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Embrional and larval development of F_1 generation of green frogs different combinations

[Pp. 123—162, 9 text-figs.]

Rozwój embrionalny i larwalny pokolenia F_1 różnych kombinacji żab zielonych

Эмбриональное и личиночное розвитие поколения F_1 розличных комбинации у зеленых лягушек

Abstract. The author presents the results of investigations on cross fertilization of three morphological forms of green frog: *lessone*, *esculenta* and *ridibunda*. Basing on the speed of development and percentage of tadpoles' mortality he supposses that the specimens which after morphological characters are included in *esculenta* — form are probably the hybrids of other two green frog forms.

I. INTRODUCTION

From different branches of investigations which I made in order to clarify the relationship among some forms of green frogs which were usually considered as belonging to the species *Rana esculenta* LINNAEUS, only one part was published, concerning morphological analysis of 276 specimens caught near Poznań during their breeding season (BERGER 1966). I found that this frog population was divided into three morphological groups without intermediate individuals. The characters which served as a basis for distinguishing these groups, are usually ascribed to forms: *R. lessonae* CAMERANO, *R. esculenta* LINNAEUS and *R. ridibunda* PALLAS, which are very common in the whole of Central Europe (BOULENGER 1898, SCHREIBER 1912, TERENTIEV and ČERNOV 1949, BERGER and MICHAŁOWSKI 1963).

This work reports the results of observations on development of embryos and then of tadpoles up to the moment of their metamorphosis. They were

obtained by fertilization in different combinations of the three forms mentioned above. Similar investigations carried out by PFLÜGER, in 1883, were mentioned by BOULENGER (1885) and in later years similar works were carried out by BOLKAY (1901), Mandeville and Spurway (1949) and SMITH (1949). These authors studied hybridization of forms: *esculenta* and *ridibunda*. The results of their investigations however did not bring any change in opinion as to the relationship of these forms.

In European literature we find very few data concerning observations on embryonal development of green frogs. In general there is only one work (MICHNIEWSKA-PREDYGIER and PIGOŃ 1957) giving an exact description of the early stages of development of these frogs. The remaining works include either very little data as to their development (HERTWIG 1896) or are presented in a form of little use to other investigators (TAMINI 1947, DOUGLAS 1948, KAURI 1959). The authors of the latter three works, when distinguishing the embryonic stages, assumed the scheme of description elaborated for American species (POLISTER and MOORE 1937) in which the sequence of the successive stages was different than in some European species.

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II. MATERIAL AND METHOD

Investigations on fertilization of three green frog forms were carried out on the individuals caught in amplexus, in some ponds situated on the left bank of the Warta river, in Poznań. In 1963 they originated from one environment (BERGER 1966) and in the following years they were caught in different places. The total number of 180 individuals belonging to three forms were hybridized: 66 *lessonae* (35 ♂♂ and 31♀♀), 73 *esculenta* (35 ♂♂ and 38 ♀♀), 41 *ridibunda* (21 ♂♂ and 20 ♀♀). In 1963, 32 individuals were used, in 1964 — 104, and in 1965 — 44.

I present the general method of fertilization of all forms of green frogs, basing on the plan in Table I, while the details, that often deviate from the plan, will be described later on. To facilitate the use of names of different combinations, I introduced symbols, i. e. letters for females and Arabic figures for males.

Before starting fertilization of eggs I checked the females as to whether they were ready to lay eggs, by pressing the belly of each female. If the eggs were ripe, they came out through cloaca at slight pressure. Then, following the method of MICHNIEWSKA-PREDYGIER and PIGOŃ (1957), I prepared the sperm. After killing the male I took out the testes and crushed them in a few ccm of aquarium water. A few minutes later I checked activity of the spermatozoa.

Having prepared the sperm suspension of all forms of green frogs, I began artificial fertilization.

In the prepared petri dishes, placed according to the plan (Table I), I squeezed from each female three portions of eggs, approximately equal in number. On each portion I poured the sperm suspention of the males belonging to various

Table I

The general plan of cross fertilization of three green frog forms

Male \ Female	*lessonae* a	*esculenta* b	*ridibunda* c
lessonae — 1	1a	1b	1c
esculenta — 2	2a	2b	2c
ridibunda — 3	3a	3b	3c

forms. The moment when the sperm came in contact with the eggs was considered as the moment of fertilization. A few minutes later the eggs were submerged in aquarium water. The water was changed after 15 minutes and the dishes were placed in the rooms in which they were to develop.

In addition to the complete series (9 types of fertilizations) I prepared different incomplete series including 1 or 3 types of combinations. I also made many natural fertilizations, placing in a separate aquarium the suitably chosen pairs which spontaneously laid eggs.

To facilitate orientation in different series within the same types of fertilizations, the following signs were introduced. The complete series including 9 combinations of the same specimens were asigned S, the series including 3 combinations (eggs from one female always) were assigned with a capital letter corresponding to the symbol of the given female form (Table I). The germs of each series as well as of single matings within the same type of fertilization obtained successive ordinal numbers. The development of eggs of various series and of single combinations proceeded in different temperatures: low (L) — 15° C, midle (M) — 19—20° C and high (H) — 28—29° C.

Due to an insufficient number of thermostats, the dishes with the fertilized eggs were placed side by side on special tables in rooms with more or less constant air temperature, so that fluctuation of water temperature was always the same in all dishes.

The constant temperature in the rooms supplied very favourable conditions enabling continuous observations, and, consequently, precise determination of different moments of development. Such observations, with series including

9 combinations, developing with different speed (Tables II—V, XIII), would be impossible if only thermostats were used.

One of the most remarkable advantages of simultaneous fertilization of all combinations was that comparative observations could be carried on during the development of germs, although this method of fertilization was rather troublesome. The greatest difficulty consisted in finding simultaneously mature females. In the laboratory, I had always 10 to 20 or even many more females of each form, caught in amplexus, but only in exceptional cases I succeeded in choosing the individuals equally mature at the same time. Therefore, when I found a mature femal I placed her in a separate aquarium and waited until the females of the remaining forms matured.

I distinguished the embryonic stages following the data from the works by WITCHI (1956), MICHNIEWSKA-PREDYGIER and PIGOŃ (1957) and GOIN and GOIN (1962). Basing generally on the same morphological features, these authors distinguished 25 embryonic stages in anurans. The sequence of the individual stages is given here after MICHNIEWSKA-PREDYGIER and PIGOŃ, and the English names after GOIN and GOIN (1962).

The development of eggs, even originating from the same pair, was usually very different. That is why I assumed the moment when the determined features appeared in at least 4 or 5 germs as the beginning of the given stage. Since many germs died in different stages, for unknown reasons, I had to give up the method of MOORE (1949) who considered as the beginning of the given stage the moment when about 50% of germs reached this stage.

There was no special difficulty in distinguishing most of embryonic stages in green frogs, so the moment of their appearance could be given very exactly, especially for the stages 1 to 6, 10 to 18, 20 and 23 to 25. The beginning of stage 18 I assumed after MICHNIEWSKA-PREDYGIER and PIGOŃ as the moment of exit of the larva from the gelatinous envelope. The interval between the first movements of germs in the gelatinous envelopes (stage 18 according to GOIN and GOIN but not distinguished by MICHNIEWSKA-PREDYGIER and PIGOŃ) and their hatching (stage 20 according to GOIN and GOIN) ranged from 20 to 30 hours.

Determination of the remaining stages needs some explanations. The stages 7 to 9 include blastula. In this period the limits between cells undergoing cleavage are very indistinct and one should have much practice and experience to catch the right moment. Determination of commencing moments of different stages of neurula is somewhat obscure, particularly in stages 14 to 16. The greatest difficulties however were in distinguishing stages 19, 21 and 22. It appeared that the embryos of some combinations, firstly those from ridibunda female, were in most cases quite opaque. For this reason it was sometimes impossible to observe the moment of heart beat or of tail fin circulation. It was also very difficult to distinguish the beginning of stage 22 as it often coincided with stage 23. Thus the moments of beginning of these stages are often erroneously recorded (usually these stages began earlier) and they were not reported in cases of great doubt (Tables II, III). As the moment determining primary larval

period (stage 25) I assumed the moment of disappearance of the outside gill in spiraculum.

The tadpoles (larvae) for further reaging were transferred to aquaria placed side by side in the open air in a special enclosure for rearing adult frogs. In a case of a series all tadpoles were placed in similar aquaria. Water was replaced daily from a tank or a tap. All aquaria were carefully washed every week.

The tadpoles were fed with a meal (powdered nettle, powdered milk, powdered liver and $CaCO_3$), prepared according to KUCIAS (1961). In addition they were given letuce and dead tadpoles of amphibians, mostly of green frogs.

In 1963, the first series S1M was prepared on May 18, using 3 females and 1 male of each form. From each live female I squeezed about 100 eggs on one dish (on each dish about 300 eggs from 3 females) and they were fertilized with sperm of one male. In a similar way, on May 21, the series S2M was fertilized using 2 females *lessonae* and *esculenta* and 1 female *ridibunda* and 1 male of each form. From each live female I took 30 to 50 eggs on each dish. All dishes with fertilized eggs of complete and incomplete series were placed side by side on the table in the laboratory. The water temperature in the dishes was about 19° C, in extreme cases ranging from 17·2° to 20·0° C, daily deviations being as high as 1° to 2° C.

In 1964, on May 12, for the first series I used 3 females and 3 males of each form. From each live female I squeezed about 30 eggs on one dish. They were fertilized in a similar way as in 1963, except that the sperm of each male was prepared separately and poured on the eggs of only one female. In such a way I obtained on each dish the desired combinations but originating from three different pairs. These frogs were used for three complete series which developed in three different temperatures.

The dishes with the eggs of the series S3L were placed in a dark room with low temperature (15° C), depending to a certain degree on the outside temperature. In extreme cases it ranged from 13·5° to 17·1° C but in the period of 24 days' observations the daily fluctuation did not exceed 0·5° C. On the first day the water temperature was 16° C, but the next day it started decreasing. On the 7th day it reached 13·5° C, remained at this level for 3 days and then slowly increased reaching 17·1° C on 21st day, and remained at this level to the end of development of the embryos.

The dishes with the eggs of series S3M were placed on the table in the room with more or less constant temperature of approximately 23° C for the whole period of development of the embryos. The water temperature (checked every 2—3 hours) deviated from 19·5° to 20·5° C but usually it was about 20° C. Only twice during the whole period it overreached these limits, once reaching 18·8° and once 22·5° C.

The eggs of series S4H, placed in the thermostat in 28° C, after a few hours of development were destroyed (Table V).

This method of fertilization appeared to be disadvantageous. The eggs squeezed from the living females were often deformed or damaged when passing

Table II

Comparison of rates of embryonic development in different combinations of green frogs. Series S4L (15° C)

Stage \ Female / Male	*lessonae*			*esculenta*			*ridibunda*		
	les.	*esc.*	*rid.*	*les.*	*esc.*	*rid.*	*les.*	*esc.*	*rid.*
1—2. Unfertilized — Gray crescent									
3. Two-cell	4·10	4·08	4·09	3·58	3·58	4·00	4·16	4·16	4·16
4. Four-cell	5·20	5·32	5·30	5·26	5·43	5·45	5·55	5·56	5·55
5. Eight-cell	6·33	6·43	6·55	6·51	7·15	7·15	7·20	7·28	7·25
6. Sixteen-cell	7·50	8·00	8·01	8·13	8·33	8·50	8·37	8·40	8·40
7. 32-cell	10·10	10·30	10·30	10·45	11·07	11·07	10·43	10·55	10·57
8. Mid-blastula	12·25	12·45	12·50	12·40	13·15	13·15	14·25	14·25	14·25
9. Late blastula	21·40	21·40	21·40	24·15	24·15	24·15	23·45	23·45	23·45
10. Dorsal lip	34·52	35·10	37·35	45·55	46·10	47·10	58·10	50·55	54·35
11. Mid-gastrula	46·10	46·10	49·45	48·17	50·55	53·05	69·15	69·40	69·40
12. Late gastrula	48·25	49·40	53·15	55·30	56·00	58·30	76·10	76·05	75·05
13. Neural plate	64·40	67·40	71·40	75·45	75·42	76·20	94·45	100·25	106·25
14. Neural folds	77·00	87·40	88·40	94·40	100·30	108·10	107·55	127·15	119·50
15. Rotation	92·40	94·40	108·15	108·05	119·55	120·30	127·10	144·00	127·25
16. Neural tube	108·10	108·15	120·00	119·50	132·30			156·25	
17. Tail bud	127·20	131·40	142·40	143·30	150·30	156·30	151·40	187·25	187·25
18. Hatching	195·13	228·10	216·40	228·00	265·30	295·30	347·25	324·25	324·25
19. Heart beat	239·50	265·40	265·40	252·00	301·30	311·30	361·15	348·25	348·20
20. Gill circulation	264·40	278·40	288·40	288·30	311·30	348·30	367·15	361·25	361·25
21. Tail fin circulation. Mouth open	315·40	315·40	346·40	361·30	368·30	407·30	383·15	392·05	392·05
22. Cornea transparent	361·40	373·40	383·40	383·30		446·00		434·25	
23. Opercular fold	372·10	392·10	404·00	407·30	446·00	480·30	406·25	456·25	464·25
24. Operculum closed on right	441·40	455·40	480·40	456·30	502·30	528·30	456·15	504·25	504·25
25. Operculum complete	464·10	489·55	513·40	498·30	548·30	576·30	480·15	552·25	552·25

Table III

Comparison of rates of embryonic development in different combinations of green frogs. Series S4M (20° C)

Stage	Female / Male	*lessonae*			*esculenta*			*ridibunda*		
		les.	*esc.*	*rid.*	*les.*	*esc.*	*rid.*	*les.*	*esc.*	*rid.*
1—2. Unfertilized — Gray crescent										
3. Two-cell		2·02	2·05	2·02	1·57	1·59	1·58	2·04	2·03	2·03
4. Four-cell		2·36	2·42	2·37	2·31	2·43	2·41	2·45	2·46	2·45
5. Eight-cell		3·14	3·22	3·15	3·30	3·31	3·31	3·25	3·24	3·25
6. Sixteen-cell		3·53	4·11	3·54	4·05	4·05	4·03	3·54	3·52	3·54
7. 32-cell		4·39	4·40	4·39	4·43	4·43	4·42	4·37	4·37	4·38
8. Mid-blastula		6·58	7·00	7·00	7·18	7·18	7·18	6·45	7·12	6·47
9. Late blastula		11·10	11·10	11·10	18·00	19·00	19·00	11·05	11·05	11·05
10. Dorsal lip		18·10	18·17	18·30	28·55	27·45	28·00	21·05	21·05	20·55
11. Mid-gastrula		22·30	23·10	23·15	36·30	36·00		26·55	26·45	26·45
12. Late gastrula		28·55	29·55	29·40	47·10	47·00	36·45	32·35	32·35	32·35
13. Neural plate		37·40		37·40		68·00	52·30			37·05
14. Neural folds		45·10	45·25	47·20	71·00	76·15	61·00	47·20	47·00	47·25
15. Rotation		52·10	54·30	52·10	79·00	79·00	71·30	58·05	58·05	58·05
16. Neural tube		62·10	66·10	68·10	83·00	92·30	73·00	68·50	69·05	71·40
17. Tail bud		73·00	78·20	76·25	108·00	108·00	93·30	76·15	78·25	76·55
18. Hatching		106·10	118·10	117·00	143·00	148·30	124·45	116·05	124·05	125·05
19. Heart beat		118·10	124·55	119·10			155·30	126·05		
20. Gill circulation		125·10	131·10	132·10	155·30	175·00	175·15	131·20	131·25	142·35
21. Tail fin circulation. Mouth open		151·10	166·30	166·40	191·00	191·00	191·15	166·35		191·05
22. Cornea transparent		175·10								
23. Opercular fold		190·40	190·50	197·10	236·00	239·00	228·00	190·35	214·05	239·05
24. Operculum closed on right		198·10	242·10	244·10	264·40	283·00	276·00	226·45	234·05	264·25
25. Operculum complete		224·40	272·10	261·10	295·00	311·00	311·00	260·05	276·05	299·05

through the anus. When they stuck to the dry bottom of the dish, these deformations did not recover, making further development difficult or even impossible.

When preparing the next series S4L, S4M, on May 13, I introduced a change in the method. For reproduction I used 3 females *lessonae* and *esculenta*, and 2 females *ridibunda*, and 4 males of each form. The sperm from all males of one form was mixed in one vessel and sperm suspension was poured into dishes. Then I killed simultaneously all females of one form, and from each of them I squeezed a more or less equal portion of eggs directly into the sperm suspention on the dishes. Later fertilization was carried out in this way.

The individuals mentioned above were used for two complete series which developed in two various temperatures.

In 1965, I used one female and one male from each form for the first complete series. For further observation however there remained only the eggs from *esculenta* female, which developed in 20° C (B3M). This unsuccessful experiment is described in detail elsewhere.

III. RESULTS

The results of development of different combinations from the moment of fertilization to metamorphosis (complete resorption of the tail) are presented in the Tables, and the detailed analysis is given below.

1. Rate of development

Periods of development of embryos of individual combinations are distinctly differentiated (Tables II—IV). The tadpoles of combinations 1a developed most rapidly in all tested temperatures and those of several cambinations developed the slowest. The development of germs of the same type of fertilization and in the same temperature was very similar. Only the tadpoles of combination 3b deviated greatly from this regularity. The hybrid reciprocal combinations (2a—1b, 3a—1c and 3b—2c) had approximate periods of development, in some cases almost identical (Table IV).

It results from Table V that the initial rates of development of the eggs of the same female were very approximate, although they were fertilized with the sperm of males of various forms. This phenomenon was observed from the first cleavage of eggs (Tables II, III) to the medium period of neurula (stages 13 to 15). The most marked differentiation in the development of germs from the females of various forms occurred in the period of gastrula. In this period the germs from each female reached stage 10 at different moments (Table V). The difference in development of germs from various females was very great — it ranged from a few to several hours, whereas that among the germs from one female was quite small — several minutes. In the period of tailbud (stages 17 to 24) the development of the latter germs also began to differentiate.

The general tendency of development of germs of all combinations including *lessonae* form was a distinct acceleration of development, at least in the final stages, while in the combinations with *ridibunda* form the development of germs was retarded.

The tadpoles of different combinations reached metamorphosis in various periods of time (Table VI). As a rule, a few metamorphosed specimens appeared simultaneously. In the group of tadpoles reaching metamorphosis in the shortest time, there were first of all the tadpoles of combinations 2a, 3a and 1c, and their periods of development were almost the same. The tadpoles of 2b and 3b were the slowest in reaching metamorphosis. To this group belonged also the

Table IV

Recorded moments of reaching stage 25 by germs of various combinations. Time in hours and minutes. Development of differentiated eggs of *esculenta* females is given in Table XIII

Series	Combinations								
	1a	2a	3a	1b	2b	3b	1c	2c	3c
in 15°C									
S3L	479·15	—	—	513·05	579·05	—	489·30	—	578·30
S4L	464·10	489·55	513·40	498·30	548·30	576·30	480·25	552·25	552·25
10L	454·40								
7L					559·00				
in 19°C									
S1M	255·45	291·15	328·05	310·15	336·45	391·05	276·55	328·10	341·10
S2M	263·22	278·02	277·42	276·54	334·12	302·17	300·05	342·05	318·35
A1M	262·40	274·50	274·50						
1M		301·15							
1M				322·00					
in 20°C									
S3M	221·40	257·40	254·40	—	—	—	272·30	280·00	300·30
S4M	224·40	272·10	261·10	295·00	311·00	311·00	260·05	276·05	299·05
B3M				264·00	323·30	323·30			
11M	216·00								
12M	216·00								
8M					325·40				
10M					325·30				
12M					293·40				
3M						262·00			
3M								285·15	
4M								282·15	
in 29°C									
A2H	114·20	123·10	124·20						
13H	132·20								

Table V

Comparison of periods of time up to reaching three stages by the germs of all combinations. Time in hours and minutes

Series	No. of Stage	Combinations								
		1a	2a	3a	1b	2b	3b	1c	2c	3c
S4L (15°C)	3	4·10	4·08	4·09	3·58	3·58	4·00	4·16	4·16	4·16
	10	34·52	35·10	37·35	45·55	46·10	47·10	58·10	50·55	54·35
	25	464·10	489·55	513·40	498·30	548·30	576·30	480·15	552·25	552·25
S2M (19°C)	3	1·57	1·58	1·58	1·59	2·10	2·05	2·03	2·05	2·05
	10	25·10	25·12	25·29	27·07	27·32	27·37	34·55	34·55	32·05
	25	263·22	278·02	277·42	276·54	334·12	302·17	300·05	342·05	318·35
S4M (20°C)	3	2·02	2·05	2·02	1·57	1·59	1·58	2·04	2·03	2·03
	10	18·10	18·17	18·30	28·55	27·55	28·00	21·05	21·05	20·55
	25	224·40	272·10	261·10	295·00	311·00	311·00	260·05	276·05	299·05
S3H (28°C)	3	1·36	1·36	1·36	1·38	1·39	1·39	1·42	1·45	1·45

tadpoles 1a, clearly retarded in comparison to those of other combinations of the same series. The tadpoles of remaining combinations (1b, 2c, 3c) showed very great deviations in various series. Sometimes they metamorphosed the earliest of the whole series while at another time their metamorphosis was significantly delayed.

When comparing the periods of development of tadpoles from the moment of fertilization to metamorphosis, one can state that tadpoles of all combinations

Table VI

Development of tadpoles to complete metamorphosis. Days calculated beginning from three different stages (1, 18, 25) of embryonic development

Series	Number of days from stage	Combinations								
		1a	2a	3a	1b	2b	3b	1c	2c	3c
S4L (15°C)	fertilization	79	77	77	85	99	89	79	76	79
	hatching	71	67	68	76	88	77	65	63	66
	25 stage	60	57	56	64	76	65	59	53	56
S4M (20°C)	fertilization	71	68	68	65	71	76	68	69	65
	hatching	67	63	63	59	65	71	63	65	60
	25 stage	62	57	57	53	58	63	57	57	53

S4M metamorphosed much earlier than the tadpoles S4L. In extreme cases this difference ranged from 7 days in 2c to 28 days in 2b. The periods of metamorphosis appear quite different when we begin our calculation from stage 25. In this case only the tadpoles 1b and 2b of series S4L are delayed by 11 and 18 days respectively, when compared with the tadpoles S4M, while the period of development of the remaining combinations in both series is distinctly equalized, and in 3 cases (S4L: 1a, 3a, 2c) is even significantly shorter.

2. Viability and development of progeny

Different methods of investigations on development of germs were used in various years. To enable a comparison of results obtained in particular years, they are presented in chronological order.

In 1963 detailed observations on the development of germs were not carried out (compare the method and Table XII: B1M) and for this reason there are only the results of rearing tadpoles of all combinations obtained. On July 11, photos were made of the tadpoles of series S1M (Fig. 1) taking 3 specimens

Table VII

Results of rearing tadpoles to complete matamorphosis in 1963. All metamorphosed specimens kept for further rearing

Series and combination	Initial number on June 2	Per cent of mata-morphosed specimens	Dates of metamorphosed		Date of death of the last specimen	Maximal length on July 11, mm
			the first	the last		
S1M + S2M						
1a	72	58·3	25·7	21·8	1· 6·64	58
2a	110	55·4	27·7	17·7·64		60
3a	17	70·7	21·7	5·8		67
1b	105	84·8	26·7	7·9	1· 2·65	60
2b	56	—			29· 8	23
3b	25	40·0	29·7	20·9		46
1c	20	55·0	28·7	15·8		55
2c	42	81·0	4·8	26·8	22·12·64	54
3c	7	85·7	30·7	9·8		53
A1M						
1a	22	—				
2a	17	35·3	23·7	27·7	15· 9	
3a	35	82·9	23·7	5·8	16· 5·65	
2a—1M	4	100·0	25·7	27·7		
2a—2M	40	95·0	6·8	12·9		
1b—1M	80	96·2	4·8	5·9	26· 9	

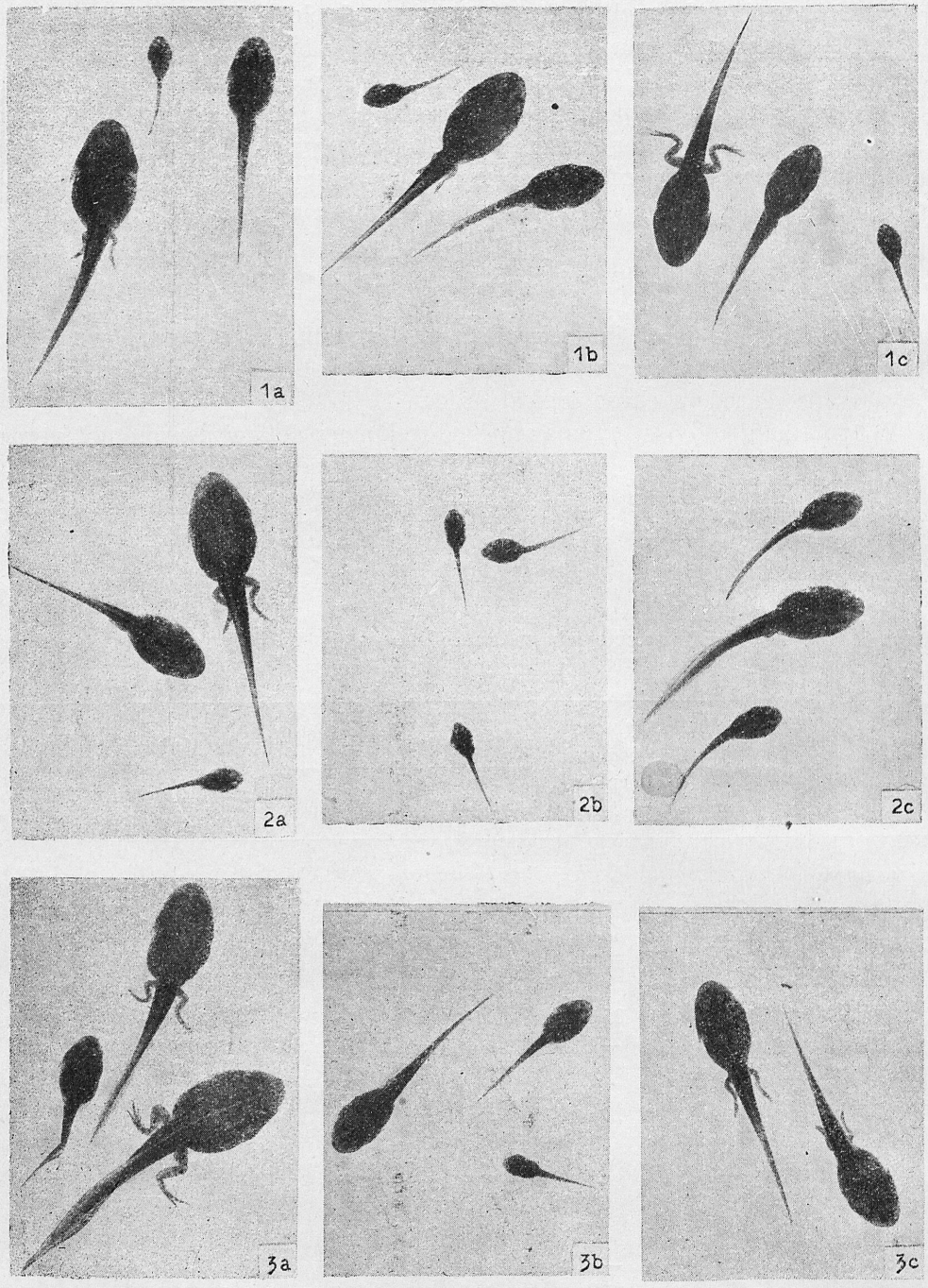

Fig. 1. Tadpoles of all combinations of series S1M, in 1963, 54 days after fertilization. From each aquarium 3 tadpoles: the biggest, the smallest and average

Phot. *Z. Pniewski*

from each combination. Combination 3c is represented by 2 tadpoles, the only ones obtained in this series. At the beginning the tadpoles of the series S1M and S2M were kept separately but shortly before metamorphosis (July 19) they were placed in common aquaria. The developments of the corresponding combinations in both series were very similar.

The comparison of photos (Fig. 1) and data in Table VII reveals that among 9 combinations only the tadpoles of combination 2b were not differentiated as to their size and were clearly delayed in development, in relation to the remaining tadpoles. They were the last 3 tadpoles that remained from 34 initial

Table VIII

Development of eggs of series S4L and S4M and further rearing of tadpoles in 1964. Rearing begun: S4M on May 26; S4L on June 2; finished S4M on August 18; S4L on September 2

Embryonic development					Rearing tadpoles (larvae)					
Series and combinations	Number of eggs	Per cent of dead embryoes and unfertilized eggs	Embryoes after hatching		Initial number	% dead		% alive (preserved)		
			total number	percent of abnormal		tadpoles	with 4 limbs	tadpoles	with 4 limbs	metamorphosed
S4L										
1a	203	29·6	143	—	100	33·0	22·0	25·0	2·0	18·0
2a	210	43·8	118	2·2	100	11·0	13·0	32·0	7·0	37·0
3a	203	18·3	166	1·8	100	9·0	8·0	41·0	4·0	38·0
1b	303	6·9	282	2·1	100	65·0	4·0	20·0	2·0	9·0
2b	218	20·2	174	21·8	100	68·0	6·0	14·0	—	12·0
3b	203	15·3	172	18·6	100	34·0	—	50·0	—	16·0
1c	245	97·6	5	60·0	36	41·6	19·4	2·8	8·3	27·9
2c	229	86·1	30	66·0	10	30·0	10·0	—	—	60·0
3c	205	94·1	12	83·3	10	10·0	20·0	10·0	10·0	50·0
S4M										
1a	199	50·2	99	3·3	80	10·0	7·5	16·3	3·7	62·5
2a	196	8·1	180	3·9	80	32·5	3·7	11·3	—	52·5
3a	238	36·9	150	6·0	80	32·5	—	7·5	6·2	53·8
1b	373	71·3	107	32·7	65	21·6	1·5	10·8	—	66·1
2b	316	78·8	67	53·7	28	39·3	14·1	17·9	7·1	21·4
3b	329	32·4	192	31·2	80	13·7	2·5	36·3	7·5	40·0
1c	260	54·2	119	8·4	80	13·7	1·3	11·3	—	73·7
2c	163	50·9	80	17·5	63	50·8	1·6	4·6	1·6	42·9
3c	299	58·5	124	23·4	80	23·8	3·7	22·5	3·7	46·3

specimens of this combination, and the last of them died on August 29, being 27 mm long. The tadpoles 2b-S2M developed in identical way: from the initial number of 22 specimens the last one died on July 23.

The tadpoles of some combinations (Table VII) lived for a very long time without any sign of further development. The last tadpoles 1a and 2c of the series S1M + S2M were albinotic and the tadpole 1b, in which the spiraculum was at the right side of the body, was 125 mm long when it died. The period of metamorphosis of tadpoles of cross 2a lasted almost the whole year: the last tadpole in 1963 passed through metamorphosis on September 24 and two remaining metamorphosed in June 3 and July 17 the following year.

The results of investigations carried out in 1964 are presented in Tables VIII to XI. The series S3L and S3M are omitted here as the germs of a few combinations did not reach stage 25 (Table IV) and in most of the remaining combinations there were only a few specimens.

Detailed observations were carried out basing on the series S4L and S4M (Table VIII) and tadpoles from the remaining series were used mainly as control specimens (Tables IX to XI). However, for the reasons described below, some data in Table VIII (first two columns) can not be used for comparative analysis. In the series S4M I had to remove, from all dishes, the eggs of one *esculenta* female and of one *ridibunda* female, since it appeared that the eggs of these females after reaching stage 3 began irregular cleavage and then were subjected to cytolysis. In one *esculenta* female a part of the eggs did not begin cleavage at all.

The development of eggs of these females was quite different in series S4L. The eggs of all *esculenta* females underwent cleavage in 100% while among two *ridibunda* females, the eggs of all combinations did not begin cleavage in one and in the other the eggs reached stage 4, did not develop further, and were subjected to cytolysis. Finaly 5 to 30 developing germs remained on the dishes with the eggs of *ridibunda* female. It should be explained here, that for further rearing I placed 34 tadpoles 1c and 8 tadpoles 3c from the series S3L together with two normal tadpoles 1c and 3c from the series S4L.

In the progeny of *lessonae* females in both series, S4L and S4M, another abnormality was observed. It appeared that the development of eggs from one female was clearly delayed when compared with that of two other females. Before hatching, the difference in both series was uniform — 9 to 15 hours. Some germs of this female began to die in gelatinous envelopes in stage 17.

The facts presented above explain best the differentiation of percentages of dead germs in various combinations of these series.

For further rearing only normal tadpoles were used and for this reason in some aquaria their number was much lower than planned. The lowest percentage of metamorphosed tadpoles in series S4M was in 2b (21·4%) which had the highest percentage of dead specimens (53·6%). It should be noticed that they were in the aquarium with the lowest density of population. The tadpoles 2b-S4L developed in a similar way.

Generally it could be stated that, contrary to a similar series of the preceding year, none of the tadpoles of any combination showed, at least to the moment of metamorphosis, any decided domination or delay in development.

The tadpoles 2b from the parents of *esculenta* form, in 1963, were characterized by the lowest viability and for this reason in 1964 I paid special attention to them. In order to study their viability I prepared 22 aquaria (Tables IX, X) for the following experiment. Each aquarium was divided with a net into 2 equal parts. In one part I placed the tadpoles of different combinations 2b, and in the other there was usually the same number of tadpoles of other combinations (the control group). In addition, in 4 newly built basins I placed tadpoles 2b in three basins and a control group in the fourth basin (Table XI). In such a way the germs originating from 12 *esculenta* pairs were under control (the tadpoles 2b-S3L originated from 3 females).

Part of the 2b tadpoles remained in 2 aquaria (No. 14 and 20) without any control and in one case the tadpoles 2b-3M, from about 200 eggs of 3 females fertilized with the sperm of one male (Table IX, No. 22), were not subjected to any control. For unknown reasons, in aquarium No. 12, 17 tadpoles 1c-3M were lost on July 16—17, and in one aquarium (Table X, No. 5) the dividing net collapsed, so that the tadpoles got mixed and they could not be segregated again.

The tadpoles 2b-S4X (Table X), distinguishing by the highest viability, were obtained in the following way. Into a part of sperm that remained unused, I squeezed about 300 eggs from one female. The sperm and eggs were taken from the individuals which were used to prepare the series S4L and S4M, that is why the symbol of this combination is similar. I divided these eggs into two parts: one of them remained in the laboratory, and the other I took home. Among 102 eggs at home, 5 did not develop further and from 97 I obtained excellently developing tadpoles. I divided them into 3 parts (Table X: No. 1—3). The first part (33 specimens) was kept at home till the complete metamorphosis, in tap water changed every day. I preserved 6 of them on June 24 and among the remaining 27 only 16 passed through metamorphosis and 11 died before climatic metamorphosis. The first tadpole metamorphoses on July 10, in 57 days from fertilization, and the last on July 27. At the end of May the remaining 2 parts were taken back to the Institute where their development in the open air was much slower and a relatively low percentage of them reached metamorphosis. The first tadpole of these two groups metamorphosed on July 17 and the last died in December.

Much slower development was observed in the group of tadpoles which remained in the Institute. It was not until July 30 that the first of them metamorphosed. This is proof that the tadpoles 2b-S4X had better conditions for development at home. In the same way as the germs 2b-S4X I raised 20 germs 2b-1M and 25 germs 2b-5M at home, but they all died as tadpoles.

Table XII presents the results of investigations on development of germs and tadpoles in 1965. In this Table the series B1M from 1963 is also listed and

Table IX

Development of tadpoles of different series 2b in common aquaria with control tadpoles, in 1964. Tadpoles 2b were kept till death of the last specimen, control ones to August 18

Cross 2b	No. aquarium	Control cross	Initial number of tadpoles	Number of dead specimens		Date of death of the last specimens	Number of alive specimens (preserved)			Total	
				tadpoles	with 4 limbs		tadpoles	with 4 limbs	metamorphosed	dead	alive
	1	—	3	3	—	2· 8	—	—	—	3	—
		1a—S3L	3	—	—		—	—	3	—	3
2b—S3L	2	—	20	7	13	23·10	—	—	—	20	—
		2a—S3L	20	7	—		5	1	7	7	13
	3	—	6	5	1	3·10	—	—	—	6	—
		3a—S3L	6	1	—		—	—	5	1	5
	4	—	24	24	—	3· 1·65	—	—	—	24	—
		2a—S4M	24	1	—		—	1	22	1	23
	5	—	40	40	—	15· 1·65	—	—	—	40	—
		3a—S4M	30	3	—		9	8	10	3	27
2b—1M	6	—	25	25	—	12· 9	—	—	—	25	—
		1b—2M	25	1	—		3	3	18	1	24
	7	—	20	20	—	13· 3·65	—	—	—	20	—
		3c—2M	20	1	—		—	—	19	1	19
	8	—	20	20	—	26· 6	—	—	—	20	—
		3c—2M	20	2	—		18	—	—	2	18
2b—4M	9	—	23	23	—	19· 6	—	—	—	23	—
		1a—3M	30	4	9		4	2	11	13	17
	10	—	30	18	12	19· 4·65	—	—	—	30	—
		3a—1M	30	1	—		—	—	29	1	29
	11	—	30	15	15	5· 1·65	—	—	—	30	—
		3a—1M	20	2	—		6	2	10	2	18
2b—5M	12	—	28	10	18	27· 3·65	—	—	—	28	—
		1c—3M	25	—	—		—	—	8	?	8
	13	—	20	14	6	10·10	—	—	—	20	—
		2c—2M	20	5	1		—	—	14	6	14
	14	—	78	66	12	16· 4·65	—	—	—	78	—

Table IX — continued

Cross 2b	No. aquarium	Control cross	Initial number of tadpoles	Number of dead specimens		Date of death of the last specimens	Number of alive specimens (preserved)			Total	
				tadpoles	with 4 limbs		tadpoles	with 4 limbs	metamorphosed	dead	alive
2b—7L	15	—	30	30	—	19· 6	—	—	—	30	—
		1a—10L	30	2	—		10	8	10	2	28
	16	—	20	20	—	17· 6	—	—	—	20	—
		1a—10L	20	6	—		14	—	—	6	14
	17	—	20	19	1	22·10	—	—	—	20	—
		1b—S3L	20	3	2		2	—	13	5	15
	18	—	14	14	—	11· 6	—	—	—	14	—
		1b—S3L	19	—	—		19	—	—	—	19
2b—8M	19	—	22	22	—	17· 6	—	—	—	22	—
		1a—4M	22	2	—		20	—	—	2	20
	20	—	10	8	1	July 65	—	—	1	9	1
2b—9M	21	—	18	18	—	19· 6	—	—	—	18	—
		3a—S3L	18	3	5		2	2	6	8	10
2b—3M	22	—	13	9	4		—	—	—	13	—

in the three first columns the approximate values are given for this series. It is worth noticing, however, that the data in the fourth column for this series clearly indicate very high mortality of the germs from the eggs of this female.

In 1965, among 6 obtained combinations 2b, only one (2b-12M) was preserved earlier, on October 11, the remaining were kept to the death or metamorphosis of the last specimen. The tadpoles of other combinations which were raised longer, usually were liquidated when at least one part of them reached climactic metamorphosis. In 1965 I caught 3 pairs of different forms in amplexus in the moment of laying eggs. From them I prepared 3 complete series, as mentioned already, which had to develop in three different temperatures. However, for unknown reasons, none of 385 eggs from *lessonae* female and 378 from *ridibunda* female passed through first cleavage, while out of 391 eggs of *esculenta* female over 90 % of the eggs on each dish developed further. Only the series B3M was kept for observation (Table XII).

Table X

Results of rearing tadpoles 2b-S4X with control crosses in 1964. Tadpoles 2b were reared in 4 aquaria to death of the last specimen, control ones to August 18

No. aquarium	Control cross	Initial number of tadpoles	Number of dead specimens				Number of alive (preserved) specimens			Total specimens	
			tadpoles	with 4 limbs	metamorphosed	date of death of the last specimen	tadpoles	with 4 limbs	metamorphosed	dead	alive
1	—	33	—	11	—	28· 7	6	—	16	11	22
2	—	38	23	7	2	28·12	—	—	6	32	6
3	—	21	9	3	—	23·12	—	—	9	12	9
	1c—2M	21	—	—	—		1	—	20	—	21
4	—	30	15	—	—		11	3	1	15	15
	3c—2M	30	—	—	—		21	7	2	—	30
5	—	30									
	3b—1M	30	9	12	20	22· 3·65	2	3	14	41	19
6	—	97	30	17	—		47	—	3	47	50

Table XI

Results of rearing tadpoles in basins in 1964. Most tadpoles 3c-1M were eaten by the individuals of F_1 generation of green frogs from 1963

No. of basin Combinations	1 3c—1M	2 2b—2M	3 2b—5M	4 2b—6M
Capacity of basin in litres	250	50	200	350
Approximate number of fertilized eggs	3000	1000	1000	3000
Number of alive specimens:				
tadpoles	26	—	1	—
with 4 limbs	60	—	—	—
metamorphosed	15	—	—	—
Aproximate date of death of the last tadpole	—	11·6	20·10	15·6

The tadpoles of some combinations only were kept for further raising. All germs that reached the stage 25, or, if their number exceeded 100, only a part of them were reared. It results from Table XII that the percentage of fertilization of the eggs of all combinations in most cases was over 90. The most interesting are data concerning development of the tadpoles 2b, including 6 combinations. Their common character is a very high mortality which in

5 cases reached 96·4 to 100%, and a very low percentage of specimens, that passed metamorphosis. The metamorphosed individuals were not raised any longer.

Three years' observations on development of different green frog combinations showed that the lowest viability and very high differentiation in this regard characterised the tadpoles of combination 2b, while those of all other combinations, when raised further, reached complete metamorphosis.

Basing on differentiation in viability of tadpoles 2b, we can divide them into a few rather distinctly marked groups.

1. To the first group I should include the germs which died in early stages and did not reach hatching. But this group, although it includes the germs of a few females, is not clearly distinguished because similar characters occurred also in germs from other females.

2. The germs of the second group developed in the normal way but all died shortly after reaching stage 25. The germs of 5 females belonged to this groups (Table IX: 9M; Table XI: 2M, 6M; Table XII: B3M, 10M).

3. In the third group there are combinations in which the tadpoles lived very long but none of them developed legs, only in the biggest specimens some very small legs in but stage appeared. The eggs laid spontaneously by two pairs (Table IX: 1M, 4M) as well as the eggs from at least 2 females from 1963 belong to this group (Table VII: S1M, S2M).

The tadpoles 2b-1M can serve as an example of development. They were placed in 5 aquaria with the control groups. Among 119 control tadpoles which in different combinations began to metamorphose from July 20 to August 1, only 8 specimens died, while all 129 tadpoles 2b died. The photos (Fig. 2) illustrate best the delayed development of these tadpoles.

During the development of this group of tadpoles, numerous individuals appeared among them, which looked quite normal but could not swim. When excited to move, they turned round in one place without swimming or swam irregularly turning round the axis like a corkscrew. These individuals very often lay on their back on the bottom without showing any distress in this unnatural position. The tadpoles with similar behaviour were observed also in 2a-S1M in 1963. It seems that in the tadpoles of this type a disturbance of the sense of balance occurred in some period of development.

4. In the fourth group I listed the tadpoles which did not reach complete metamorphosis although some of them died as almost metamorphosed individuals. These tadpoles originated from 4 pairs which mostly laid eggs spontaneously (Table IX: 5M, 7L, S3L, 3M). A detailed description of particular cases is needed to throw light upon their mortality.

On May 16, two pairs laid eggs spontaneously: 2b-7L about 5^{00} and 1a-10L about 6^{20} o'clock in the morning. A part of the eggs from each pair were taken on the dishes which were placed side by side in the temperature 15° C. Their development much differed. The germs 1a-10L reached stage 25 on June 4, and the germs 2b-7L on June 8 (Table VI). The photo (Fig. 3), made on June 3,

Table XII

Development and further rearing of tadpoles of various combinations in 1965. Series B1M from 1963. In the lower part of Table-development of differentiated eggs of *esculenta* females (compare Table XIII)

Series and combinations	Embryonic development					Rearing tadpoles					
				embryoes after hatching			% dead		% preserved		
	number of eggs	% of first cleavage	% of dead embryoes	total numb.	% of abnor.	initial number	tadpoles	with 4 limbs	tadpoles	with 4 limbs	metamorphosed
B1M (19°C)											
1b	100	50·0	96·0	2	—	2	—	—	100·0	—	—
2b	100	50·0	96·0	2	100·0	—					
3b	100	50·0	100·0	—		—					
A2H (29°C)											
1a	247	92·7	36·4	139	5·3	—					
2a	218	97·7	65·2	71	2·8	—					
3a	221	98·6	47·9	112	0·9	—					
B3M (20°C)											
1b	40	95·0	23·7	29	6·9	27	11·1	—	81·9	3·5	3·5
2b	25	100·0	16·0	21	14·3	18	100·0	—	—	—	—
3b	42	97·6	36·6	26	26·9	19	21·1	—	21·1	21·1	36·7
1a—11M	100	100·0	1·0	99	—	—					
1a—12M	100	98·0	6·1	92	—	—					
1a—13M	65	89·2	13·5	51	15·7	—					
2b—10M	100	91·0	14·3	78	16·7	78	100·0	—	—	—	—
2b—12M	225	100·0	31·5	154	6·5	100	45·0	3·0	50·0	—	2·0
3b—3M	235	100·0	21·3	185	44·4	—	—	—	—	—	—
2c—3M	98	61·2	13·3	52	3·9	52	5·8	3·7	69·2	—	21·3
2c—4M	141	97·2	16·9	113	3·5	100	18·0	6·0	34·0	—	42·0
1b—3M	147	91·8	22·2	105	1·9	62	25·8	3·2	38·7	—	32·3
1b—4Ma	31	93·5	100·0	—	—	—	—	—	—	—	—
1b—4Mb	31	93·5	10·3	26	—	—	—	—	—	—	—
B2M (20°C)											
1b	122	91·8	75·8	27	48·2	27	63·0	14·8	11·1	3·7	7·4
2b	120	94·2	74·3	29	86·3	29	96·6	3·4	—	—	—
3b	117	91·1	73·6	28	64·3	28	89·2	—	7·2	—	3·6
B2H (29°C)											
1b	124	87·8	98·2	2	50·0	—	—	—	—	—	—
2b	154	85·7	93·2	9	11·0	—	—	—	—	—	—
3b	210	85·7	97·7	4	25·0	—	—	—	—	—	—
2b—11M	204	94·1	67·7	62	40·4	57	96·4	1·8	—	—	1·8
2b—13M	130	98·5	55·5	57	38·6	49	98·0	—	—	—	2·0

Table XIII

Development of differentiated eggs of *esculenta* females in 1965. Mean and exteme dimensions (compare Table XII)

Symbol of developing eggs	Number of eggs				Diameter of eggs in mm		Reaching St. 25 by germs in hours and minutes		Length of tadpoles in mm					
	in development (Tab. XII)		measured (preserved)		smaller	larger			in St. 25		on August 2			
											smaller		larger	
	smaller	larger	smaller	larger			smaller	larger	smaller	larger	number	length	number	length
1b—3M	133	14	58	5	1·10—1·28—1·50	1·80—2·01—2·20	265·00	242·00	9·2	11·5	45	26—45	9	50—61
1b—4M	—	—	—	34	—	1·45—1·57—1·65	—	—	—	—	—	—	—	—
1b—4Ma+b	31	31	53	53	1·15—1·25—1·35	1·40—1·57—1·75	—	244·00	—	11·6	—	—	—	—
1b—B2M	115	7					289·05	246·05	8·7	11·8	8	41—56	2	59—60
2b— „	113	7	130	8	0·95—1·31—1·75	1·95—2·09—2·20	290·00	290·00	8·4	11·7	1	58	1	67
3b— „	111	6					289·05	—	8·5	—	4	42—56	—	—
2—11M	193	11	—		—	—	337·00	270·00	8·0	12·2	—	—	3	44—86
2—13M	123	7	28	6	1·30—1·41—1·50	2·00—2·09—2·20	320·00	295·00	8·5	12·2	21	14—23	2	31—82

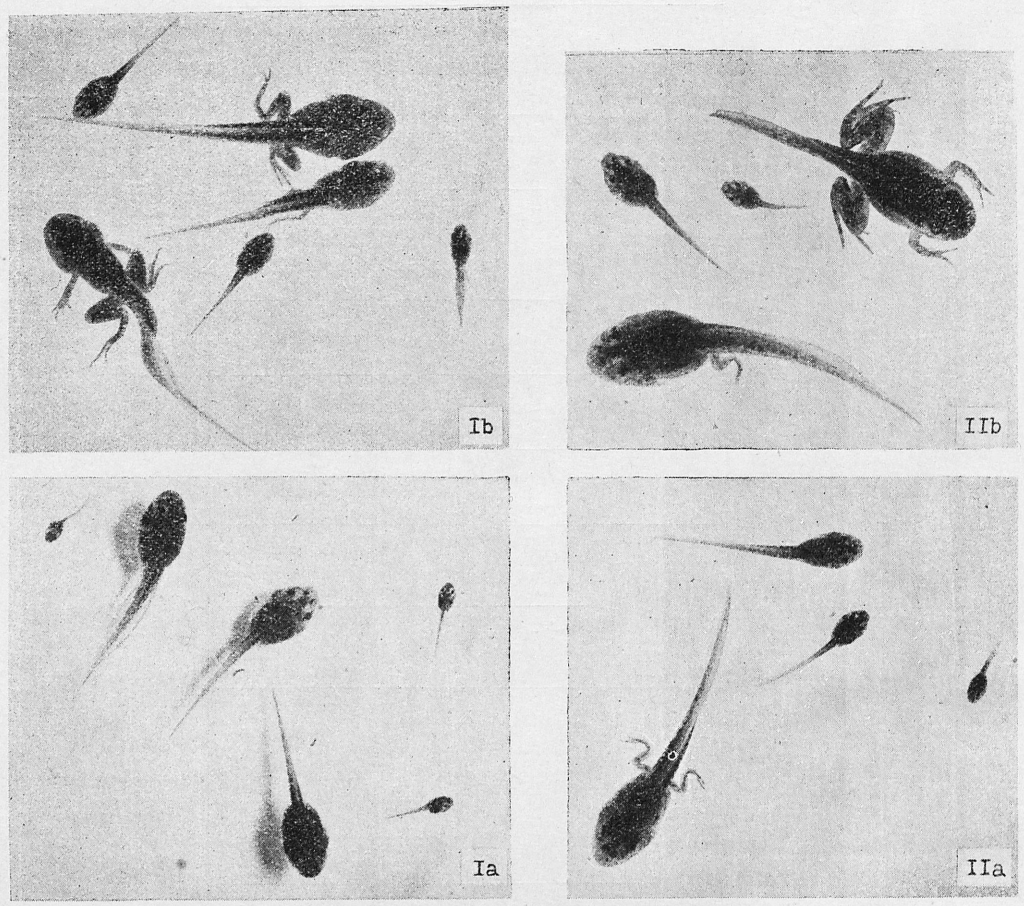

Fig. 2. Comparison of size of tadpoles of combination 2b-1M with control tadpoles 3c-2M (Ia, b) and 2a-S4M (IIa, b) reared in common aquaria. Photos: Ia — June 9, Ib — July 7, IIa — June 7, IIb — July 17. In each photo tadpoles 2b (smaller) and the same number of control ones (bigger)

Phot. *Z. Pniewski*

illustrates best the difference between the larvae of both combinations. For further raising I took 84 tadpoles 2b and placed them in 4 aquaria, adding a control group to each aquarium; two of them belonged to the tadpoles 1a-10L, mentioned above (Table IX: No. 16 to 19). The results were as follows: out of an initial number of 84 tadpoles 2b, only one remained to June 19 and died on October 22 as almost metamorphosed frog. Among the whole amount of 89 control tadpoles, 13 specimens died before August 18, and the rest metamorphosed or were preserved.

In the remaining combinations 2b included in group 4, the tadpoles were more viable. Although their development was slower (Fig. 4) in comparison with that of the control ones, a few or several almost metamorphosed individuals appeared in each aquarium. However, all of them died before reaching complete

metamorphosis, and many died at earlier stages. The sequence of their death was similar as in the precedent group only the time of dying was more varied. Numerous specimens died at the beginning of summer 1965, without reaching complete metamorphosis.

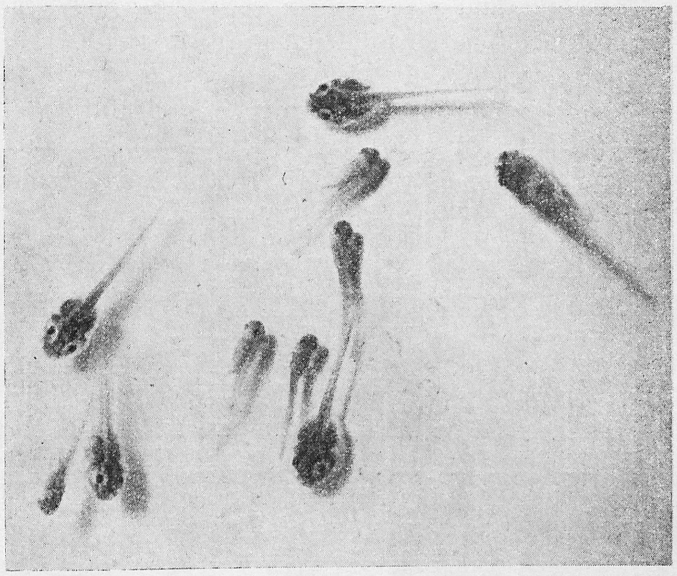

Fig. 3. Larvae of combination 2b-7L (smaller) and 1a-10L (bigger), 510 hours after fertilization (development in 15° C)

Phot. *Z. Pniewski*

When the first, almost completely metamorphosed tadpoles began to die, I thought that, due to generally lower viability in the tadpoles 2b, they were drowned because they had no strenght to get on the pieces of bark placed in the aquarium. In order to help them I prepared quite shallow aquaria with sandy islands in the middle and I even fed three 1—2 mm tailed specimens 2b-5M. In spite of such care, however, they died. Such treatment was not applied to the control tadpoles and they survived.

5. The most interesting group was the last one, including the eggs of 2 females, as in this group some metamorphosed and very viable individuals appeared (Table IX: 8M; Table X: S4X).

32 tadpoles remained from about 60 that developed from spontaneously laid eggs 2b-8M. All the tadpoles 2b which were placed with the control tadpoles 1a-4M, died before June 15, but in an aquarium without any control 7 tadpoles remained and 2 of them, characterized by much greater size, reached metamorphosis. One of them passed through metamorphosis on July 31, 1964, but died in July 1965, and the other did not reach complete metamorphosis and died on August 19, its body being of abnormal shape (Fig. 9).

The eggs of the other female developed into numerous very viable indi-

viduals. In all aquaria containing the tadpoles originating from this female, metamorphosed specimens appeared among the tadpoles 2b as well as the control groups. In any case the development of the tadpoles 2b (the tadpoles from this female reared at home are not included here) was clearly retarded, as illustrated in photos (Fig. 5). On May 29, I brought to the Institute 21 tadpoles 2b from my home and placed them with the control tadpoles 1c-2M. The tadpoles of both combinations did not differ in size at this time, but on June 9 the difference was already visible which was very evident a month later. The tadpoles 2b began their metamorphosis on July 17 (12 specimens died) and the tadpoles 1c-2M on July 13 (none died).

A rather obscure position is that of the germs from 3 pairs (Table XII:

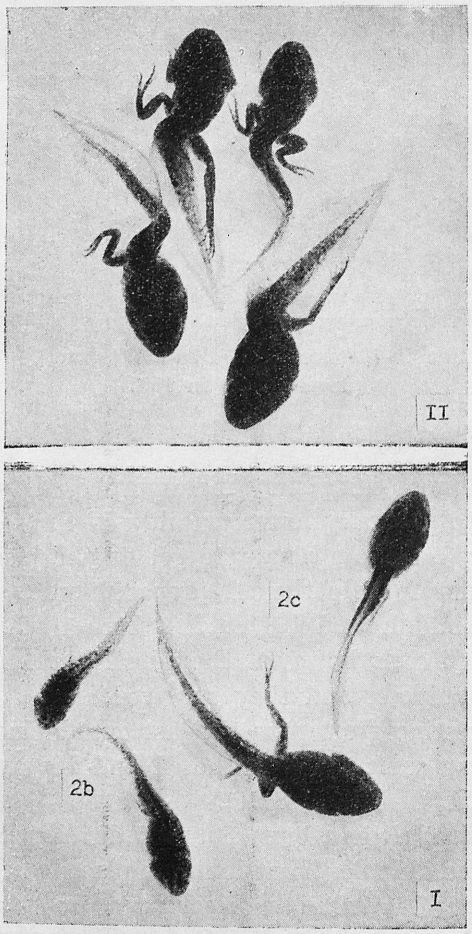

Fig. 4. Comparison of size of tadpoles of combination 2b-5M with those of 2c-2M reared in common aquarium. Photos: 8 7 1964. I — normal tadpoles 2b and 2c, II — abnormal tadpoles 2c

Phot. *Z. Pniewski*

12M, 11M, 13M), not mentioned till now. Four of them reached metamorphosis but they were not kept any longer. In the classification presented above they should be ranged in the fifth group.

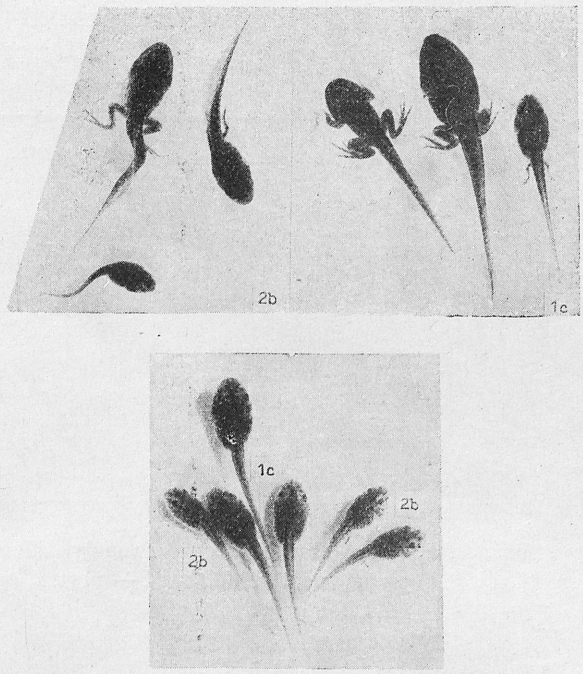

Fig. 5. Comparison of size of tadpoles 2b-S4X with tadpoles 1c-2M reared in common aquarium. Lower photo on June 9, upper on July 8

Phot. *Z. Pniewski*

3. Irregularities in development

Different abnormal features appeared in germs and tadpoles of all 9 combinations. Some abnormalities were clearly connected with some properties of eggs which I would like to explain first. These observations concerned only the eggs of *esculenta* females in 1965.

I noticed that round numerous eggs of 3 *esculenta* females (Table XII: B2M, B3M, 1b-4M) opaque sheaths appeared after fertilization, making further observations of development impossible (Fig. 6b). I did not notice, however, that they had any unfavourable influence on development of germs in embryonic period and consequently not pursue further observations on development of the tadpoles.

The eggs of some females, strongly varied in size, were more interesting (Fig. 6a, c). The progeny which developed from them, differed much in many features (Table XIII). I observed a distinct differentiation in eggs of 5 *esculenta* females. Four of them laid eggs spontaneously. The germs originating from

bigger eggs developed much more rapidly and were bigger than those from smaller eggs. When the smaller germs reached stage 25 the difference in length was 4 to 5 mm.

The development of germs is closely connected with the degree of differentiation of eggs. In 2b-3M, for instance, the eggs greatly varied (Fig. 6a) forming 2 groups. Most of the smaller tadpoles died shortly after reaching stage 25,

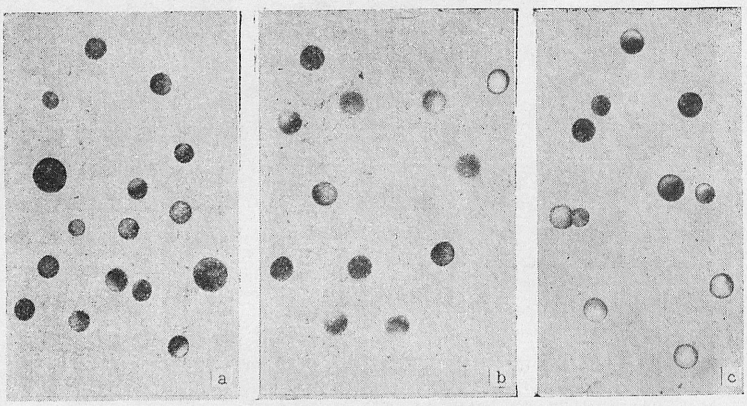

Fig. 6. Eggs of *esculenta* females. Series: a) 2b-13M — eggs strongly differentiated, b) B3M — numerous germs surrounded with opaque sheaths, c) 1b-4M — normal eggs and double eggs in common envelope

Phot. *Z. Pniewski*

and the rest in August (Table XIII), while one big tadpole (the other was abnormal) reached great size and was very viable after metamorphosis. The eggs of the female of series B2M were less differentiated and so were the tadpoles that developed from them. In further development they were undistinguishable. A high mortality was observed in them and especially in the tadpoles 2b and 3b.

The germs of combination 1b (3M, 4M) which generally were characterized by higher viability, require special discussion. From the total amount of 105 tadpoles 1b-3M, I reared 50 smaller and 12 bigger specimens. On July 9 the smaller tadpoles were 23 to 31 mm long (mean 27·2 mm) and the bigger ones 36 to 49 mm long (mean 43·8 mm). On August 2 (Table XIII) the smaller were 26 to 45 mm long (mean 36·5 mm) and the bigger 50 to 61 mm (mean 56·3 mm). In the period of metamorphosis they also formed two groups but not very distinctly separated. The bigger tadpoles metamorphosed mostly in August, while the smaller ones in October.

Differentiation of the eggs 1b-4M was of another kind (Table XIII). For every 40—50 normal eggs there was one abnormal pair (Fig. 6c). They were in a common gelatinous envelope and one was always of normal size while the other was smaller (1b-4Ma + b). In 31 such pairs (Table XII) only 2 pairs did not pass through cleavage. All small germs were subjected to cytolysis and died in over 80% in the period of gastrula (stage 11 to 13) with a great yolk-plug

(exogastrulation). None of the germs reached hatching. Among 29 bigger germs 26 reached stage 25 and no abnormal features were observed.

In embryonic development special attention should be paid to the germs which died in gastrula with symptoms of exogastrulation. Embryos of this type appeared in large numbers first of all among the germs from *esculenta* females, but with special intensity in combinations 2b. They often constituted 50%, and among the germs developed from the smaller eggs even 80%, of the dead germs. Sometimes such germs developed further and died in stage 17 or 18 with a small yolk-plug in gastropore or at the side of the body; in rare cases they even reached stage 24, as was found in 1b-S4M (Fig. 7a—b). It is not excluded that some of them overcame this abnormality and developed further.

The germs showing this type exogastrulation appeared also in the remaining combinations but were not numerous. Among all combinations 1a, two such specimens were in S3M and in combinations 1c only one in S4M, but all three died in stages 23 or 24.

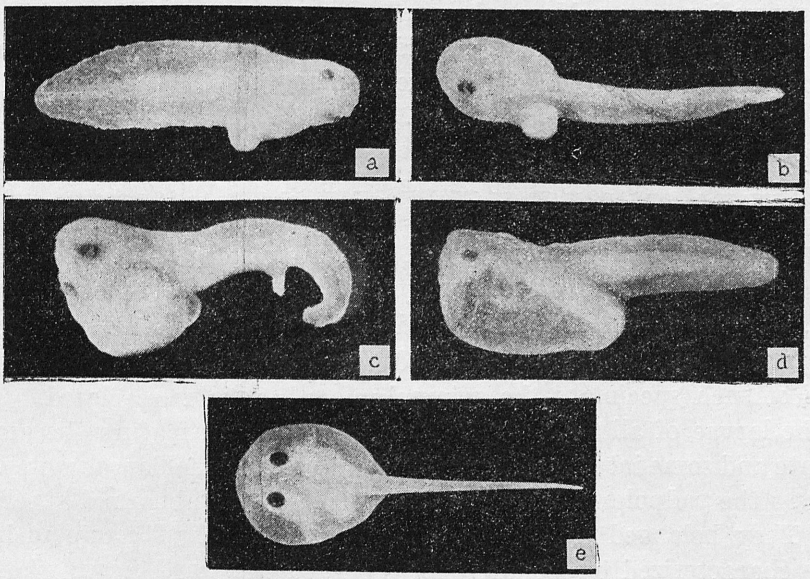

Fig. 7. Abnormal larvae form eggs of *esculenta* females

Phot. *Z. Pniewski*

All larvae with abnormal exterior features, which reached hatching, can be divided into 3 clearly distinguishable groups.

1. In the first group there were the larvae with different visible deformations of the body (asymmetry, scoliosis). The larvae of this type were more or less numerous in the progeny of all 9 type of fertilizations. In some specimens these irregularities disappeared in further development and they became normal tadpoles. For experiment, I placed all abnormal larvae 2b and 3b from the

series S4M in separate aquaria. Among them there were also larvae with yolk plug and numerous larvae with the features of the second group. To June 30 from 36 larvae 2b only 4 normal tadpoles remained (the rest died) and from 60 larvae 3b only 1 tadpoles survived. A similar phenomenon but in reverse occurred in normal larvae, among which some single tadpoles with some irregularities appeared.

2. In the second group I included the specimens with some monstrous features (Fig. 7c—d). In the larvae of this type in the body cavity an edema appeared in stage 17 already, insignificant at first, and grew to great size making swimming impossible. In such larvae the gills developed very weakly. The larvae died after some time; they never reached stage 24. Among them many individuals had some underdevelopment in the tail web.

Such larvae were numerous or even very numerous in almost all combinations 2b and 3b; they appeared more rarely in some combinations 2c and 3c. Among 82 abnormal larvae in 3b-3M (Table XII) there were 41 of this type and in addition 10 to 12 larvae with the symptoms of this abnormality, while in 2c-4M all four larvae were strongly swollen. In the remaining combinations this abnormality was seldom observed. In combinations 1a only one such specimen appeared in S3M. It should be mentioned, that individuals with monstrous features were also observed among the larvae which developed from bigger eggs of *esculenta* females.

3. In the third group there are the larvae with a different type of edema (Fig. 7e). Already in stages 20 to 22 the germs of this type became almost quite transparent and obtained a round, flattened shape of body. After reaching stage 25 they stopped developing and died after some time. They developed, together with the larvae of former type, from the smaller eggs of *esculenta* females. In some cases (Table XII and XIII: 2b-11M, 2b-13M) almost all abnormal larvae had these features. In detailed examination it was found that in some of such specimens the spiraculum and anus were closed.

In the larvae of all mentioned types the abnormal features became visible rather late and some intermediate specimens to the normal larvae were observed.

Among the tadpoles left for further rearing some abnormal specimens appeared. They belonged to two different types. The first group included the tadpoles characterized by a more or less curved tail. In the case when the tail was not much deformed, the tadpoles could swim rather easily (Fig. 5), but when these tail deformations were very severe (Fig. 4) the tadpoles could not move in the water or their swimming was of little effect. The individuals with insignificant deformations of the tail occurred in all 9 combinations but those with strong deformations were exclusively in combinations 2c, 2b, and less numerous in 3c. Such deformations, at least the less marked ones, had not much influence on their metamorphosis. For instance in an aquarium with 20 tadpoles 2c-2M (Table IX) 14 were strongly deformed (Fig. 4); 4 of them died, while 10 metamorphosed and some of them are still alive.

The other kind of abnormality in the tadpoles consisted in unusual shape

of the body. In some tadpoles with hind legs developed well enough, the lymph sacs (sacci lymphatici subcutanei) situated in tibia and femur began to enlarge. With the growth of tadpoles the lymph sacs on the body also slowly enlarged (Fig. 8) reaching great size before metamorphosis (Fig. 9). The tadpoles of

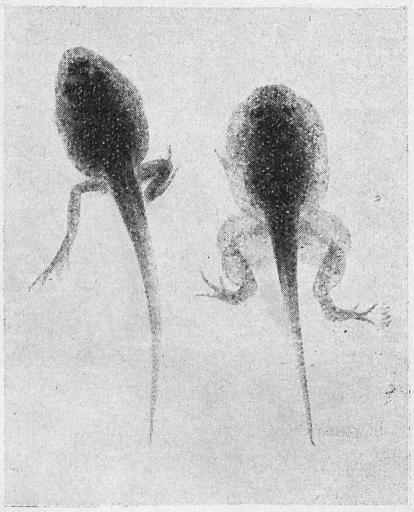

Fig. 8. Tadpoles of combination 2c-S4M, normal and abnormal specimen
Phot. *Z. Pniewski*

this type swam with difficulty but in the final phase of metamorphosis they became very active. They all died. There were very rarely specimens in which the lymph sacs did not enlarge so much and these tadpoles sometimes passed through metamorphosis.

Such abnormality was found among the tadpoles of combinations 2b-S4M (11 specimens), 2b-S4X (7 specimens) and single specimens in 2b-5M, 2b-8M, 2c-S4M, 3c-S4M. It is interesting that such individuals occurred only in 1964. In other years and combinations no such specimen was found.

Other abnormal features were very much differentiated and occurred sporadically and, it seems, without distinct connection with any combination. In 9 specimens of some combinations of the series S1M (1b, 2b, 3b, 2c) and in 2b-B3M the spiraculum was closed; in 3 specimens in 2b and 3b of the series S1M the spiraculum was at the left side, and in one at the belly side of the body.

The most interesting observations concern the sequence of appearance of the features of some embryonic stages. During observation I found in all germs 2c-3M (Table XII) in stage 17 the heart beat and gill circulation. In about 24 hours after this observation the first larvae left the gelatinous envelopes. A similar phenomenon occurred among the germs 2c-4M, in which also the heart beat was in stage 17 but gill circulation was noticed only after hatching.

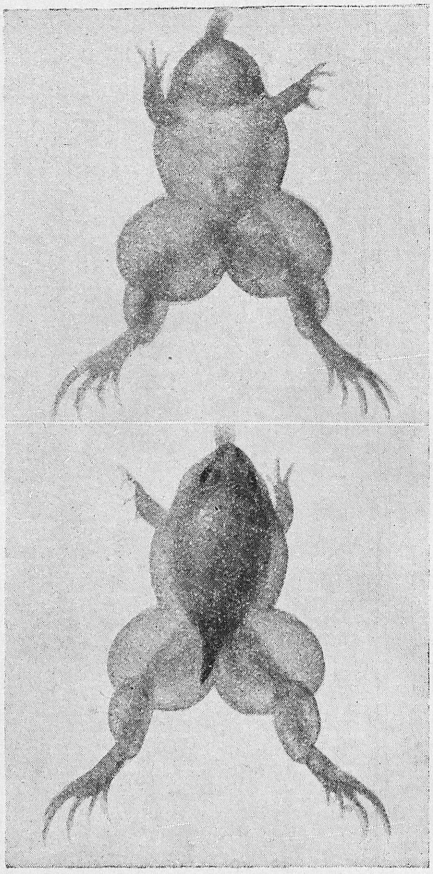

Fig. 9. Abnormal specimen of combination 2b-8M in the final period of metamorphosis. Photo 5 hours after death

Phot. *Z. Pniewski*

IV. DISCUSSION

In the European herpetological literature there is almost no data on hybridization and on observations of early stages of green frog development, that could be fully utilized without any doubt. The values of the results of the cited reports (HERTWIG 1896, TAMINI 1947, DOUGLAS 1948, SMITH 1949, MICHNIEWSKA-PREDYGIER and PIGOŃ 1957, KAURI 1959) as well as of other works are open to discussion as we are not sure whether the authors had in fact at their disposal the forms they described.

From the data presented in part III it results clearly that the mentioned three morphological forms of green frogs; *lessonae*, *esculenta* and *ridibunda*, can be mated with each other without any restriction, giving viable progeny. In the observations of their development two most important problems arose

which require a detailed discussion. They are: rate of development and viability of the germs of individual combinations.

Influence of temperature on the rate of development of embryos of different species and even of different forms of the given species of *Anura* is widely known (MOORE 1949). In my investigations the differentiation of temperature however was too slight and was not always applied to all combinations (Table IV) so any general conclusion could not be drawn.

In each series stage 25 was reached most rapidly by the larvae of combination 1a and most slowly, almost always, by those of 3b or 2b (Tables II, III, IV). In extreme cases the differences in time within the same series were from 79 hours in S2M to 136 hours in S1M. On the basis of these data, quite intelligible are the differences reported by various investigators, as MICHNIEWSKA-PREDYGIER and PIGOŃ (1957) and DOUGLAS (1948), in the development of green frog embryos in the same temperature. These authors undoubtedly investigated individuals of different forms.

The periods of development till the moment of reaching metamorphosis by tadpoles of various combinations are also distinctly differentiated (Table VI). It seems however that this differentiation is independent of the differentiation which was observed in their embryonic development. The germs 1a in embryonic period, in all tested temperatures got well ahead in reaching stage 25, at least a few days earlier, in comparison with the germs of all remaining combinations, but their development in the larval period was distinctly restrained and retarded. Longer periods of time for reaching complete metamorphosis were observed only in tadpoles 2b and 3b which also in embryonal development were the last to reach stage 25. This problem is difficult to interprete.

It seems that the cytoplasm of eggs had distinct influence on the rate of development of germs and on their viability. This influence was quantitative as well as qualitative.

The influence of cytoplasm of the eggs from females of different forms on development in early embryonal stages results from the fact that the development of the germs of 3 combinations originating from 1 female is very approximate, while the difference in the development of the germs from females of various forms is very great (Table V). It is only in the period of tailbud, that the development of the germs from one female begins to differ. It seems that in this period the influence of cytoplasm vanishes and genetic factors localized in the nuclei of sex cells begin to play an increasing role (BRACHET 1964). Doubtless due to this fact, in the final effect the hybrid reciprocal combinations reach stage 25 in almost equal time. Unintelligible however are the cases of earlier cleavage of eggs of *esculenta* female (S4L, S4M) or, in another case, of those of *lessonae* female (S2M), as well as the fact that in two series (S2M, S4M) kept in the same temperature only the eggs of *esculenta* female reached stage 10 in the same time, while there was a difference of about 7 hours in the eggs of *lessonae* and about 14 hours in those of *ridibunda* females.

The size of eggs seems to influence not only the rate of development of the germs (Tables XII, XIII) but also their viability. The best example is that of the tadpoles of combinations 2b (11M, 13M) in which the complete metamorphosis was reached by one tadpole developed from the big egg.

Determination of the degree of viability in the germs of individual combination to the period of hatching is rather difficult. It appeared that the percentage of fertilized eggs in all observed cases was very high (Table XII) and progeny of all combinations reached stage 25 without any difficulty. Although their viability was distinctly differentiated, due to the lack of distinct percentage data as to their mortality, there was no possibility to compare in this respect the individual combinations. The data in Table VIII can not be used for this purpose, as mentioned in part III, and the values in Table XII are not sufficient. For this reason we shall begin our discussion by an analysis of development of tadpoles, which provide reliable data as to viability of progeny in all combinations.

From the comparison of the development of tadpoles (Tables VII, VIII, XII) it results that their mortality is very high in all combinations. A general conclusion could be that their development occurred in unfavourable environmental conditions, about which it is difficult to discuss because of lack of specific data. However it is worth while noticing that the differentiation in viability was very high among various matings in the same combinations, the best example being the tadpoles of 4 crosses in combination 2a (Table VII). These tadpoles development in the very same aquaria, all washed every week and with a daily change of water, thus environmental conditions should be very similar. We can state however that the percentage of metamorphosed specimens is not proportional to the number of tadpoles. We come to a similar conclusion when comparing data of corresponding combinations among different series.

It results from the works of many authors (Mecham 1957, Blair 1964a, Michałowski 1964) that the percentage of metamorphosed larvae is not only an index of their viability but it also illustrates the general genetic differentiation between the individuals being mated. If we consider the values presented in the Tables from this point of view, we shall find a distinct regularity in development on the tadpoles from individual combinations. We are going to analyse this regularity.

In 1963, (Table VII) by fertilizing the eggs of 5 *esculenta* females with the sperm of males of the same form, I obtained in two series 56 tadpoles 2b, all of which died as tadpoles with symptoms of a distinct restraint in their development (Fig. 1). When mating the same individuals with the individuals of *lessonae* and *ridibunda* forms, I obtained 282 tadpoles; 194 of them metamorphosed and many are still alive as mature frogs.

In the following years the situation was generally repeated only viability of the tadpoles in combination 2b was clearly differentiated.

In 1965, (Table XII) in all combinations 2b a very high mortality of tadpoles

was observed (for a total of 6 cases, in 5 cases it exceeded 96%). In 3 combinations however some single individuals metamorphosed and were very viable.

The most interesting results were obtained from the experiment carried out in 1964. In 22 aquaria (Tables IX, X) and 4 basins (Table XI) with control groups I studied viability of 504 tadpoles and several thousands germs originating from 12 *esculenta* pairs. Among them only the tadpoles from one female were very viable and reached complete metamorphosis (Table X), while among the tadpoles originating from 11 females only one passed through metamorphosis (Table IX: 2b-8M), but died in July 1965. The rest of tadpoles died in different stages of development. It should be pointed out that the tadpoles 2b were reared till they died or the last specimen metamorphosed, while the control tadpoles, among which in every combination a high percentage passed through metamorphosis, were preserved on August 18 or earlier.

It results from the data presented above, that when the eggs of 35 *esculenta* females were fertilized by the sperm of males of the same form, only one female gave tadpoles with a relatively high percentage of metamorphosed and viable specimens. Among the remaining 34 females, 3 gave 1 tadpole each and one female gave 2 tadpoles which reached complete metamorphosis. It is worth recalling, that these tadpoles developed from exceptionally big eggs and perhaps their great viability, as mentioned above, was connected with the large amount of egg deutoplasm. All germs from the remaining 30 females died in different stages of development.

The above mentioned examples seem to prove that viability of the tadpoles of combination 2b is very low when compared with that of the remaining combinations. Their mortality can not be always explained by unfavourable environmental conditions, which at least in each aquarium were identical. The control tadpoles had always higher viability and in each aquarium a high percentage of them reached complete metamorphosis.

Further information as to viability in individual combinations are supplied by observations on frequency of some types of abnormal germs. Firstly all the germs with symptoms of exogastrulation and larvae with edema (Fig. 7) are concerned. According to many authors (MECHAM 1957, HERTWIG, WEISS and ZIEMANN 1959, WEISS 1960) these types of abnormalities in the germs are a distinct index of genetic incompatibility of parents. Occurrence of such specimens is very interesting. The highest number and frequency were in combinations 2b and 3b and much lower in 2c and 3c. In some cases over 80% of germs died in gastrula with symptoms of exogastrulation and among abnormal larvae sometimes all belonged to this type.

It is possible that a similar indicative value is of the tadpoles with unusually enlarged lymph sacs (Fig. 8, 9), which occurred in high numbers in different combinations 2b and single in 2c-S4M and 3c-S4M. Tadpoles with similar features are known in crosses of toads (BLAIR 1964a). Such phenomena found in some fish species are also said to be connected with hybridization (DIETRICH

1938), but as it results from the works of other authors (GRIMM 1953, JARA, TABORSKI 1962, JARA 1963) such edemata occur in larvae of amphibians fed with little varied food.

On the basis of these examples we should rather adopt the opinion that lower viability of progeny of green frogs belonging to *esculenta* form is not the result of unfavourable environmental conditions but that the individuals of this form show genetic incompatibility when mated. We could presume that the individuals with various genotypes belong to *esculenta* phenotype, or, that these individuals are hybrids of different forms of green frogs.

Accepting such a conception we are not able however, basing on the results of previous investigations, to explain some facts observed. Although the germs with exogastrulation and the larvae with the symptoms of edema occur most often and are most numerous in the combinations 2b, their appearance in all other combinations, even as single specimens, is incomprehensible. Equally incomprehensible is the problem of tadpoles with unusually enlarged lymph sacs (Fig. 8, 9) not only in combinations 2b and hybrid combination 2c but also in „homospecific" combination 3c.

The remaining observations which were omitted up to now in discussion as less important, are also very interesting and it is not excluded that these observations can serve as a starting point for further investigations.

Basing on the observations presented, we come to the conclusion that in laboratory rearing the lowest viability among 9 combinations characterized the progeny 2b from *esculenta* parents. We have some difficulty in deciding about the combination with the highest viability as it can not be ascribed to the progeny of „homospecific" combinations 1a and 3c but rather to two pairs of hybrid reciprocal combinations 2a-1b and 3a-1c, as clearly results from analysis of numerous Tables. A comparison of photos (Fig. 1) seems to indicate that in development the pair 3a-1c dominates. This problem needs a more fundamental elaboration because it is not excluded that in the tadpoles from those pairs the „hybrid vigor" occurs, which is often found in the progeny obtained when crossing different forms (VOLPE 1960, MAYR 1963).

Very interesting are also unexplicable cases of lack of development in eggs and mortality of germs originating from some females of different forms. These phenomena almost always included all eggs of the given female, independently of the form of the male. A conclusion could result from this observation that they are closely connected with some obscure properties of females. Perhaps these phenomena were caused by keeping the female with ripe eggs sometimes for 2 days after the moment of laying the first eggs. In the further phase of development of germs this phenomenon probably depends on environmental temperature, as the results of development of the eggs from *esculenta* and *ridibunda* females seem to indicate (Table VIII).

The succession in occurrence of individual embryonic stages, found during the observations on development of germs of different types of fertilization of green frogs (Tables II, III), agrees with data of MICHNIEWSKA-PREDYGIER

and PIGOŃ (1957). For this reason especially interesting is the fact that in all germs of hybrid combination 2c-3M (Table XII) the sequence of succession of some stages is different in the period of tailbud. In these germs first the heart beat appeared then gill circulation, and only about 24 hours later hatching of larvae followed. Normally the hatching of larvae should precede heart beat. It is worth recollecting that a similar phenomenon was observed in the germs 2c-4M, only gill circulation occurred shortly after hatching.

If we compare the time difference between appearance of stages 18 and 19 (hatching and heart beat) in the germs of all combinations (Tables II, III) we shall find an interesting regularity. It appears that only in the larvae from *lessonae* females these differences are very clear in all combinations, while in the remaining larvae, particularly in those from *ridibunda* females, they are very slight. In S4M we could not even catch the moment of first heart beat because it coincided with hatching (compare description in method).

From the above observations it would result that development of germs in these groups of combinations proceeds in some periods in another way and the distinct difference existing among them appears only in hybrids. This problem however should be more thoroughly elaborated because, as MICHAŁOWSKI (1964) supposes, the phenomenon that the features which were absent in parents appeared in hybrids could consist in a kind of atavism and would provide some information as to common progenitors.

The above presented observations seem to indicate that the problem of hybridization in the group of green frogs is more complicated than it would result from KAURI'S opinion (KAURI 1954). According to this author out of 3 forms of green frogs living on Bornholm island two, namely *R. lessonae* and *R. ridibunda* are geographic races of the same species, while the third, *esculenta*, is an intermediate form resulting from crossing of the two former forms.

What are the connections and dependences among the individual genotypes, as we may call each combination or at least hybrid reciprocal combinations, further investigations will demonstrate, in particular those on the individuals of F_1 and F_2 generations.

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STRESZCZENIE

Autor omawia wyniki badań prowadzonych w latach 1963—1965 nad krzyżowym zapładnianiem trzech form morfologicznych żab zielonych: *Rana lessonae*, *R. esculenta* i *R. ridibunda*. Zapłodnienie przeprowadzono według załączonego schematu otrzymując 9 różnych kombinacji: 3 typowe i 6 hybrydowych. Zapłodnienia seryjne przeprowadzono na tych samych osobnikach. Do krzyżowania użyto 180 osobników wszystkich form złowionych w amplexus.

Autor zwraca uwagę przede wszystkim na szybkość rozwoju zarodków i ich żywotność.

Z przeprowadzonych eksperymentów wynika, że rozwój jaj wszystkich kombinacji bez żadnych trudności osiąga 25 stadium, jednak szybkość rozwoju poszczególnych kombinacji jest różna.

Najniższą żywotność wykazuje potomstwo osobników *esculenta*. Przejawia się ona w występowaniu dużej liczby okazów anormalnych, wysokim procencie śmiertelności wśród kijanek, a przede wszystkim w różnym stopniu ich żywotności: 1) kijanki albo giną krótko po osiągnięciu 25 stadium; 2) albo żyją długo, jednak nigdy nie dochodzą do okresu metamorfozy; 3) albo giną w końcowym okresie metamorfozy; 4) albo osiągają przeobrażenie i są żywotne. Żywotność kijanek formy *esculenta* pochodzących od 10 par skontrolowano w 22 akwariach przegrodzonych siatką, w których rozwijały się razem z kijankami innych kombinacji. Kijanki kontrolne zawsze osiągały przeobrażenie, natomiast kijanki kombinacji *esculenta* wszystkie wyginęły w różnych okresach rozwoju. Przeobrażały się tylko niektóre kijanki pochodzące od dwóch samic. Na ogólną liczbę 35 samic *esculenta* zapłodnionych samcami własnej formy, tylko od jednej samicy otrzymano dość wysoki procent przeobrażonych kijanek. Z pozostałych samic od trzech otrzymano po jednej kijance, a od jednej samicy dwie kijanki, które się przeobraziły i były bardzo żywotne. Kijanki od tych czterech samic rozwinęły się z wyjątkowo dużych jaj. Wszystkie zarodki od 30 pozostałych samic wyginęły w różnym okresie rozwoju.

Kijanki wszystkich krzyżówek należących do pozostałych 8 kombinacji, jeśli były hodowane, zawsze osiągały przeobrażenie i były żywotne.

W oparciu o powyższe dane autor sądzi, że osobniki, które ze względu na cechy morfologiczne są zaliczane do formy *esculenta*, są prawdopodobnie hybrydami powstałymi ze skrzyżowania innych form żab zielonych.

РЕЗЮМЕ

Автор обсуждает результаты проведенных в 1963—1965 годах исследований по вопросу скрещивания трёх морфологических форм зелёных лягушек: *Rana lessonae*, *R. esculenta*, *R. ridibunda*. Оплодотворение проведено было согласно прилагаемой схеме, получая в результате 9 различных комбинаций: 3 типичные и 6 гибридных. Серийное оплодотворение проведено было на тех же особях. Для скрещивания употреблены 180 особей всех форм, выловленных в amplexus.

Автор обращает внимание прежде всего на быстрое развитие зародышей и на их жизнеспособность.

Из проведенных опытов следует, что развитие яиц у всех комбинаций без всяких трудностей достигает 25 стадии, однако быстрота развития у отдельных комбинаций разная.

Самую низкую жизнеспособность обнаруживает потомство особей *esculenta*. Она проявляется в появлении большого количества анормальных екземпляров, в высоком проценте смертности головастиков и прежде всего в разной степени их живучести: 1) головастики либо погибают вскоре после достижения 25 стадии; 2) либо живут долго, однако никогда не достигают периода метаморфоз; 3) либо погибают в последнем периоде метаморфоз; 4) либо достигают периода метаморфоз и являются жизнеспособными. Жизнеспособность головастиков формы *esculenta*, происходящих от 10 пар, была проконтролирована в 22 аквариумах, перегороженных сеткой, в которых они развивались вместе с головастиками иных комбинаций. Контрольные головастики всегда достигали периода метаморфоз, головастики же комбинации *esculenta* все погибли в разные периоды развития. Периода метаморфоз достигали только некоторые головастики, происходящие от двух самок.

На общее количество 35 самок *esculenta*, оплодотворённых самцами собственной формы, только от одной самки получен довольно высокий процент головастиков метаморфизованных. Из числа остальных самок от трёх получено по одному головастику, а от одной самки — два головастика, которые метаморфизовались и были очень живучи. Головастики от этих четырёх самок развились из исключительно больших яиц. От 30 остальных самок зародыши погибли в разные периоды развития.

Головастики всех гибридов, принадлежащих к остальным 8 комбинациям, если разводились, всегда достигали периода метаморфоза и были жизнеспособны.

Опираясь на приведенные данные, автор считает, что особи, которые ввиду их морфологических признаков относятся к форме *esculenta*, вероятно являются гибридами, происходящими от скрещивания иных форм зелёных лягушек.

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