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Principles of studies of European water frogs

[With plates XXIV—XXVIII and 4 text-figs]

Zasady badania żab zielonych Europy

Abstract. For the last 24 years (1963—1986) the author has been working on European water frogs. He has crossed more than 740 pairs of water frogs, and has counted and measured the eggs from over 600 females of all six European species and their five hybrids which live in wild, and other hybrids received in crossing experiments in laboratory. He has reared over 20 000 metamorphosed froglets of which more than 4000 individuals were devoted for further rearing to study their various features during their ontogenetic development. During this long period he has elaborated numerous rearing and investigative methods which he has gathered in this paper.

I. INTRODUCTION

In the last 20 years water frogs of Europe have aroused general interest in biologists of different specialities. This interest resulted from the fact that the most numerous frog of Europe, *Rana esculenta* LINNAEUS, is not a species as it was gererally supposed, but only a hybrid of *Rana ridibunda* PALLAS and *Rana lessonae* CAMERANO. Further studies have shown that *Rana esculanta* is an exceptional hybrid. It reproduces by hybridogenesis, i. e. in its meiosis the phenomenon of independent segregation of chromosomes does not appear and unrecombined chromosomes of one parental species are transmitted to gametes (TUNNER, 1974; UZZELL and BERGER, 1975). At present we know three kinds of such hybrids. One of their parental species is *R. ridibunda* and the second one is one of the three related species (Table I) which are sympatric and syntopic with hybrid. The hybrid lives with *R. bergeri* (GÜNTHER et al., 1988) throughout the Apenninic Peninsula and Sicily; with *R. perezi* SEOANE in southern France and north-eastern Spain; and with *R. lessonae* from the Atlantic coast in France and throughout all Central Europe to the Volga river in the Soviet Union.

Till 1960 we believed that only one (KAUNI, 1959) or two (MERTENS and WERMUTH, 1960; TERENTJEV, 1962) species of water frogs existed, but at present we know that only in Europe there are 11 taxons (Table I): six species, three hybridogenetic hybrids, and two interspecies hybrids (GRAF et al., 1977; UZZELL and HOTZ, 1979; TUNNER and HEPPICH, 1982; HOTZ and UZZELL, 1982; SCHNEIDER et al., 1984; GÜNTHER et al., 1988). Other species live in North Africa (HEMMER et al., 1980) and in Tadzhikistan in Soviet Union (ALEXANDROVSKAYA and KOTOVA, 1985).

I began the study on water frogs in 1951 (BERGER, 1955) and the experiment on their hybridization in 1963 (BERGER, 1967). In the course of time the number of crosses, using forms with different genotypes and origin, enlarged and the rearing of adult frogs and their progeny, which is the basis in the study on the relation of species, changed completely. During 24 years (1963—1986) I have crossed more than 740 pairs of frogs, and have counted and measured the eggs from over 600 females of all six European species and of all hybrids which live in nature (Table I) and of other hybrids which have been obtained in captivity. From fertilized eggs I have reared over 20 000 metamorphosed froglets of which more than 4000 individuals were devoted for further rearing for the purpose of the study of their various characteristics.

During this long time I have elaborated numerous rearing and investigative method which are published in different journals, often difficult to access. Some of these methods have only historical value at present; others, with passing years were completely changed, and those which have been elaborated during the last years are not published yet.

In the present paper which came in existence through the influence of my friends — Hansjurg HOTZ and Thomas UZZELL — and I thank them for that heartily in this place, I have gathered all my experience paying special attention to the breeding and investigative methods.

Table I

Six species of European water frogs and their five hyberids which live in nature. All 11 taxa have been bred in Poznań for some years. F_1 — first generation, B_n — back crosses

Parental species		Hybrids	
Female	Male	Genomes and generation	Origin and persistance in nature by:
ridibunda-RR	*lessonae*-LL	RL F_1	hybridization
		RL, RRL, RLL B_n	hybridogenesis
ridibunda-RR	*perezi*-PP	RP B_n	hybridogenesis
ridibunda-RR	*bergeri*-BB	RB B_n	hybrigodenesis
ridibunda-RR	*shqiperica*-SS	RS F_1	hybridization
ridibunda-RR	*epeirotica*-EE	RE F_1	hybridization

II. MARKING AND BIOMETRY

All frogs in the rearing place have to be marked individually for the purpose of their quik identification, because only in this way we can investigate their different characteristics during their ontogenetic development. American zoologists worked out (NACE et al., 1973) an easy marking method by toe amputation for *Salientia* in which regeneration does not occur. In the individuals which are devoted for rearing we clip the required toes as follows. The shin of the toe should be tightened and pulled in the direction of the body, so after clipping off the digit, the skin will cover the wound. For disinfection purposes the wounded digit can be moistened in alcohol. By simple addition of figures which mark the digits we can find the number of every individual easily (Fig. 1). In this way we can mark individually 9999 frogs; when we also utilize the first toes of hind legs (digitus primus) the number of marked frogs will grow to 19 999 or even to 39 999. Digitus primus of right leg (20 000) is clipped as the last one because its length has the fundamental meaning in the identification of some forms of water frogs.

In order to qualify the phenotype of individual frog we perform numerous measurements of its body (TERENTJEV, 1950) from which the most important

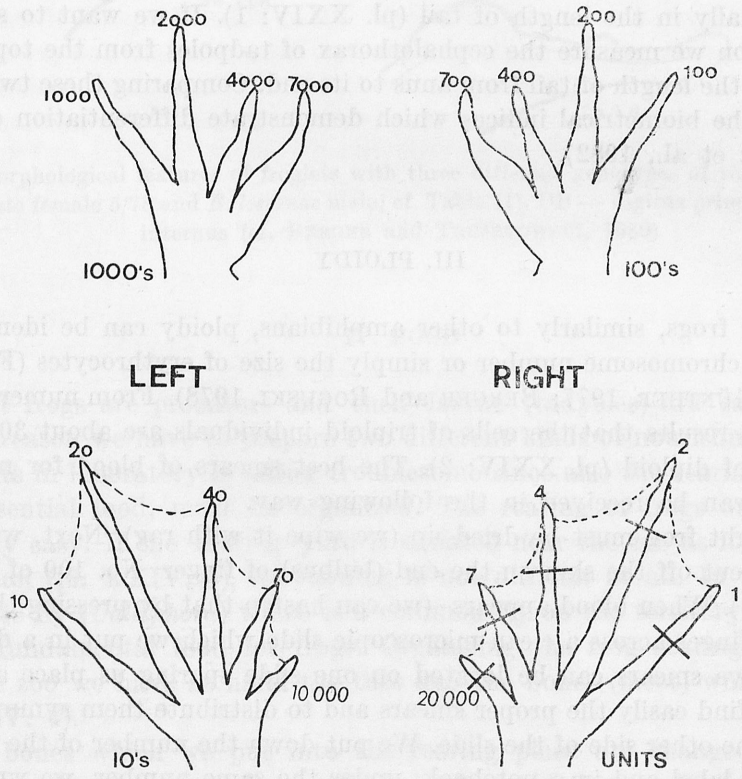

Fig. 1. Toe clipping in water frogs (according to NACE et al., 1973, changed)

four ones are very useful for preparing biometrical indices (BERGER, 1966, 1975): 1. L — body length (longitudo corporis) measured from the end of snout to anus, 2. T — length of tibia (longitudo tibiae) measured by the bent femur and tarsus, 3. DP — length of the first toe of hind limb (digitus primus), 4. CI — length of internal metatarsal turbercle (callus internus).

The water frog forms of Central Europe can be identified correctly very easily by comparing the digitus primus with callus internus without any measurements. After reaching some experience we can identify the ploidy and foresee the composition of genomes in all individuals of *esculenta* phenotype to which belong three different genotypes or phenotypes (Table I; Fig. 2).

The tadpoles reared in the laboratory are nor marked individually. We can mark them, however, in groups, for the purpose of counting their number in the studied body of water. We clip about 1/3 tail of the caught tadpoles which are later released in the pond. Their tails will regenerate but will never reach their previous size and shape. At the recatching (after 10—15 days) we separate all the marked tadpoles from the unmarked ones. Using these data and basing on Lincoln index we can estimate the number of all tadpoles inhabiting the studied pond.

When we cross one female with some males (or reversely) which belong to different phenotypes, the developed tadpoles differ frequently in the size of body, especially in the length of tail (pl. XXIV: 1). If we want to show this differentiation we measure the cephalothorax of tadpoles from the top of head to anus and the length of tail from anus to its end. Comparing these two lengths we receive the biometrical indices which demonstrate differentiation of tadpoles (BERGER et al., 1982).

III. PLOIDY

In water frogs, similarly to other amphibians, ploidy can be identified on the basis of chromosome number or simply the size of erythrocytes (FANKHAUSER, 1945; GÜNTHER, 1977; BERGER and ROGUSKI, 1978). From numerous measurements it results that the cells of triploid individuals are about 30% larger than those of diploid (pl. XXIV: 2). The best smears of blood for measuring blood cells can be received in the following way.

The caught frog must be dried up (we wipe it with rag). Next, with sharp scissors, we cut off the skin on the end (bulbus) of finger No. 100 of the right hand (Fig. 1). When blood appears (we can hasten that by pressing the finger) we run the finger across a clean microscopic slide which we put in a dry place. Ten or twelve smears can be located on one slide sparing us place and time. In order to find easily the proper smears and to distribute them symmetrically, we square the other side of the slide. We put down the number of the specimen on the stick label and in a notebook, under the same number, we write down its body length and other features. We ought to be aware that the size of the

cells is correlated with the size of the frog: cells of a small frog are much smaller than those of a large one.

Determining the ploidy of a tadpole we put it on a hygroscopic rag or blotting--paper and clip off its tail and run it across a microscopic slide. It is very difficult to obtain good smears from tadpole tdils and for this reason we must make them very carefully and without hurry. Cytological slides (karyotype) for couting the number of chromosomes can also be made out of the regenerated tails which appear in tadpoles after 4—5 days (BERGER and ROGUSKI, 1978). The ploidy of individuals are designated from the size of cells which we measure. The area of one side of erythrocyte is counted from the $ab\pi^2/4$ formula, where a and b denote the length and width of the cells. In order to determine the ploidy of a given individual it is enough to measure 3—4 cells; for statistical counting, however, at least 10 cells must be measured (UZZELL and BERGER, 1975).

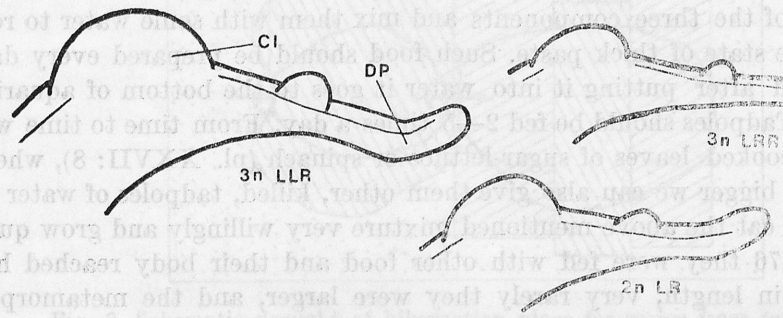

Fig. 2. Morphological features of froglets with three different genotypes of the same parents (*R. esculenta* female 5/78 and *R. lessonae* male; cf. Table II). DP — digitus primus, CI — callu internus (cf. BERGER and TRUSZKOWSKI, 1980)

IV. FOOD

Adult frogs are predators and their larvae (tadpoles) are saprophag and for this reason we have to prepare two different kinds of nourishment. Feeding the adults in laboratory is rather troublesome since also the rearing for insects, their essential food, must be organized. The rearing of frogs out-of-doors is relatively easy. If the rearing yard is situated near the zoo as it was the case in Poznań (pl. XXIV: 3), its running is not difficult at all. In this situation bluebottle fly (*Calliphora*) which is a common fly on the territory of a zoo will be the fundamental food for frogs. Organizing the frog rearing out-of-doors near the zoo we have at hand the flies and the bones (meet) which lure them (pl. XXV: 4).

The bones which we put into the rearing place are changed every 2—3 days for sanitary reasons. During this short period thousands of larvae of the fly will appear on the bones, but the frogs do not like them. If we want, however,

to enlarge the living food (flies) for frogs, we leave the bones with the larvae and wait for their metamorphosis. If the rearing yard is not situated in such a suitable place as a zoo, and the number of frogs is too large (more than 50 individuals) the frogs should receive additional food, otherwise — because of shortage of food — the females may not lay eggs next year. The best animals for feeding are earth worms, butterfly larvae, colorado beetles (larvae and insects), tadpoles and froglets of water and brown frogs (pl. XXV: 5) which live nearby. The froglets and tadpoles are put in basins from which they will be caught by frogs. Other animals are put in a special place (cf. pl. XXVI: 6).

Feeding tadpoles is much easier. After the experiments in 1977 (BERGER and PNIEWSKI, 1981) it was found that the best food for tadpoles is a mixture of three components: pea of puree type, yolk of hard-boiled eggs and powdered nettle. The preparation is very easy. The leaves of nettle (*Urtica dioica*) are picked up in early spring and after drying they are pulverized in a coffee-mill. The other two types of food are very easy to prepare. We take more or less equal parts of the three components and mix them with some water to receive a pulp in the state of thick paste. Such food should be prepared every day. It is heavy and after putting it into water it goes to the bottom of aquaria (pl. XXVI: 7). Tadpoles should be fed 2—5 times a day. From time to time we can give them cooked leaves of sugar-lettuce or spinach (pl. XXVII: 8), when the tadpoles are bigger we can also give them other, killed, tadpoles of water frogs.

Tadpoles eat the above mentioned mixture very willingly and grow quickly. In 1963—1976 they were fed with other food and their body reached hardly 50—70 mm in length, very rarely they were larger, and the metamorphosed froglets were rather small reaching only 16—23 mm of body length. Since 1977 they received the novel mixture and after 30—35 days they reached 60—90 mm of length, and the metamorphosed froglets were, as a rule, larger (20—27 mm of body length).

V. THE REARING OF FROGS

If we want to have good conditions in the rearing yard it should be located in a sunny place. The area measuring about 3×3 m, or manifold larger (pl. XXIV: 3) is fenced by plastic plates 90—100 cm high. As a fence you should never use the wire net through which the drogs can go away or can be seriously wounded (BERGER 1970a). The outer fence must be higher than the inner partitions which should be about 80 cm high. Only small frogs can get over such a partition because of adhesion on rainy days. For this reason a plastic fence with rough surface is the best one.

In the centre of every separate enclosure we build a basin 100—150 cm in diameter, and about 30 cm deep. The basin may be built of concrete, or a plastic container can be fixed inside the basin, or the pit can be covered with thin plastic. In the centre we put some turf. Then the basin is flooded with water and some

plants, the best of which are *Strotiotes aloides* or *Cerathophyllum* are put in it. The plants and the grass of turf will form a shelter for frogs in water and places for laying eggs. In one or two spots in the rearing yard we put a piece of plank, or a plate, or a trunk of wood under which we make a hollow which will act as a shelter for frogs on hot and cold days as well as during the autumn ground frost. This shelter can at the same time be the entrance to the winter quarters (cf. Fig. 3; pl. XXVI: 6).

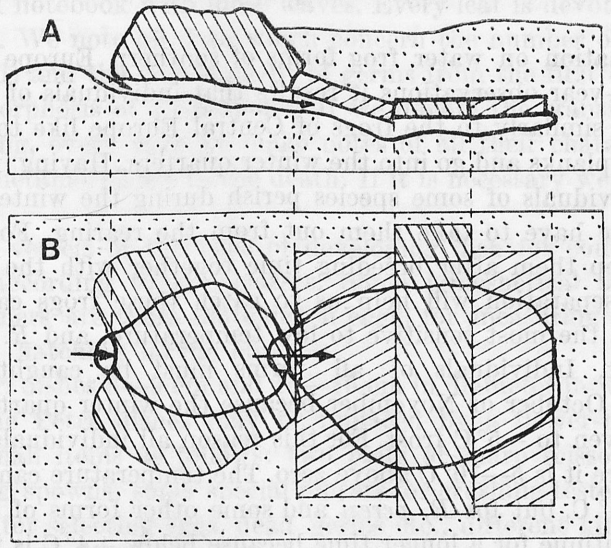

Fig. 3. Schematic draught of hibernation place for water frogs (original)

Winter is the most difficult period for some species of water frogs. For that reason we must learn the fundamental information of their biology. The native forms, to which in Central Europe belong *R. ridibunda*, *R. lessonae* and their hybrydogenetic hybrid, *R. esculenta*, cause the least trouble. *R. ridibunda* is the inhabitant of large water bodies (lakes, rivers, claypits) in which it hibernates. *R. lessonae*, however, lives in small bodies of water which frequently dry periodically; it leaves them in early autumn and hibernates in soil (BERGER, 1982). *R. esculenta* is an eurytopic form. It inhabits all types of water and hibernates with *R. ridibunda* in water or with *R. lessonae* on land.

For these three forms the above mentioned quarters (BERGER, 1970a) must be prepared. We examine them every year in the middle of August or so. We do it at that time because the frogs must get accustomed to the new enviroment and also because numerous individuals of *R. lessonae* and *R. esculenta* bury in the soil at the end of that month. For this reason we deepen and widen the shelter (Fig. 3); next we put some planks in the hollow. Under the hollow we leave a slit 3—5 cm high. We cover the place with 5—10 cm stratum of earth and in late autumn with leaves. Nearly all of *R. ridibunda* frogs will go into these winter quarters because they do not like to hibernate in a small basin. The

entrance to the winter quarters must be carefully covered to make any issue of frogs impossible. It is important as the yearlings like to wonder within the rearing yard to the end of November (BERGER, 1982). Some frogs of *lessonae* and *esculenta* phenotype frequently buried themselves in the ground at different hours of the day, during the whole summer (pl. XXVIII: 10). In autumn they buried much deeper in different parts of the rearing and spent winter in this way. To help them to hibernate in good condition we dig the earth in one or two places to 10—20 cm deep and before winter (in November) we cover it with leaves.

Our information on water frog forms of southern Europe is scanty. From my own many-year observations it results that individuals of all forms as well as the hybrids similarly to the frogs of Central Europe like to hide during the summer under planks and go into the winter quarters. Having in mind, however, that most individuals of some species perish during the winter bacause of low temperature we have to take them out from the rearing. For this reason we should not keep them after breeding time together with the frogs of Central Europe. This separation will help us to catch these frogs easily.

R. perezi is the most sensitive to low temperature, and *R. shqiperica* is the most resistant. Individuals of all forms must be caught before winter. We do that in October or November because the winter quarters protect them against light even to −5° C frost. For this reason all individuals are moved over to a place where it is 5—10° C above zero. The temperature can drop for a short time even to 4° C, but for *R. perezi* and some other forms of southern Europe this cannot continue for a longer time because below +4° C is injurious or even lethal for frogs.

The frogs are put into containers with plump garden soil in which they will burrow. We put leaves on the surface of the earth, ideally of lime (*Tilia*). From time to time one must look into the containers and water the leaves in order to keep moisture in the soil.

About the middle of April we open the entrance to the winter quarters and prepare the frogs for rearing out-of-doors. All frogs which have hibernated in the container are measured, especially the length of their body, and for special study also tibia, digitus primus and callus internus the size of which change during ontogeny (cf. BERGER, 1970b). We carry them to the ready basin and put them according to the earlier prepared plan of their crossing. Next we catch all the frogs which have hibernated out-of-doors and in winter quarters. After having measured them we also put them into the prepared fenced place.

VI. BREEDING TIME AND CROSSING EXPERIMENTS

Before we start crossing the frogs we have to prepare the following equipment:

1. A general notebook in which we will note the most important data con-

cerning the rearing yard and frogs. On the first page you draw a plan of rearing for each year (pl. XXIV: 3) and in the place of every basin you put symbols of the animals (cf. Table I) which we plan to cross. On every page of the notebook we note all data which concern one pair only: the successive number of the female and the year of laying the eggs (cf. Table II), symbols and sex of the crossed frog and if possible their individual number (Fig. 1), number of eggs and their measurements with division into size classes.

2. A detailed notebook with loose leaves. Every leaf is devoted for progeny of one pair only. We note all data which concern the number of eggs (unfertilized and cleaved) and the development of germs from the first cleavage to the death or metamorphosis of all tadpoles. If we devote the transformed froglets for further rearing, every year we make notes of all their characteristics from the moment of metamorphosis to the death. If it is necessary we add additional leaves.

3. Some flat vessels of the type of developing dish for counting eggs and dividing them according to size classes. Some himesherical little containers (the best containers are those for chicken eggs) for measuring eggs, gathering the dead germs, hatching larvae etc.

4. Many Petri dishes 8—9 cm and 15—16 cm in diameter in which the germs will develop (the Petri dishes may be replaced by a flower-pot).

5. Several small items as follows: two pairs of sharp scissors for marking frogs and cutting spowns, some special pincers for crushing testes, some pincers with sharp ends for selecting eggs, dead germs etc., different kinds of pipettes, syringes with needles for injection, one homogenizor for crushing the pituitary glands (hypophysis), several aquaria and different plastic containers, special fishing-net for catching frogs and tadpoles in basins, and a fishing-rod with barbless hooks to catch adults in wild.

Breeding time of water frogs begins in the Poznań region about the middle of May and goes on with interruptions to the end of June. The breeding time in my rearing yard begins usually earlier because of the advantageous microclimate. During that time the basins must be examined very often to spot and collect the laid eggs or to catch the pairs in amplexus (pl. XXVII: 9). The spotted pairs can be left in the place or can be caught and put into aquaria with plants. After laying eggs the individuals must be identified and the eggs taken out and counted. If we plan to cross the female or male with other individuals the pair must be separated after laying 2—3 spowns (300—800 eggs). The frogs are washed in tap water and put into separate aquaria with selected frogs which are ready to lay gametes. As a rule, these new pairs will lay gametes spontaneously.

The female that is to cross with some males is put into a dry container (without water). That is very important because the gelatineous envelopes of eggs in oviducts will begin to swell after 3—7 hours if the female is in water. The eggs like that are not suitable for fertilization and it is very difficult to take them out (to squeeze out) of a female. The eggs, however, should be taken

out if the female is very valuable for us, otherwise she can die. The female being in a dry container and in a cool place (15—20°C) can be crossed within 1—4 days. You can also put her into a refrigerator at +4°C but her eggs, however, not always are able for cleavage next day.

Any female lays some thousand eggs, as a rule (BERGER and UZZELL, 1980), and therefore you may cross them with a large number of males. The matter with males is more complicated because they are not always ready to lay gametes. Before crossing experiments you ought to prepare some Petri dishes 8—9 cm in diameter. All males should be washed in tap water, killed, next you take out their testes and put them into dishes which are put obliquely. Testes should be crushed precisely with pincers in about 3 cm^3 of aquarium water, and next you pour water (10—20 cm^3) and the ready suspension is left for some minutes. To every dish a label with a symbol and successive number of the female and a symbol and successive alphabetic letter of the male is attached (BERGER, 1976). These data are later written down into both notebooks. With a suitable pincer you take a drop of every suspension and put it onto a microscopic slide to prove the presence of spermatozoa. This operation is very important because some hybrid males are sterile and we are able to replace them by another males with the same genotype.

After the sperm activity has been confirmed, the eggs are stripped from the female by holding her in such a manner that pressure is applied to her abdomen with the force directed foward her cloaca, thus squeezing the eggs from the ovisacs into suspension. You squezze 200—400 eggs on every dish. This goes very easily. A spiral pattern of the eggs is formed in the Petri dish to prevent the eggs from clumping (pl. XXXVIII: 11). In the other hand you hold scissors or suitable pincers to cut up a portion of eggs. All eggs should be plunged into suspension and spread out in a single layer. After about 20 minutes the successfully fertilized eggs will rotate so the black animal hemisphere is uppermost. At that time you pour off the suspension and replace it with fresh water. Sperm suspension can be used with good results to fertilize the eggs of at least one or two other females.

If you want to cross the frogs in early spring the method given by RUTH (1934) should be applied. This method is based on giving the frog a pituitary injection from another frog. In the case of water frogs it is the only method which accelerates their ovulation because no gonadotropic hormone gives positive results. In experiments we can take the pituitary from any individual of water frogs. One pituitary of a female is equivalent to two male pituitaries. The frog is decapitated, the jaw is removed and the palate is uppermost. The one end of the scissors is put deep into the foramen magnum and both lateral processes of os pterygoideum under which pituitary gland is placed as a little pink body. The os pterygoideum is bent up and the pituitary is removed with pincer, next we put it into about 0.5 cm^3 of destilled water and homogenize it. The injection should be done in the coelomic cavity.

The male is injected at first with one gland from a small female or a large

male. After 24 hours the manipulation is repeated and the female is simultaneously injected with three female glands if she is large or with two ones if she is smaller. You put the pair into an aquarium, the male will take the female in amplexus and within 24 hours, sometimes even 48 hours, the pair should lay gametes. If they do not we check the female by slight pressure of the abdomen. If the eggs are not observed at the cloaca we should repeat the procedure with injection for the female, because of underdose applied to the female the first time she has been injected. We can also guess that the eggs have been laid earlier or that the females is still juvenile.

In some females which are prepared for crossing many eggs may be crushed at the first experiment. To avoid that we press her stomach stronger. This accelerates stripping the eggs and causes the loosing of cloacal sphincter muscle, eventually we must cut it. After this intervention the eggs will strip without damage.

When we cross a male which we do not want to kill, as he is very important for us, we inject him a pituitary gland of one female, next we put him into a dry container. After one hour the spermatozoa should appear in his cloaca. We prove that with a thin pipette, the end of which has to be melted in fire to avoid damage of his inner organs. Next the pipette is placed into the cloaca and s small drop of liquid is put on a microscopic slide. When the spermatozoa are present you take a little water and place it into the cloaca. We repeat this procedure (rinse the cloaca) 2—3 times. The liquid is gathered onto small finger bowls and the eggs are stripped from the female. As a rule the eggs will cleave in 100%. We can repeat this procedure with male several times.

If we plan to carry out the genetic studies the bodies of both parental frogs and their progeny should be frozen at leat in —20°C or lower.

VII. EGGS

The eggs of water forg females are frequently differentiated into size classes which concern in particular the females of *esculenta* phenotype. The size of eggs is connected with their ploidy and for this reason all gametes of every female must be looked through very carefully.

The eggs of *R. esculenta* females are divided into three discrete size classes, as a rule (Table II; pl. XXVIII: 12), but the number of eggs and ploidy of various classes are different in different populations and in different individuals. In mixed *lessonae-esculenta* populations (L—E system; UZZELL and BERGER, 1975) from near Poznań all small eggs which are not numerous, and medium eggs which contain normally more than 95% of all gametes are haploid ova with a *ridibunda* genome (R.) The large eggs, however, are diploid ova and contain, as a rule, the genomes of both parental species *R. ridibunda* and *R. lessonae* (RL). The large size class of some *R. esculenta* females has two size subclasses (BERGER, 1979): the large smaller eggs — type A, and the large larger eggs — type B (Table II). The eggs of type B apparently are always diploid and have genomes of both

Table II

Examples of varied egg sizes of *Rana esculenta* females. The eggs form one (1—2), two (3—4), three (5—6), and four (7—8) discrete size classes. Two females (9—10) laid eggs which are differentiated, but they are not divided into classes. N/y — successive number of female (N) and year (y) of laying the eggs

Female		Diameter of eggs (mm)															
No.	N/y	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.0	2.1	2.2	2.3	Total
1.	15/74						2	43	5								50
2.	18/68					2	4	15	25	15	1	1					50
3.	2/82								4	27	9		7	16	1	1	63
4.	12/65			3	27	19	7	51	22	2							131
5.	11/81		1	29	1	28	1	5	23	1							89
6.	26/86					8	2	10	17	4	4	8	19	7	2	2	83
7.	7/76	1	25	3	26	27	7	15	7	2	6	3					122
8.	5/78		7	9	23	121	49	6	17	7	3	2	3	1			248
9.	9/76			15	24	26	36	34	42	25	8	4					214
10.	11/76			5	11	37	63	28	34	24	12	16					230

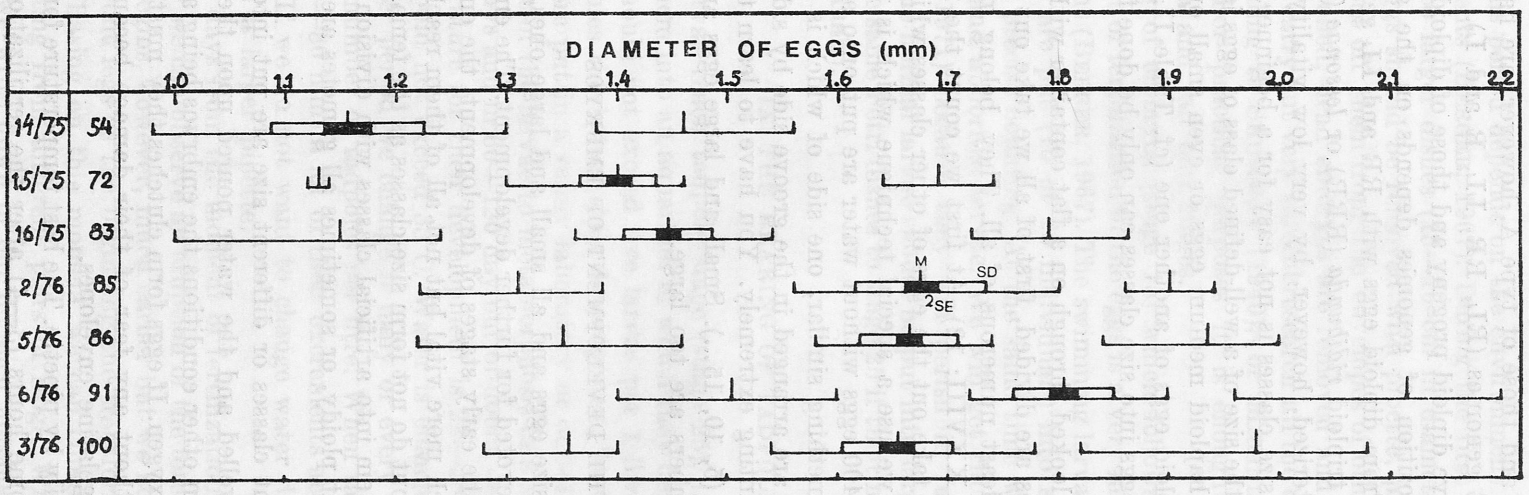

Fig. 4. Linear diagrams of size classes of eggs of *Rana esculenta* females. First column = number of females, second column: their body length in mm. Ranges and means (M) of measurements of small, medium and large eggs are denoted. Standard deviation (SD) and double standard errors of the mean (2 SE) are given for medium eggs only (cf. BERGER and ROGUSKI, 1978)

parental species (RL), and those of type A, however, are haploid and diploid gametes with different genomes (RL, RR, LL, R and L).

The haploid eggs give diploid progeny and those of diploid ova give triploid offspring whose composition of genomes depends on the genome of sperm (RL+R or RL+L). From diploid eggs with RR and LL genomes in suitable crosses we can cereive triploid *ridibunda* (RRR) or *lessonae* (LLL) frogs. Such individuals are characterized, however, by very low vitality.

Dividing eggs into size classes is not easy for a beginner. We ought to be aware of the fact that the size of a well-defined class of eggs is relative. Sometimes we can find that haploid medium eggs or even small ones of one female are larger that the diploid eggs of another one (cf. Table II; Fig. 4). For this reason the division of eggs into size classes can only be done for gametes of the same female.

The eggs should be looked through on a flat container with a white bottom. If we find that gametes are divided, first of all we take out the eggs of those classes which are the least numerous of all. They belong normally to small and large gametes (pl. XXVIII: 12). At first we count the medium size eggs and simultaneously we take out the eggs of other classes which have not been seen earlier. Counting we use a special technique which is precise and saves our time. About 200—400 eggs without water are put onto a white and damp developing dish, or something similar, one side of which is slightly upraised. In such a dish all eggs are arranged in the groove side by side into 2—4 lines. This simplifies the counting extremely. You have to learn to count the eggs in the system of fives (5, 10, 15...). Small and large eggs are counted in the same way if their numbers are too large.

VIII. DEVELOPMENT OF EMBRYOS

A part of medium-size eggs and all small and large ones, if their numbers are not too large, are devoted for further development. The embryos from small eggs die, as a rule, in the early stages of development, the embryos from large ones, however, are much more vital but not all of them reach stage 25. If the eggs are differentiated but do not form size-classes as the females 9/76 and 11/76 (Table II) we divide them into artificial classes with division of 0.1 mm. Such eggs can have different ploidy, or sometimes all gametes are diploid (Berger et al., 1978).

The eggs of different classes or different size are put into separate dishes. The eggs should be levelled and the water poured upon them should not be more that 1 cm deep. In other conditions the embryos being at the bottom can die because of lack of oxygen. If eggs form clutches they must be cut into small pieces with scissors without any fear of their damage because they are very well protected by the gelatine envelopes.

Water is changed daily by pipette. The best temperature for the development of embryos is 28°C. Some hours (3—4) after the fertilization the eggs begin

to cleave, but this period depends on the environment temperature and on the genotype of parents (BERGER, 1967). During the first cleavages we transport the embryos onto larger dishes (15—16 cm in diameter). After 12—20 hours the uncleaved eggs should be removed with pipette, pincers, or sharp scissors with which we cut off them from the envelopes of other eggs. The dishes are very often controlled, dead embryos are removed and their embryonic stages are noted. If we carry out very careful research all the embryonic stages should be noted according to SHUMWAY (1940), or MICHNIEWSKA-PREDYGIER and PIGOŃ (1957). For practical purposes it is enough to note the blastula, gastrula, neurula and tail bud stages.

The hatching of embryos (stage 18) which follows at 20°C in 5—6 days from the moment of fertilization of the eggs is a very important moment during their development (BERGER, 1967). The swimming larvae are caught with a special pipette and are put (10 or 20 individuals) into small containers for the purpose of their precise counting. At the same time we divide them into two groups: normal and abnormal larvae. We will get rid of the abnormal larvae soon (BERGER, 1971) because they will perish in further development (pl. XXVIII: 13). The normal larvae should be put into larger containers or we can leave them in the same Petri dishes to stage 25 if their number is small (no more that 100 individuals).

IX. THE REARING OF TADPOLES

When the larvae reach tadpole stage (stage 25), i. e. when they begin to eat, you place them into an aquarium in which they develop to metamorphosis. Their number should not exceed five larvae per 1 litre of water. The smaller number of tadpoles the better their development. The aquaria in which there are also plants are put in a sunny laboratory or out-of-doors in a sunny place. The development of the larvae to metamorphosis in a sunny room is completed within 35—50 days and in 60—120 day out-of-doors, depending on the weather. The best temperature for their development is 24—28°C and the temperature of the water should not exceed 33°C. Water is changed rarely when tadpoles are small, in later period we change it daily or every two or three days. This depends on the number of tadpoles per litre. When tadpoles reach the size of a large body (60—80 mm) a lot of their excrements accumulate on the bottom of the aquaria. If we do not want to change water daily we can remove the excrements with a larger pipette.

After 30—40 days from the moment of fertilization of eggs in tadpoles reared in laboratory we can observe an eruption of the forelimbs. The metamorphosis in tadpoles begins, however, much earlier but we accept the moment of the appearance of front legs as the beginning the transforming, because it is very easy to notice. Basing on this moment we can compare the development of tadpoles originating from different pairs of frogs. When the eruption of the forelimbs is on we write down in our detailed notebook which limb appeared

first: left or right. According to my own observation I can tell that in hybrid tadpoles the right hand appears first and in non-hybrid the left one.

Individuals with front limbs must be caught and placed into 1—2 litre containers with a lid (tiny frogs can go away). About half of the container should be filled with water in which a twig of *Stratiotes aloides*, on the leaves of which the frogles will set during metamorphosis, must be put. Such froglets can be placed in an aquarium with little water (2—3 cm deep) but we must put many twigs of *Elodea canadensis* on the bottom. Water must be changed in all containers every 1—2 days. At the end of metamorphosis (froglets with 2—5 mm tails) we measure every individual in the same way as adults. Next, from all or selected individuals we make dry blood smears in order to determine their ploidy. Individuals devoted for rearing are marked and placed out-of-doors. No adults can be present in the fenced yard where the froglets are since they can catch the froglets within some days (cf. BERGER, 1967). If tadpoles and, later, metamorphosed froglets live in good conditions most males and many females of certain forms can reach maturity in the autumn of the same year and next year they are ready to lay gametes.

The froglets which are not devoted for rearing are etherized and preserved in 3% formalin, later we will study their sex. If they are used for genetic studies we freeze them at −20°C or lower temperature.

When we plan to investigate the sex proportions in some crosses we devote many more tadpoles (50—100 individuals) for rearing. We put them into large basins which should be placed in a sunny room (cf. pl. XXIV: 3). In those basins there is no place for adults which are dangerous for the little ones. All tadpoles in the basins should be caught on the day when the first individuals with forelimbs appear. They are killed and put into 3% formalin and after some time we can tell their sex basing on the shape of their gonads (BERGER, 1971). Only in small tadpoles the gonads are not seen (they are not differentiated). In living individuals the gonads are hardly observable and for that reason to determine their sex in a such state one should have a very large practice.

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STRESZCZENIE

W okresie 24 lat (1963—1986) autor prowadził badania nad żabami zielonymi Europy. W tym okresie skrzyżował ponad 740 par żab, pomierzył oraz policzył jaja ponad 600 samic należących do sześciu gatunków oraz ich pięciu mieszańców żyjących w naturze i innych mieszańców wyhodowanych w laboratorium. Autor wyhodował ponad 20 000 przeobrażonych żabek, z których ponad 4000 przeznaczył do dalszej hodowli w celu badania ich właściwości w rozwoju ontogenetycznym. Podczas tego długiego okresu autor opracował liczne metody hodowlane i badawcze, które zostały omówione w obecnej pracy.

Edited by Dr. Z. Szyndlar

Plate XXIV

1 — Tadpoles fathered by *Rana bergeri* male and mothered by *R. lessonae* (right) and *R. ridibunda* (left) females on the day 42 of their development

2 — Erythrocytes of diploid (A) and triploid (B) froglets from the same parents

3 — General view of enclosure for rearing of water frogs in Poznań on the back-groud of the Zoo. There are 17 separate enclosures in the breeding time. At the bottom of the photo there are many flat basins for rearing tadpoles for the study of their sex

Phot. Z. Pniewski

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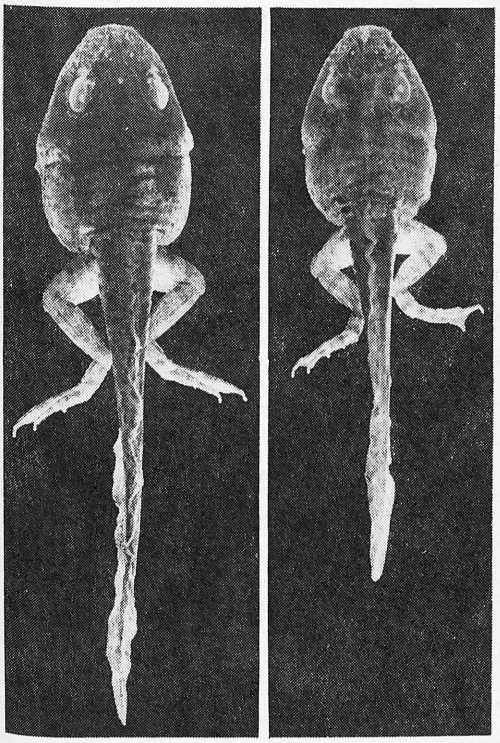

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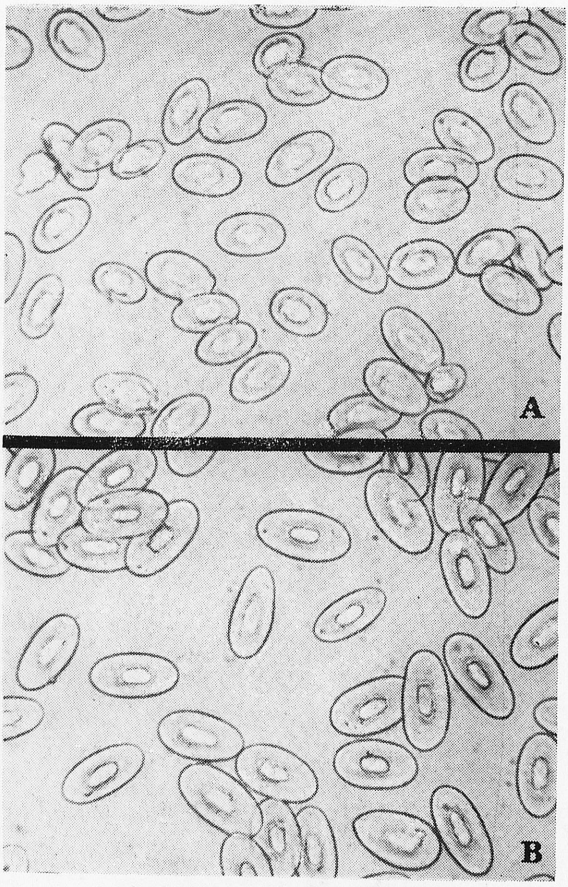

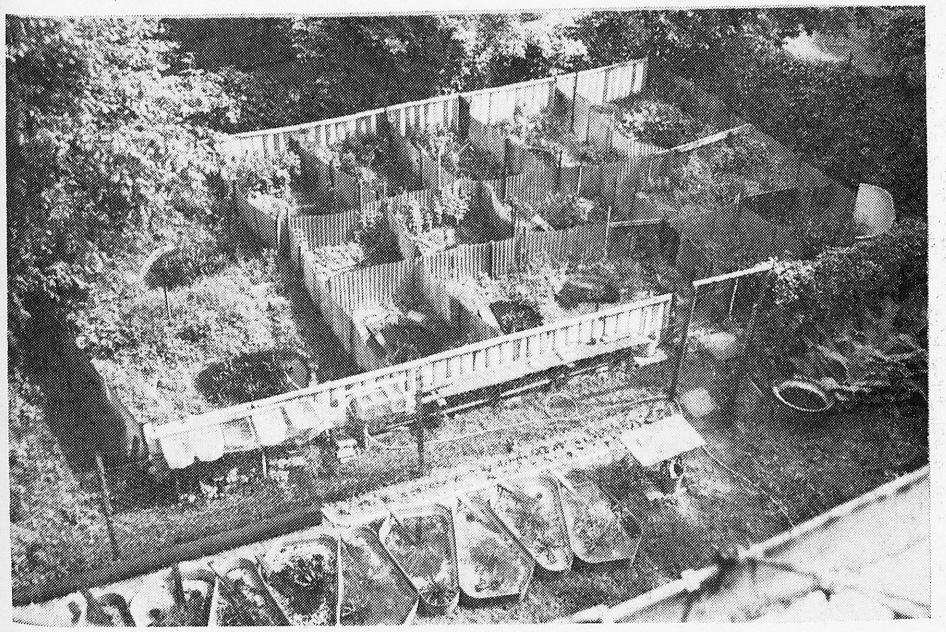

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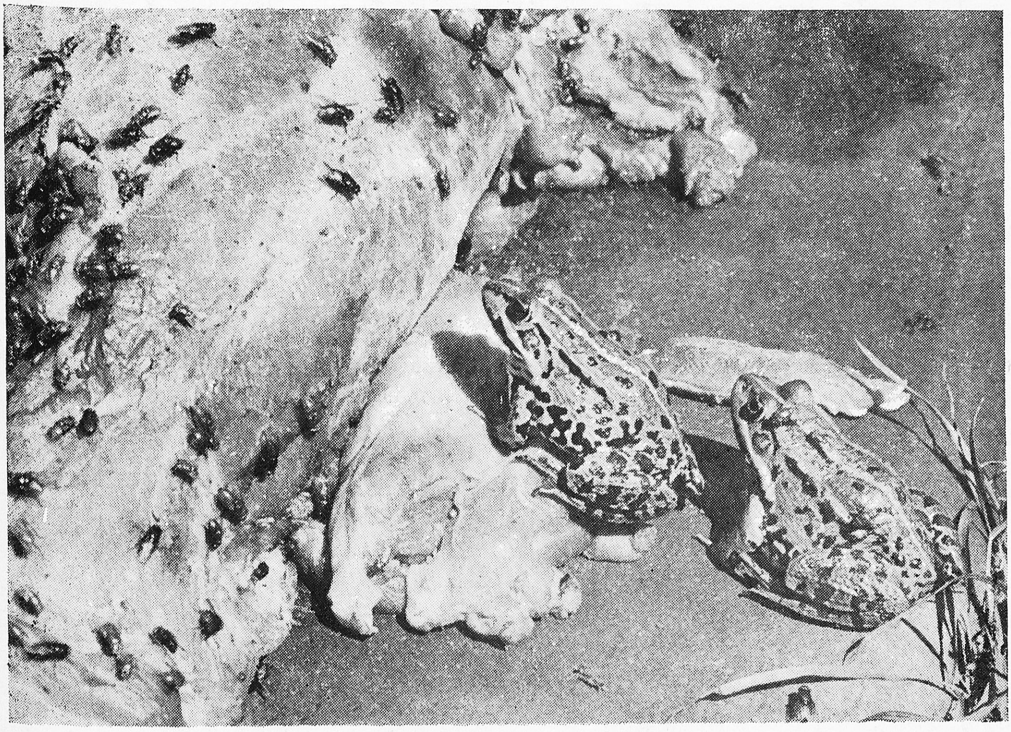
Plate XXV

4 — Water frogs catching flies (*Calliphora*)
5 — Adult frogs at feeder: on plate there are tadpoles of water frogs

Phot. Z. Pniewski



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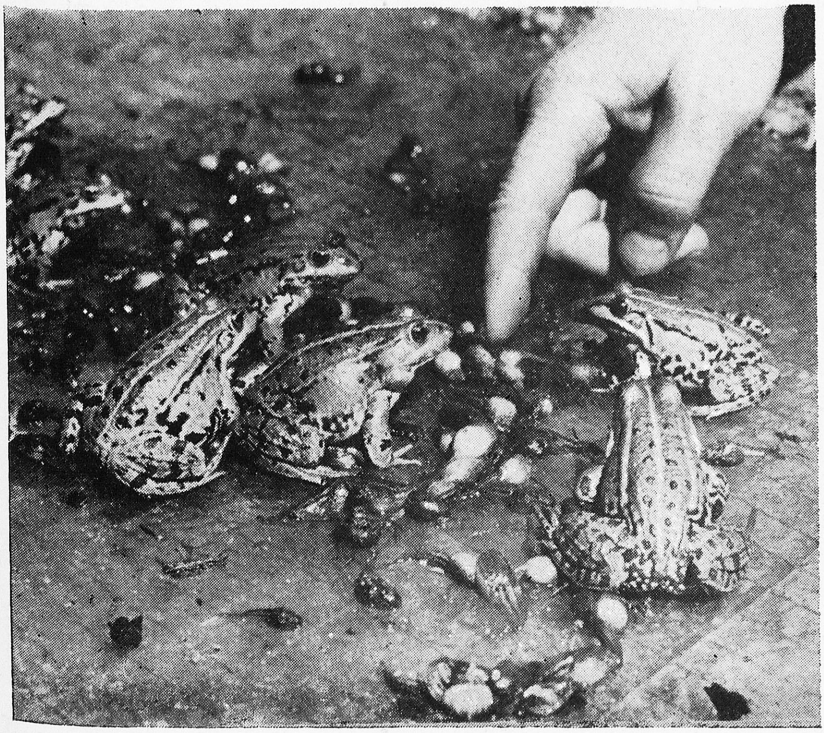

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Plate XXVI

6 — Shelter for frogs. In the further part of shelter there is a hibernating place (cf. Fig. 3)
7 — Tadpoles of water frogs in a laboratory aquarium

Phot. Z. Pniewski

6

7

Plate XXVII

8 — Tadpoles of water frogs eating a leaf of letuce
9 — Breeding time of water frogs in capativity. The eggs are attached to the leaves of *Stratiotes aloides*

Phot. Z. Pniewski

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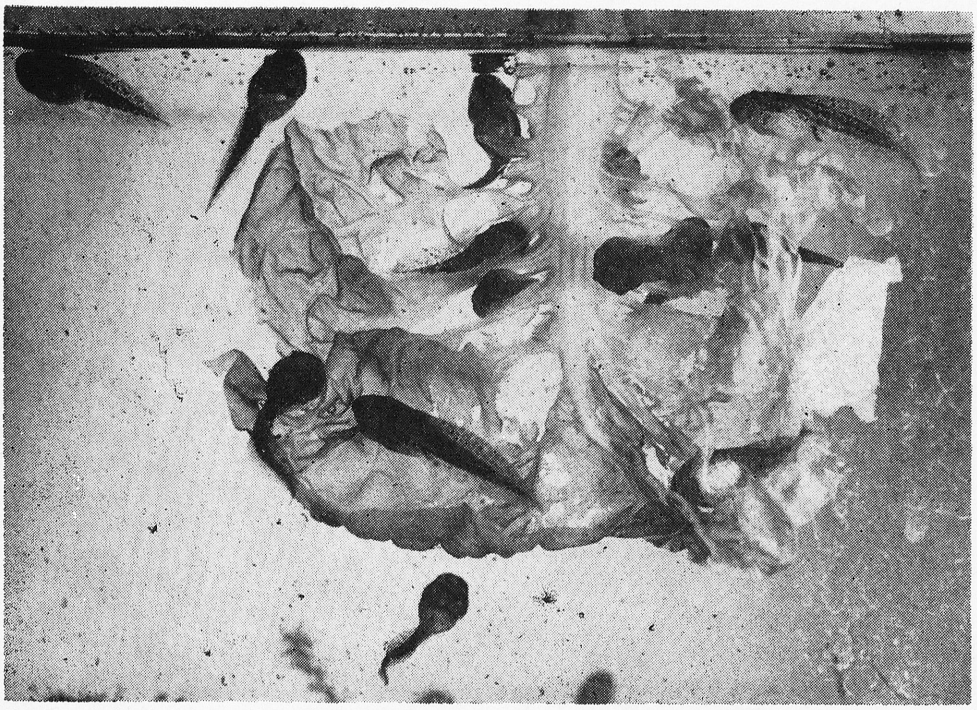

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Plate XXVIII

10 — *Rana esculenta* female burrowed in the ground in summer
11 — Eggs of *Rana lessonae* female at the moment of laying (A) and 20 minutes later (B)
12 — The eggs of *Rana esculenta* female which form three size classes. Among the medium-sized eggs there are some albino ones. Some large eggs are present; two small eggs are marked
13 — Abnormal germs parented by *Rana esculenta* individuals

Phot. Z. Pniewski

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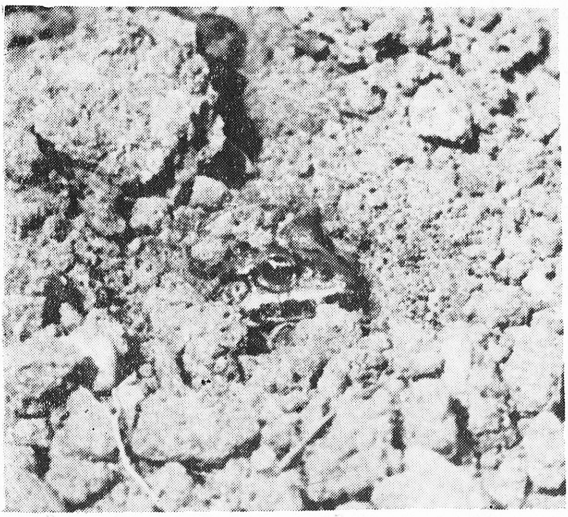

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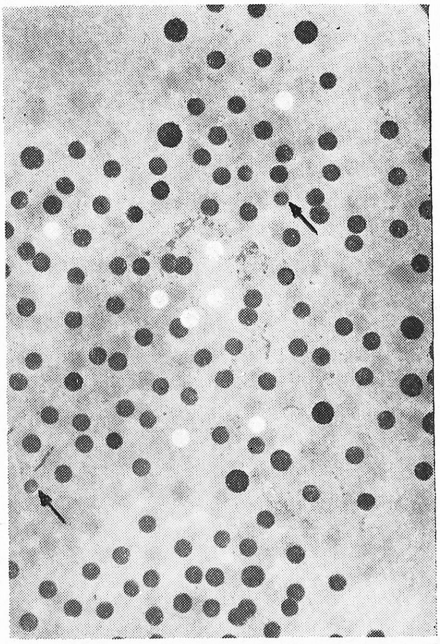

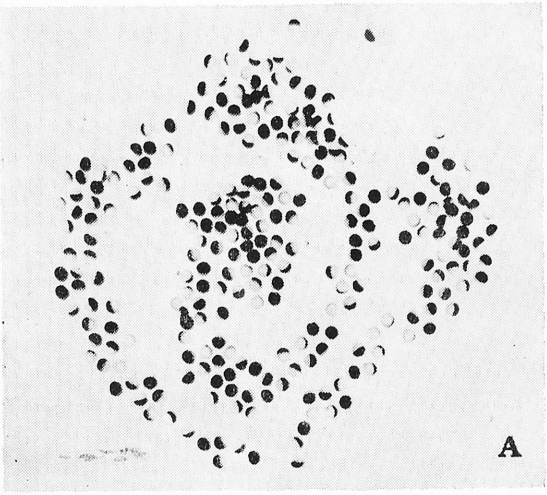

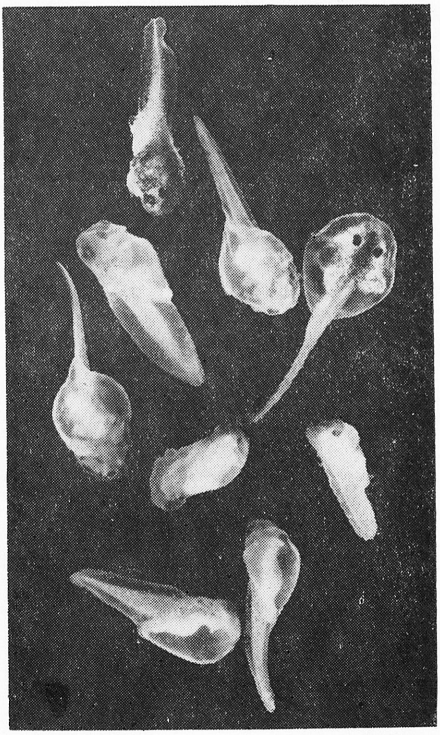

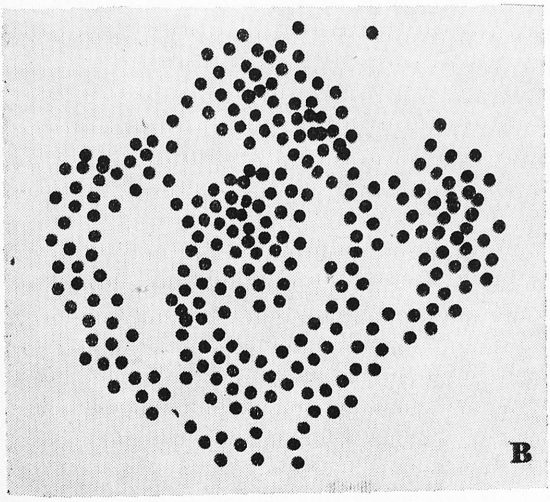

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L. Berger