

On June 1st of the next year one of the above two females spawned 678 eggs after a pituitary injection and mating with the same male. Out of these eggs 661 cleaved normally and 543(80.1%) became swimming tadpoles.

In June and September of the year 1970, a female and a male collected from the vicinity of Kyongiu, North Kyongsang, were mated after a pituitary injection. They spawned 452 eggs on June 24th. Out of these eggs 422 cleaved normally and 411(90.9%) developed into swimming tadpoles. By the next mating the same female and male spawned 292 eggs on September 3rd. Two hundred and seventy-nine eggs cleaved normally and 251(86.0%) became swimming tadpoles.

In the same year, a female and a male collected from South Chungchong Province in spring of this year spawned three times after pituitary injections. They spawned 73 eggs by the first mating on August 4th, 133 eggs by the second on September 3rd and 160 eggs by the third on November 30th. Out of these eggs, 70, 131 and 142, respectively, cleaved normally and 69(94.5%), 127(95.5%) and 139(86.9%), respectively, became normal tadpoles.

On November 1st, 1971, two pairs of toads from the vicinity of Seoul spawned 328 and 520 eggs after a pituitary injection. Of these eggs, more than 90% cleaved normally and became normal blastulae. Five days after spawning, 258(78.7%) and 399(76.7%) eggs hatched, and later 250(76.2%) and 370(71.2%) developed into normal, metamorphosed toads.

From the breeding experiments described above it is quite sure that healthy offspring can be obtained at any time all the year round from toads collected in the field.

2. Offspring generations

a. F_1 generation

Fertilized eggs of oriental fire-bellied toads required only one year in order to develop into sexually matured males or females when they were raised at about 25°C all the year round. These toads became nearly full size at the age of two years. The number of eggs laid by a F_1 female at a time differed in

general according to her size.

Females of F_1 generation produced in 1964 were examined in terms of body lengths and egg numbers of the first spawn at the age of 1, 2, 3 or 4 years (Table 4). The first spawning was always induced to occur in May or June. The eggs of the 1-year-old females were remarkably fewer than those of the 2-year-old females, as their bodies were larger. Moreover, the eggs of the 1-year-old females were about 2.0 mm. in diameter, that is, distinctly smaller than those of the 2-year-old females, which were about 2.5 mm. In contrast with this, there were no great differences in these respects among the 2-, 3- and 4-year-old females.

TABLE 4

Number and developmental capacity of eggs laid by each female as the first spawning at the age of 1, 2, 3, or 4 years

Age	Item \ Female no.	S64F$_1$f1	S64F$_1$f3	S64F$_1$f4	S64F$_1$f6	S64F$_1$f9	S64F$_1$f10
1 year old (1965)	Body length (mm.)	45.8	40.2	41.0	42.0	39.0	35.6
	No. of laid eggs	101	71	89	66	62	0
	No. of cleaved eggs (%)	91(90.1)	54(76.1)	60(67.4)	55(83.3)	52(83.9)	
	No. of hatched tadpoles(%)	52(51.5)	23(32.4)	53(59.6)	34(51.5)	32(51.6)	
2 years old (1966)	Body length (mm.)	51.2	49.8	51.9	50.0	48.2	49.0
	No. of laid eggs	396	375	531	447	276	345
	No. of cleaved eggs (%)	344(86.9)	312(83.2)	387(72.9)	347(77.6)	202(73.2)	301(87.2)
	No. of hatched tadpoles(%)	235(59.3)	196(52.3)	285(53.7)	209(46.8)	162(58.7)	203(58.8)
3 years old (1967)	Body length (mm.)	54.6	53.2	54.2	54.8	50.0	51.5
	No. of laid eggs	356	223	511	562	238[1]	323
	No. of cleaved eggs (%)	232(65.2)	183(82.1)	387(75.7)	492(87.5)	200(84.0)	288(89.2)
	No. of hatched tadpoles(%)	192(53.9)	172(77.1)	293(57.3)	374(66.5)	192(80.7)	192(59.4)
4 years old (1968)	Body length (mm.)	55.0	54.1	54.2	54.9	54.2	52.0
	No. of laid eggs	526	389	232[1]	343[1]	492	251[1]
	No. of cleaved eggs (%)	490(93.2)	321(82.5)	[2]	[2]	405(82.3)	[2]
	No. of hatched tadpoles(%)	356(67.7)	232(59.6)			356(72.4)	

[1] Obtained by pressing the abdomen and inseminated artificially
[2] Used for another experiment at the embryonal stage

A female toad seems to spawn several times in a year. In our laboratory many females were induced to spawn more than three times by pituitary injections. A F_1 female, No. S64F$_1$f4, spawned three times, on June 16th, 21st and 24th, 1965, by artificial induction at the age of 1 year (Table 5). In the next year this female spawned four times, on May 20th, June 24th, July 24th and August 10th by the same method. From this female 210 and 927 eggs in total were obtained at the ages of 1 and 2 years, respectively. Another F_1 female, No. S64F$_1$f3, spawned 223, 201 and 152 eggs on March 7th, May 6th and June 10th, 1967, respectively. The F_2 individuals developed from these eggs were very similar in viability during their life to those of the F_1 individuals which had been produced from the parents collected in the field, except the period of time before gastrulation.

TABLE 5
Number and developmental capacity of eggs laid by F_1 females after a pituitary injection

Male no.	S64F1m		
Female no.	S64F1f4	S64F1f4	S64F1f3
Date of fertilization	Jun. 16, '65	May 20, '66	Mar. 7, '67
No. of laid eggs	89	531	223
No. of cleaved eggs (%)	60(67.4)	387(72.9)	182(81.6)
No. of hatched tadpoles (%)	53(59.6)	285(53.7)	172(77.1)
Date of fertilization	Jun. 21, '65	Jun. 24, '66	May 6, '67
No. of laid eggs	66	166	201
No. of cleaved eggs (%)	64(97.0)	123(74.1)	182(90.5)
No. of hatched tadpoles (%)	45(68.2)	106(63.9)	168(83.6)
Date of fertilization	Jun. 24, '65	Jul. 24, '66	Jun. 10, '67
No. of laid eggs	55	105	152
No. of cleaved eggs (%)	52(94.5)	75(71.4)	[1]
No. of hatched tadpoles (%)	40(72.7)	70(66.7)	
Date of fertilization		Aug. 10, '66	
No. of laid eggs		125	
No. of cleaved eggs (%)		89(71.2)	
No. of hatched tadpoles (%)		80(64.0)	

[1] Used for another experiment at the embryonal stage

b. F_2 generation

In the breeding season of the year 1967, two F_2 females were examined in terms of reproductive capacity. On June 9th, 425 eggs were obtained from a female, No. S65F2f1, by the first spawning induced by injection of pituitary hormones (Table 6). This female was 2 years old. Of these eggs, 307 cleaved normally and 292(68.7%) became normal, swimming tadpoles. On the 12th of the same month, 181 eggs were taken out of the oviducts of another female, No. S65F2f2, 2 years old, by pressing the abdomen after a pituitary injection. By artificial fertilization 150 out of them cleaved regularly and 128(70.7%) hatched normally. On November 1st, 1971, a pair of F_2 toads spawned 161 eggs after a pituitary injection. Of these eggs, 117(72.7%) cleaved normally and 93(57.8%) became hatched tadpoles.

TABLE 6
Number and developmental capacity of eggs laid by F_2 females after a pituitary injection

Male no.	S65F2m1		S65F2m2
Female no.	S65F2f1	S65F2f2	S65F2f3
Date of insemination	Jun. 9, '67	Jun. 12, '67	Nov. 1, '71
No. of laid eggs	425[1]	181[2]	161[1]
No. of eggs for insemination	425	181	161
No. of cleaved eggs (%)	307(72.2)	150(82.9)	117(72.7)
No. of hatched tadpoles (%)	292(68.7)	128(70.7)	93(57.8)

[1] Laid by natural spawning
[2] Obtained by pressing the abdomen

c. F_3 generation

The reproductive capacity of F_3 females was examined in the seasons of 1969

TABLE 7
Number and developmental capacity of eggs laid by F_3 females after a pituitary injection

Male no.	$S67F_3m1$	$S67F_3m3$		
Female no.	$S67F_3f2$	$S67F_3f1$	$S67F_3f2$	$S67F_3f3$
Date of insemination	June 15, '69	July 1, '70	July 1, '70	July 1, '70
No. of laid eggs	152[1]	239[1]	174[1]	348[1]
No. of eggs for insemination	152	72	49	101
No. of cleaved eggs (%)	113(74.3)	56(77.8)	39(79.6)	83(82.2)
No. of hatched tadpoles (%)	101(66.4)	52(72.2)	35(71.4)	74(73.3)

1) Obtained by pressing the abdomen

and 1970 by making use of three females and two males. On June 15, 1969, 152 eggs were taken out of the oviducts of a female, No. $S67F_3f2$, after a pituitary injection (Table 7). This female was 2 years old. By artificial insemination 113(74.3%) eggs cleaved normally and 101(66.4%) became normal tadpoles.

On July 1st of the next year, three females, 3 years old, were used to examine the reproductive capacity of F_3 generation. After a pituitary injection, 239, 174 and 348 eggs were taken out of their oviducts, respectively. A part of these unfertilized eggs were inseminated to test their developmental capacity. As a result, 77.8~82.2% of eggs cleaved normally and 71.4~73.3% became normal tadpoles.

d. Outcrossing

All the data presented in Tables 5, 6 and 7 are the results of inbreeding. In the F_1 females, 53.7~83.6% (mean 67.7%) eggs became normal tadpoles (Table 5), while in the F_2 and F_3 females, 57.8~70.7% (mean 65.7%) and 66.4~73.3% (mean 70.8%) did (Tables 6 and 7), respectively. As these comparatively low percentages of hatched tadpoles were considered to be due to inbreeding, two females of inbreeding were mated with a male from the field in order to ascertain this assumption.

In the year 1970, the F_1 female No. $S64F_1f4$ and the F_2 female No. $S65F_2f1$ were mated with males collected from Kyongiu in 1969 after injecting frog-pituitary hormones (Table 8). Both females were five and six years old. From the F_1 female 52 eggs were laid on July 5th as the third spawning in this year. Although the number of eggs was small, their development was good; 49 cleaved

TABLE 8
Number and developmental capacity of eggs laid by F_1 and F_2 females mated with a male from the field

Male no.	K69Pm	
Female no.	$S64F_1f4$	$S65F_2f1$
Date of insemination	July 5, '70	Feb. 27, '70
No. of laid eggs	52[1]	215[2]
No. of cleaved eggs (%)	49(94.2)	202(94.0)
No. of hatched tadpoles (%)	46(88.5)	195(90.7)

1) Obtained by pressing the abdomen as the third yield in a year
2) Obtained by pressing the abdomen as the first yield in a year

normally and 46(88.5%) became normal tadpoles. From the other F_2 female 215 eggs were obtained on February 27th. Out of these eggs 202 cleaved normally and 195(90.7%) developed into normal, swimming tadpoles. These results seemed to show that the females of inbred generations were good in reproductive capacity.

DISCUSSION

Anurans are one of the most popular animals found in every region of the world. They have been good materials for biological researches and instruction. Innumerable papers concerning various branches of biology are issued every year. However, there are only meager reports in the field of anurans genetics. This state of affairs principally depends upon difficulty in raising them to sexual maturity and obtaining their offspring. As far as the present authors know, DÜRKEN (1935 and '38) was the first investigator who succeeded in rearing frogs since the eggs stage to the sexual maturity in the laboratory. His nine hybrids between female *Rana arvalis* and male *Rana temporaria* were reared for four years, and four of them were reared for a long period of seven years, although it was ascertained that they were all males and quite sterile. TCHOU and others (1938, '40) reared two parthenogenetically developed females of *Rana nigromaculata* for five years. These frogs, however, did not lay eggs. In toads, BLAIR (1941) reared male hybrids between female *Bufo w. woodhousei* and male *Bufo terrestris americanus* until their sexual maturity. One of the hybrids was kept for six years (BLAIR, 1946). By the backcross of these hybrids with female *Bufo terrestris americanus* a number of tadpoles were produced. In the year 1941, KAWAMURA reported on the production of matured triploids in *Rana nigromaculata.* The same author (1942) also obtained matured interspecific hybrids between two Japanese brown frog species.

After those days, various kinds of interspecific hybrids, auto- and allopolyploids, and nucleo-cytoplasmic hybrids, gynogenetic diploids, etc. in several *Rana* species have been produced and reared to their sexual maturity by KAWAMURA and his collaborators, although these frogs were often confronted with many crises. From many of these frogs several generations of offspring have been obtained hitherto. Except for the experiments carried out in our laboratory, there were only a few which reported that the genus *Rana*, the most common frogs in the temperate zone of the Northern Hemisphere, was raised since the egg stage to sexual maturity, even in the years after 1947, owing to difficulties in rearing metamorphosed frogs. Among these reports, there were those by TING (1948) who obtained two-year-old hybrids of *Rana nigromaculata* and *Rana plancyi* and by MCKINNELL (1962) who produced a sexually matured intraspecific nucleo-cytoplasmic hybrid of *Rana pipiens.* However, the aquatic genus *Xenopus* is an exceptional animal among anurans which are hard to be sexually matured in laboratory conditions. The South African clawed toad, *Xenopus laevis,* above all other anurans, has been reared and increased in many laboratories and used

as an excellent material by numerous biologists (cf. NIEUWKOOP and FABER, 1956).

Bombina orientalis seems to be another excellent material for biological researches. While the two species of fire-bellied toads in Europe, *Bombina bombina* and *B. variegata*, were often used for biological and biochemical researches, the oriental fire-bellied toad has scarcely ever become a laboratory animal, except for researches on the chromosomes (SATO, 1938).

Bombina orientalis is very similar to *Xenopus laevis* in having the ability to lay eggs all the year round. Amplexus and spawning are reliably induced by an injection of frog pituitaries into both sexes. On the other hand, numerous unfertilized eggs can be obtained from the oviducts of a pituitary injected female through the cloaca by pressing the abdomen. The ratios of normally fertilized and developing eggs are always high in the case of either natural spawn or artificial insemination.

Although *Bombina* tadpoles are reared in the same way as the tadpoles of various *Rana* species are, metamorphosed toads can be fed on worms of Tubificidae only. As these worms are got from a gold-fish or tropical fish seller in Japan, it is very easy to rear the toads. Both males and females are sexually matured at the age of one year and become nearly full size at the age of two years, when they have been reared at ca. 25°C. Oriental fire-bellied toads have the power of resistance to severe temperatures in summer and winter as well as to infectious diseases. Accordingly, they seldom die within a period of several years after the metamorphosis.

As the toads are not aquatic, differing from *Xenopus*, much larger number of individuals can be kept in a pan than in the case of rearing *Xenopus*. Moreover, such a container is easy to handle, owing to a little quantity of water in it.

All the above-stated characteristics of oriental fire-bellied toads seem to indicate that this species is an excellent laboratory animal for researches in various branches of biology. The beautiful color pattern and good voice would qualify this species as a pet, too.

SUMMARY

1. The oriental fire-bellied toad, *Bombina orientalis* (BOULENGER) is a lovely animal easy to be raised in the laboratory.

2. Amplexus and spawning always occur reliably by an injection of mashed frog pituitaries into both sexes. Artificial insemination of eggs taken out of the oviducts is also easily done by the routine method. Naturally or artificially fertilized eggs can be obtained abundantly at any time all the year round.

3. Fertilized eggs require 25～30 days for attaining to the stage of protrusion of the forelegs at ca. 25°C. Tadpoles are fed on boiled soft vegetables like Japanese spinach. Toads are given worms of Tubificidae, such as *Limunodrilus* and *Tubifex*. Both sexes can reach their sexual maturity at the age of just one year and become nearly full size in one more year.

4. The eggs of one-year-old females are distinctly smaller in size and number than those of two-year-old females, while there are no great differences in these respects among two-, three- and four-year-old females.

5. Each females can be induced to spawn more than three times in one year by pituitary injections.

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