

STAGES IN THE NORMAL DEVELOPMENT OF RANA PIPIENS LARVAE¹

A. CECIL TAYLOR AND JERRY J. KOLLROS

Department of Zoology, The University of Chicago, Illinois

ONE TEXT FIGURE AND FIVE PLATES (TWENTY-FIVE FIGURES)

While conducting experiments on anuran larvae, both of the present authors felt a need for a simple method of determining and recording the developmental stage of the tadpole, preferably a method in which each morphologically distinct stage would be assigned a number.

Such a system was established some years ago by Dr. Ross G. Harrison for staging *Amblystoma* embryos. Illustrations for these stages have recently been published by Hamburger ('42). Glücksohn ('31) established stages for *Triton taeniatus* and *T. cristatus*, forms which differ significantly from *Amblystoma* in development. A system for the staging of frog embryos has been worked out by Pollister and Moore ('37) and by Shumway ('40, '42).

As yet no comprehensive and systematic series of stages has been published for the anuran larva, although many investigators have found it necessary to specify levels of development attained by the larvae used in their particular work. The simplest method has been that of giving measurements of the larvae, either total length, or a ratio of the length of certain parts (e.g., tail or limb) to the total body length. Since, in amphibians generally, individuals at the same level of morphological development may vary widely in size, due to

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different conditions of feeding or temperature, and also since significant morphological changes often occur with but slight accompanying change in absolute or relative measurements of body parts, criteria based on size alone prove inadequate.

Among the systems of staging based more or less upon morphological characters is that of Wintrebert ('05), who described ten stages for *Rana temporaria* and *R. viridis*. The first nine were concerned with the premetamorphic development of the hind limb, while the tenth stage included the entire metamorphic period. No figures were shown. Tschernoff ('07) established a series of eleven stages for the development of the hind limb in *Rana arvalis* (*R. temporaria*), apparently unaware of Wintrebert's work. The series started with the hind limb bud of the 10 mm. tadpole, and concluded with the limb at metamorphic climax. Tschernoff's stages were arbitrarily selected to conform to his needs in his study of the hind limb. A series very similar to that of Tschernoff's was set up by Emmett and Allen ('19) for *Rana pipiens*. They also used eleven stages, all of which were premetamorphic. According to Emmett and Allen, limb buds do not appear until late in the larval period. Their tadpoles showed only the earliest stages of limb development at 12 weeks. This is in variance with our observation that the limb buds reach equivalent stages as early as 1 week after the beginning of feeding. As in Tschernoff's case, the stages established by Emmett and Allen are not suitable for the general purpose of comparison.

Etkin ('32) described sequentially the changes occurring during anuran metamorphosis, but failed to assign stage numbers to the various changes. Later Schreiber ('37), studying *Bufo vulgaris*, proposed a sequence of eight stages. The first of these included all of the premetamorphic period, while the remaining seven were concerned only with metamorphic events. Eakin and Harris ('45) mention a series of fifteen stages for *Hyla* larvae without describing them in detail.

The purpose of the present paper is to establish a set of closely spaced developmental stages for *Rana pipiens* larvae

which will include the entire period from the end of embryonic life to the fully metamorphosed frog.

MATERIALS AND METHODS

Eggs were obtained in September from two pituitary-injected females, using the method of Rugh ('34). The egg masses were divided into small bunches containing six to twelve eggs each and kept in a water bath maintained at 20°C. When the embryos reached embryonic stage 23 (Shumway, '40), the 100 largest were distributed in pairs in fingerbowls. Several equally advanced embryos were kept at room temperature (about 25°C.) and the resulting tadpoles were used throughout the study to indicate approaching changes in the larger group. From embryonic stage 23 (Shumway) until the end of the experiment the tadpoles were observed daily. When the embryos reached stage 25 (Shumway), the fifty largest were placed in individual fingerbowls with about 200 cc. of water.

Observation and recording of data were shared by the authors. Each observed one-half of the animals daily, and the other half on the succeeding day. After some experience it became possible to subdivide the various stages further. These fractional stages were independently identified by either author when verification of the stage of a given animal was requested by the other. As a rule both of us were in agreement on the fractional stage ascribed to each animal; only exceptionally did we differ by more than one-third of a stage interval.

All observations as to developmental stage were made under a binocular dissecting microscope. The length of each tadpole was measured at the start of each new stage. Younger larvae were measured from snout to tip of tail to the nearest 0.1 mm., and those over 30 mm. were measured to the nearest 0.5 mm. Observations on the smaller larvae were made in Syracuse dishes. Only rarely did a very active tadpole require anesthesia (1.5% urethane) in order to permit its observation. In each case anesthesia lasted only a few minutes. After the

larvae reached a length of about 30 mm., observations were facilitated by the use of a dish partially filled with blackened paraffin into which a depression had been made. This depression was of such a size and depth that when the tadpole was placed into it, the animal would lie on its side with its tail resting on the flat surface of the paraffin. The developing hind limb bud was thus brought into view without the use of anesthesia and with a minimum of manipulation.

The average water temperature was maintained at about 20°C. throughout the experiment. The larvae were maximally fed on freshly boiled leaf lettuce, that is, food was present at all times. At first feces were siphoned out and well water added once daily, but after the tadpoles reached a length of 40 mm. the water was completely changed every day. At metamorphosis the water level was reduced and stones were added, permitting the young frogs to emerge from the water.

OBSERVATIONS

The larval period dates from the commencement of independent feeding. This can be detected by the appearance of food in the gut, which is distinctly visible through the belly wall. In all cases observed, feeding began shortly after the last of the embryonic stages described by Shumway. These embryonic stages will be designated by arabic numerals. Within the subsequent larval period, twenty-five stages have been defined. They are indicated by Roman numerals to distinguish them from the embryonic stages. These larval stages have been based, so far as possible, on the appearance of new structures, or on readily detectable changes in structures already present. Characters involving pigmentation, although easily seen and often quite striking, were found to vary considerably between individuals, both as to color or intensity and as to the relative time of appearance. For this reason pigmentary changes were not considered as critical characters for staging. Critical characters will be described first in the legends accompanying the photograph of each stage. Following these, in small type, are listed some of the more variable

characters, changes which appear in the intervals between stages, as well as additional notes.

The length of each animal was measured as it attained each new stage, and the time interval between stages was recorded. These figures are presented in the following table. The length of the tadpoles, expressed in millimeters is plotted against their age in days on the accompanying graph. Points on the curve indicate the larval stages. These values are presented

TABLE 1

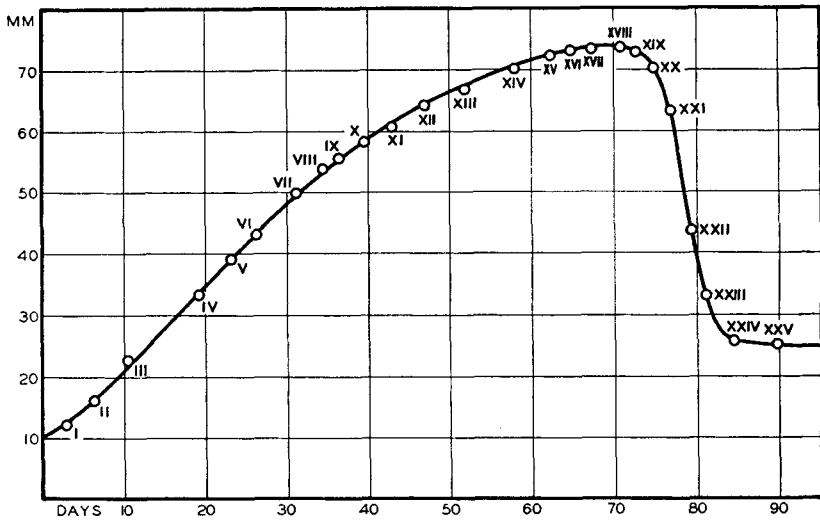
Table giving the mean length of larvae at each stage and the mean time interval between stages.

STAGE	LENGTH IN MM.	STAGE INTERVAL IN DAYS	
Embryonic	25	10.06 ± 0.4	
Limb bud stages	I	12.69 ± 0.5	3.00 ± 0.25
	II	16.55 ± 1.1	3.36 ± 0.40
	III	22.88 ± 1.64	4.28 ± 2.04
	IV	33.46 ± 2.17	8.60 ± 2.04
	V	39.19 ± 2.72	3.92 ± 1.59
Foot paddle stages	VI	43.26 ± 2.44	3.06 ± 0.81
	VII	49.91 ± 2.34	4.95 ± 0.83
	VIII	53.19 ± 2.53	2.82 ± 0.98
	IX	55.58 ± 2.75	2.44 ± 0.92
	X	58.16 ± 2.84	3.12 ± 1.11
Foot stages, or premetamorphic stages	XI	60.72 ± 3.35	3.28 ± 1.18
	XII	64.34 ± 3.35	4.06 ± 1.38
	XIII	67.49 ± 3.28	4.96 ± 1.80
	XIV	70.36 ± 3.57	6.12 ± 3.22
	XV	72.18 ± 4.05	4.46 ± 2.58
	XVI	73.06 ± 4.07	2.27 ± 1.12
	XVII	73.41 ± 3.35	2.78 ± 1.41
Metamorphic stages	XVIII	73.79 ± 2.95	3.29 ± 1.61
	XIX	73.10 ± 3.90	1.81 ± 0.76
	XX	70.28 ± 3.87	2.21 ± 0.90
	XXI	63.37 ± 5.33	1.95 ± 0.73
	XXII	43.88 ± 5.31	2.65 ± 0.69
	XXIII	32.82 ± 3.66	1.64 ± 0.63
	XXIV	25.68 ± 1.76	3.19 ± 1.21
	XXV	25.40 *	4.00 *

* Too few cases for statistical treatment.

without any thought of having them accepted as standards for the various stages, since other strains of frogs might have different growth rates and attain quite different maximum lengths, particularly if other methods of caring for the larvae were employed.

The stages lend themselves to groupings on the basis of certain characteristics. Thus, during stages I to V the limb rudiment is essentially no more than a simple bud, and this group of stages has been considered as the "limb bud" stages.



Graph showing the average length in millimeters and average age in days of larvae at each stage.

Between stages VI and X the bud is transformed first into a paddle, and then the margin of the paddle becomes indented as the several digits are marked off from each other. These stages have been called the "paddle" stages. By the end of the paddle stages the tadpole has reached 80% of its maximum length, and spontaneous and reflex activity of the hind limb has been established. The next group of stages has been termed the "foot" stages, indicating more advanced development of the hind limbs, and particularly the differentiation of

the foot. They have also been called the "premetamorphic" stages, since they precede the period of rapid transformation, or metamorphosis. They include stages XI through XVII. The "metamorphic" stages include XVIII to XXV. They are initiated by the regression of the cloacal tail piece, and end when the resorption of the tail is completed. Stage XXV represents the fully developed juvenile frog.

LITERATURE CITED

- EAKIN, R. M., AND M. HARRIS 1945 Incompatibility between amphibian hosts and xenoplastic grafts as related to host age. *J. Exp. Zool.*, vol. 98, pp. 35-64.
- ETKIN, W. 1932 Growth and resorption phenomena in anuran metamorphosis. *I. Physiol. Zool.*, vol. 5, pp. 275-300.
- EMMETT, A. D., AND F. P. ALLEN 1919 Nutritional studies on the growth of frog larvae (*Rana pipiens*). *J. Biol. Chem.*, vol. 38, pp. 325-344.
- GLÜCKSOHN, S. 1931 Äussere Entwicklung der Extremitäten und Stadieneinteilung der Larvenperiode von *Triton taeniatus* Leyd. und *Triton cristatus* Laur. *Arch. f. Entw.-mech.*, vol. 125, pp. 341-405.
- HAMBURGER, V. 1942 *A Manual of Experimental Embryology*. The University of Chicago Press.
- POLLISTER, A. W., AND J. A. MOORE 1937 Tables for the normal development of *Rana sylvatica*. *Anat. Rec.*, vol. 68, pp. 489-493.
- RUGH, R. 1934 Induced ovulation and artificial fertilization in the frog. *Biol. Bull.*, vol. 66, pp. 22-29.
- SCHREIBER, G. 1937 La definizione degli stadi della metamorfosi del *Bufo*. *Rend. R. Accad. naz. Lincei*, vol. 25, pp. 342-348.
- SHUMWAY, W. 1940 Stages in the normal development of *Rana pipiens*. I. External form. *Anat. Rec.*, vol. 78, pp. 139-144.
- 1942 Stages in the normal development of *Rana pipiens*. II. Identification of stages from sectioned material. *Anat. Rec.*, vol. 83, pp. 309-315.
- TSCHERNOFF, N. D. 1907 Zur Embryonalentwicklung der hinteren Extremitäten des Frosches. *Anat. Anz.*, vol. 30, pp. 593-612.
- WINTREBERT, M. P. 1905 Sur l'ordre d'apparition des orteils et le premier développement des membres chez les Anoures. *Compt. Rend. Soc. d. Biol.*, vol. 59, pp. 576-578.
- 1905 Essai de sériation en stades successifs des derniers temps de la vie larvaire chez les Anoures, d'après les caractères morphologiques des membres postérieurs. *Compt. Rend. Soc. d. Biol.*, vol. 59, pp. 690-692.

PLATE 1

EXPLANATION OF FIGURES

LIMB BUD STAGES

Stage I. The oral sucker elevations have completely disappeared. Four rows of labial teeth are present, one preoral and three postoral.

The pigmentation of stage 25 larvae is exclusively embryonic, being located in dark granules scattered through the epithelial cells. During the interval between this stage and stage I, chromatophores appear and become numerous on the dorsal and lateral surfaces, and extend progressively ventrad. The limb bud at stage I is visible as a faintly circumscribed elevation in the groove between the base of the tail and the belly wall. The height of the elevation is less than one-half the diameter of the disc.

Stage II. The height of the limb bud elevation (length of the limb bud) is equal to one-half of its diameter.

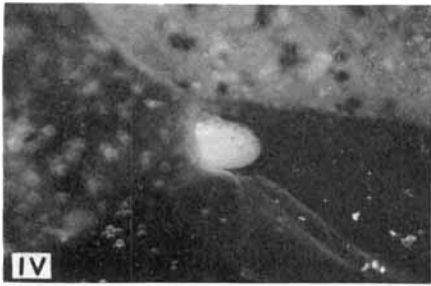
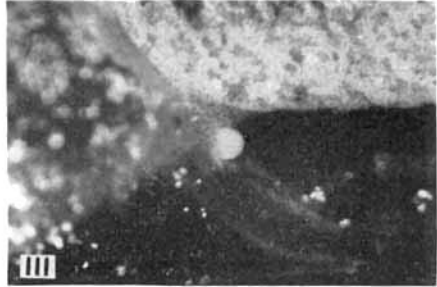
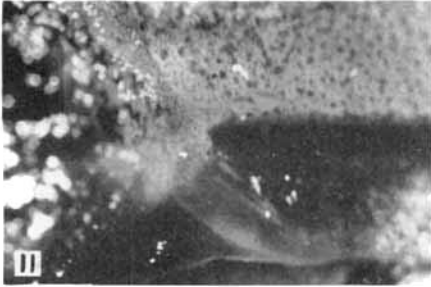
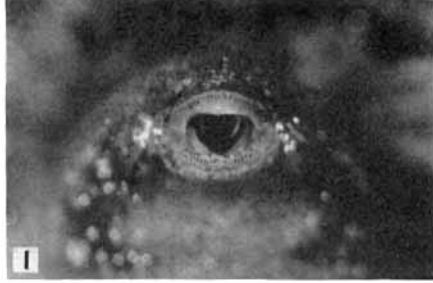
The first row of postoral labial teeth is usually divided at the middle to form a pair of crescents. On the dorsal surface of the head the lateral line system is becoming conspicuous as pigment-free lines, especially in darkly pigmented individuals. The melanophore patches covering the gill region on either side usually meet in a narrow band ventral to the heart.

Stage III. The length of the limb bud is equal to its diameter.

Stage III is followed by a period in which the limb bud grows almost equally in diameter and length. The interval between this and the following stage is comparatively long, but no obvious character appears upon which to base a division of the interval.

Stage IV. The length of the limb is equal to one and one-half times its diameter.

Stage V. The length of the limb bud is twice its diameter. The distal half of the bud is bent ventrad. There is no flattening of the tip.



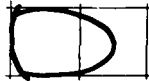
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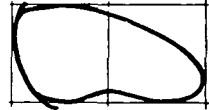
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III



IV



V

PLATE 2

EXPLANATION OF FIGURES

PADDLE STAGES

Stage VI. The distal end of the limb bud is flattened medio-laterally to form the foot paddle. There are no interdigital indentations of the paddle margin.

Stage VII. The fourth and fifth toe prominences are separated by a slight indentation of the margin of the foot paddle.

In this and the three following stages the margin of the paddle is considered indented when the region between two future toe prominences becomes slightly concave. Melanophores usually appear scattered over the bud at a much earlier stage. Soon after stage VII is reached they tend to form a distinct compact patch on the lateral surface. Guanophores appear sporadically on the limb bud.

Stage VIII. The margin of the foot paddle is indented between toes 5-4 and 4-3.

The patch of melanophores on the limb lengthens into a streak extending from above the knee bend to the foot. This character may appear as early as stage VII, or be delayed until stage X.

Stage IX. The margin of the foot paddle is indented between toes 5-4, 4-3, and 3-2.

In most cases slight spontaneous movements of the limb proper (flexion of knee and ankle) can be seen under the binocular microscope. These movements may not appear until the following stage. As a rule, shortly after spontaneous movements are observed similar movements may be elicited by stroking the limb or tail base with a hair. The melanophore streak often extends onto toes 4 and 5.

Stage X. The margin of the foot paddle is indented between all five toes. The margin of the fifth toe web (see below) is directed toward the tip of the third toe.

As toes 4 and 5 lengthen, the angle of the interdigital notch formed by the margin of the web becomes more acute. This change is used in identifying the stages from X to XIII. The half of this web adjacent to the fifth toe is, for convenience, referred to as the fifth toe web. If a line coinciding with its margin be extended, it will be seen to pass successively through the tips of toes 3, 2, and 1 and the prehallux (line AB in the drawing of stage X, and arrows in the photographs of succeeding stages).

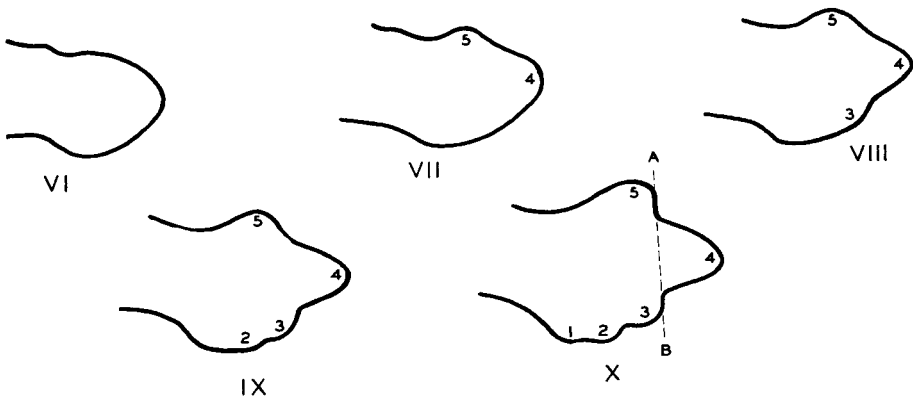
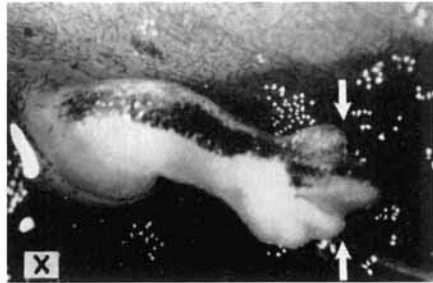
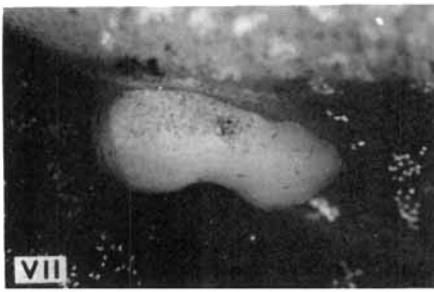


PLATE 3

EXPLANATION OF FIGURES

FOOT STAGES OR PREMETAMORPHIC STAGES

Stage XI. The margin of the fifth toe web is directed toward the tip of the second toe.

The third toe usually has a melanophore streak.

Stage XII. The margin of the fifth toe web is directed toward the tip of the first toe.

Melanophores often appear upon the second toe.

Stage XIII. The margin of the fifth toe web is directed toward the preballux.

Stage XIV. Pigment-free patches appear at the metatarso-phalangeal joints, where the proximal toe pads will later develop.

At about this same time the "brow spot" appears as a light spot in the midline slightly anterior to the level of the eyes. The nasolacrimal duct, developing as a cord of cells between the nostril and eye, is visible through the skin as a light line.

Stage XV. The proximal toe pads appear.

Proximal toe pads (subarticular pads) are the wart-like elevations which develop on the ventral surface of the toes at the metatarso-phalangeal joint (arrow).

Stage XVI. The middle toe pads appear.

This is the second row of toe pads. They form at the first interphalangeal joint on toes 3, 4, and 5.

Stage XVII. The distal toe pad appears at the second interphalangeal joint on toe four.

The cloacal tail-piece is unreduced (see photograph, and note on following stage). Reflex withdrawal of the eyeball can frequently be elicited at this stage.

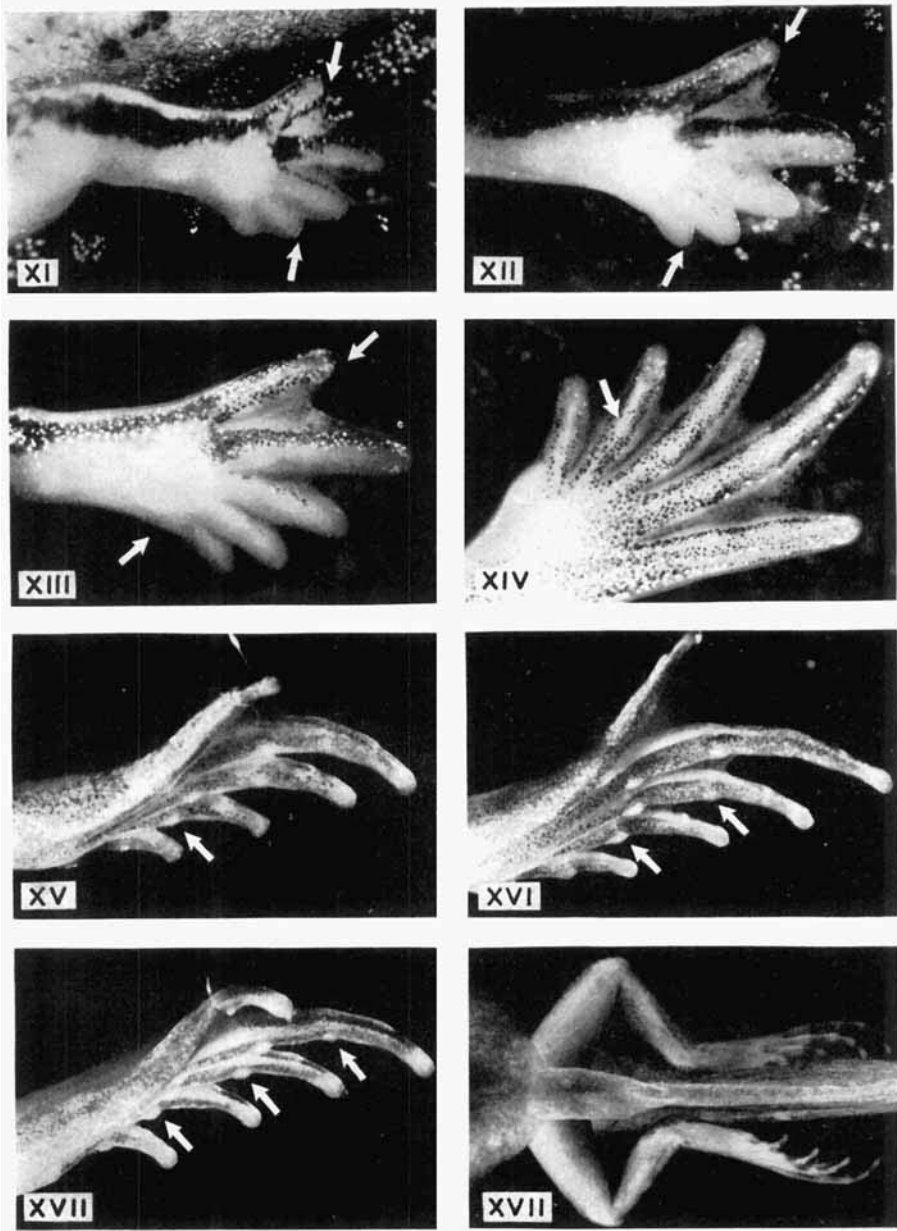


PLATE 4

EXPLANATION OF FIGURES METAMORPHIC STAGES

Stage XVIII. The cloacal tail-piece has disappeared.

In the previous stages the cloaca opens externally by an extension through the ventral tail fin called the cloacal tail-piece. It becomes progressively more prominent until stage XVII, after which it begins to regress. The complete disappearance of the tail-piece marks stage XVIII.

Stage XIX. The skin window becomes cleared.

The wall of the gill chamber at the point where the fore-legs will later protrude becomes thin and transparent. The disc-shaped clearing is referred to as the skin window (arrow).

Stage XX. One or both fore-legs have protruded.

When the fore-legs first protrude the mouth furnishings are still characteristically larval, the labial fringes being complete and the horny beak still present. During the following stage interval the horny beak is shed and resorption of the labial fringe begins at the median part of the labium.

Stage XXI. The angle of the mouth has reached a point midway between the nostril and the anterior margin of the eye.

At this point in the metamorphic period rapid changes in the mouth, pharynx and tail begin to occur. The gape of the mouth commences to widen soon after stage XX. The angle of the mouth extends progressively caudad and, when viewed from the side, passes certain land-marks which serve to indicate the attainment of stages XXI, XXII, and XXIII. The approximate extent of the gape is indicated in dorsal view by the relative position of a slight indentation of the margin of the jaw (arrow). A remnant of the labial fringe persists as a tuft at each corner of the mouth. The beginning of the resorption of the gills and the operculum is evidenced by the darkening of the opercular tissue (X). The tail assumes a darker and less transparent appearance than in the previous stage. The dorsal and ventral fins are shrunken and the length of the tail is considerably reduced, although it is still longer than the extended hind limb.

Stage XXII. The angle of the mouth has reached the level of the middle of the eye.

The extent of the gape is indicated in dorsal view by the relative position of an indentation in the margin of the jaw (arrow). The remains of the operculum are still darker in color and more restricted in extent (X). The tissue of the tail is also darker. Dorsal and ventral fins have almost disappeared, and the tail is now shorter than the extended hind limb.

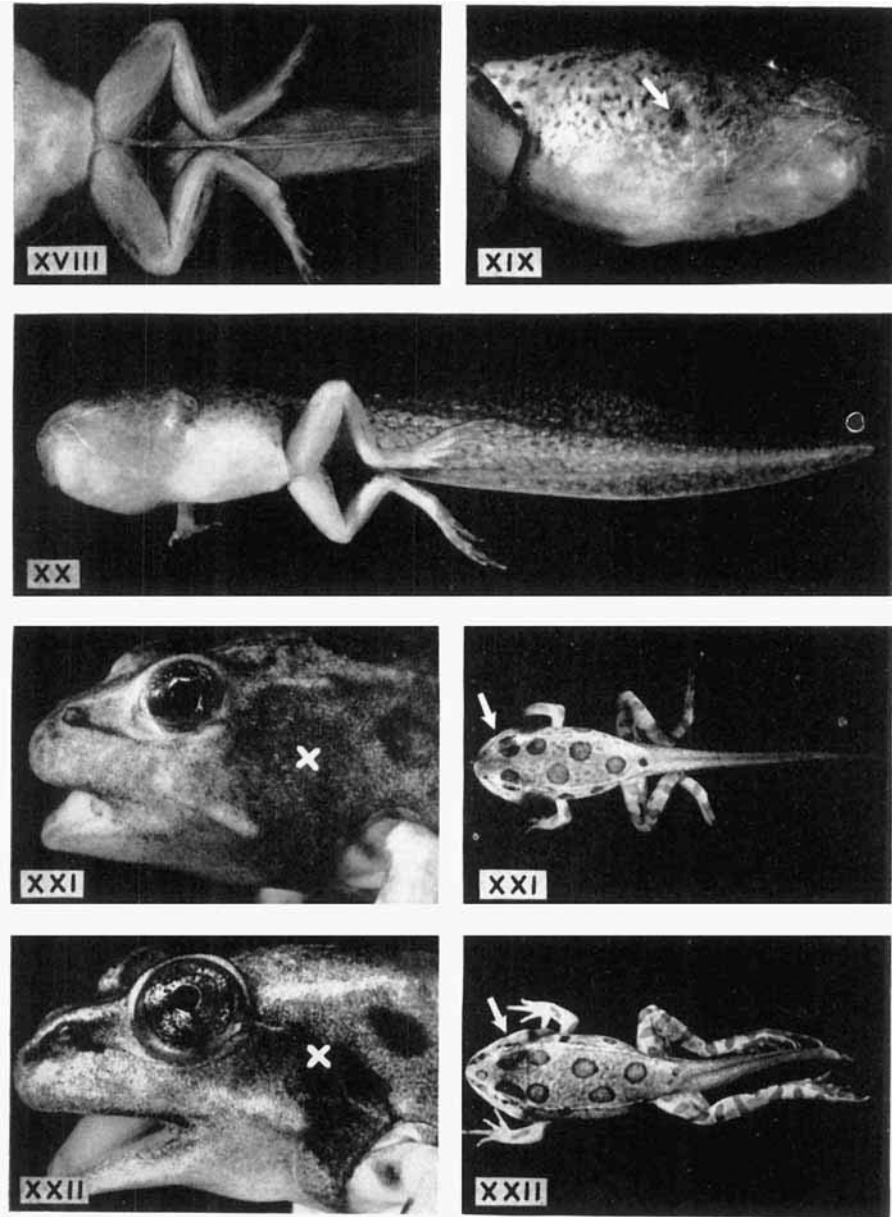


PLATE 3

EXPLANATION OF FIGURES

METAMORPHIC STAGES

Stage XXIII. The angle of the mouth has reached the level of the posterior margin of the eyeball.

No labial fringe remains. The opercular patch (X) is still further reduced.

Stage XXIV. The annular ring of the tympanic cartilage is barely perceptible under the skin.

The opercular patch has been reduced to a dark line running from the tympanic membrane to the base of the fore-leg. A stub of the tail still remains.

Stage XXV. The tail is completely gone.

The completion of resorption of the operculum and tail is indicated by the disappearance of dark tissue from the respective regions.

