

AN ABSTRACT OF THE THESIS OF

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Title THE EARLY DEVELOPMENT OF AMBYSTOMA MACRO-  
DACTYLUM BAIRD, WITH EMPHASIS ON THE LARVAL STAGES

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The embryonic and larval stages of the long-toed salamander, Ambystoma macrodactylum are described from fertilization through the development of five toes on the posterior limbs. New stage numbers are employed in the description of the hind limb.

The eggs used in the study were obtained by the injection of pituitary glands into mature female salamanders in order to stimulate them to ovulate. Fair numbers of eggs are secured in this manner if the salamanders are injected within four months after being collected. However, if injected four and one-half to five months after collection, they are found to be non-productive.

The embryonic development rate of Ambystoma macrodactylum appears to be slower than northern forms such as A. tigrinum, A. jeffersonianum, and A. maculatum but faster than A. opacum, a more southern form. However, the range of A. macrodactylum extends farther north than any of the others.

A comparison of larval development as length increases shows that A. macrodactylum reaches a state of full development with respect to limb development at a lesser total length than do A. opacum and A. jeffersonianum but at a greater length than does A. maculatum.

Pigmentation in laboratory-reared larvae is found to be lighter in color than that exhibited by A. macrodactylum larvae collected in the field. However, the same general pattern remains constant.

The use of the number of gill rakers in determining the species of a larval ambystomatid is found to be unreliable unless the stage of the larva is known.

The correlation of body dimensions at various stages of development to geographic distribution and to other species for identification purposes is not feasible until similar information for other locations and other species is obtained.

THE EARLY DEVELOPMENT OF AMBYSTOMA  
MACRODACTYLUM BAIRD, WITH EMPHASIS  
ON THE LARVAL STAGES

by

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THE EARLY DEVELOPMENT OF AMBYSTOMA  
MACRODACTYLUM BAIRD, WITH EMPHASIS  
ON THE LARVAL STAGES

INTRODUCTION

The description of amphibian development has received much attention, particularly since the turn of the century. The majority of work in this field has dealt with anuran species, and urodele development has, in general, been neglected. It was not until comparatively recently that an interest in urodele natural history began to develop. Gage (12), in describing the life history of the vermilion-spotted newt, Triturus viridescens, gave gross descriptions of larval changes based upon observations of specimens taken from ponds at various times of the year.

Jordan (16), also studying Triturus viridescens, combined some life history data with detailed descriptions of the egg, blastopore formation, fate of the blastopore, etc., but contributed very little about growth rates and descriptions of external changes during development.

During the late 1920's, R. G. Harrison (15) produced his excellent drawings of developmental stages for Ambystoma punctatum (syn. maculatum). In 46 stages, Harrison depicts the development of this animal from the fertilized egg to the actively feeding stage. This was, and still is, the best guide to salamander development

available. Although unpublished, it has been widely distributed through the courtesy of Dr. Harrison and, since his death, Yale University.

Dempster (8) collected embryos and larvae of A. maculatum at weekly intervals during the spring and summer months and examined relations between length and weight as the animals developed from the very early stages until metamorphosis.

The effectiveness of both heteroplastic and homoplastic pituitary implants in inducing ovulation among amphibians was discovered almost simultaneously by several workers in the late 1920's and early 1930's. Wolf (34) experimented with homoplastic transplants in Rana pipiens, and Adams (1, 2) transplanted Rana pipiens pituitaries into Triturus viridescens and later transplanted pituitaries from Bufo terrestris into T. viridescens, in both cases successfully causing ovulation to take place. During the following period, amphibian embryologists were able to do much more exact work. No longer having to rely on obtaining eggs from the field, they were able to determine exactly when fertilization had taken place and the exact parents of the eggs.

Previous contributions thus enabled various workers to produce developmental descriptions for each of several anuran species. However, urodeles were neglected until Anderson (3) described the normal development of Triturus pyrrhogaster, correlating external and

internal development by making histological sections of each stage.

To this writer's knowledge, there has since been no other work of this kind done with salamanders, although periodic collections from the field have yielded a general picture of larval development for several species.

This study was intended to provide a description of the early development of Ambystoma macrodactylum with emphasis upon the post-hatching stages. The earlier stages are similar to those of species previously worked upon. Growth rate tables are included. Development of the hind limb, which to this writer's knowledge has not been previously described for any salamander species, is also included.

It is hoped that information from this study may be used in comparing the larvae of Ambystoma macrodactylum with larvae of sympatric salamander species, and thus eventually, when like information is available for the other species, provide a guide for the biologist in the field.

## METHODS AND MATERIALS

The adult animals used in this study were collected approximately three miles east of Corvallis, Benton County, Oregon. They were obtained during the fall and winter of 1964 and early 1965 while they were migrating to their breeding ponds. (It was noticed, however, that many of the animals observed in this migration were small, immature specimens, particularly in the early fall.) Most of the sexually mature animals migrate during November and December, with a few appearing in January and early February. Gravid females and males with swollen vents were considered sexually mature.

In the laboratory, the animals were placed in Mason jars with screen-wire tops in which approximately one-fourth inch of water and two or three moist paper towels had been placed. The animals were then stored in refrigerators at a temperature of 9.6° C. until used.

In order to induce ovulation in the female salamanders, pituitary glands from female Rana pipiens were injected into the abdominal cavity. The method for extracting the pituitaries from the donors is described by Rugh (26, p. 102-110).

During this series of experiments, a male A. macrodactylum was injected with two female Rana pipiens pituitaries. He was then

placed in a container of filtered water with a female salamander in the hope that natural egg-laying fertilization would take place and thus a better survival percentage might be achieved. No positive results were obtained.

In this study, it was found that the injection of one female Rana pipiens pituitary followed in 24 hours by a similar injection was sufficient to induce ovulation in female Ambystoma macrodactylum. However, Reese, Kilmer, and Leveque (24) indicated that in some cases up to three pituitaries (two female and one male) were needed.

Following the injections, the female salamanders were placed in finger bowls with a moist piece of paper towel in the bottom. The finger bowls were then placed in a 9.6° C. refrigerator. Within two or three days after the second injection, ovulation was complete as was indicated by the presence of a few eggs in the bottom of the containers.

In the early stages of the study, the eggs were "stripped" from the females into a sperm suspension. This was prepared by macerating the testes of a mature male Ambystoma macrodactylum into a ten percent Holtfreter's solution. However, due to adverse effects upon the eggs resulting from this treatment, another technique was employed in later experiments. This consisted of injecting the sperm suspension directly into the cloacal opening of the ovulating female, placing her in a finger bowl about half full of charcoal filtered

water, and allowing the eggs to be deposited in the water.

Some of the eggs thus fertilized were started at 9.6° C., others were started at 13.8° C. The objective there was to obtain some information as to the effect of temperature upon developmental time. Those embryos started at 13.8° C. were observed only through the free-swimming or hatching stage. Those placed in the 9.6° C. refrigerator were observed through hatching at this temperature, then were placed in the 13.8° C. refrigerator for observation beyond the hatching stage in order to increase the rate of growth.

Unfertilized eggs were removed and the remaining embryos were checked frequently, particularly during the early stages. After hatching, the larvae were transferred to 12 inch × 6 inch × 2 inch enamel trays, containing 1.5-2.0 liters of filtered water. The water was changed daily and fresh food was added with each change of water. The food consisted of Daphnia and small Enchytraeid annelids.

The developing embryos and larvae were compared to Harrison's (15) developmental series for Ambystoma punctatum up to stage 46 (The actively feeding stage) which is as far as the Harrison series goes. New stage numbers were employed for development beyond stage 46, including the development of the hind limb.

The free-swimming larvae were measured frequently. Measurements to the nearest 0.5 millimeter were taken: total length, measured from the tip of the snout to the tip of the tail; snout-vent

length, measured from the tip of the snout to the middle of the cloacal opening; head width, measured across the widest part of the head as seen from the dorsal side; tail depth, measured from the top of the dorsal fin at its deepest point to the bottom of the tail fin at the level of the cloaca.

Measurements of the developing hind limb to the nearest 0.05 millimeter were taken when this stage of development was reached; total length of the hind limb, measured from the juncture of the limb and the body of the larvae to the tip of the longest toe. The length of each toe of the hind limb was also measured: first toe,<sup>1</sup> measured from the juncture between it and the second toe; second toe, measured from the juncture between it and the first toe; third toe, measured from the juncture between it and the second toe; fourth toe, measured from the juncture between it and the third toe; fifth toe, measured from the juncture between it and the fourth toe. In those cases where one limb bud was more developed than the other on the same animal the most advanced was measured.

During the periods they were being measured, the larvae were anesthetized with "M. S. 222" (Sandoz Chemical Co., N. Y. C.).

Drawings of several of the earlier stages were made and descriptions of those stages differing from equivalent stages of

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<sup>1</sup>"First" toe refers to the ventral-most toe when hind limb is adpressed to side of larvae.

Ambystoma punctatum were recorded. Stages 40 (hatching) through 46 were drawn and the pigmentation was described. A drawing of the fully developed larvae was also made. Following the completion of the study, the larvae were preserved.

It was found that the larvae became infected with a fungus, particularly during the development of the hind limb, which caused the limbs to assume a "fuzzy" appearance. A secondary effect was the appearance of small tubercles over most of the body. In order to combat this fungus, "Fungi-mycin" (Lambert-Kay, Inc., Los Angeles) was applied. It was effective to some extent in arresting the fungus, but did not cure it.



## OVULATION AND FERTILIZATION

Gravid female Ambystoma macrodactylum were readily stimulated to ovulate following the method described in the previous section, and good numbers of eggs were generally obtained. However, after fertilizing the eggs and placing them in the culture medium (ten percent Holtfreter's solution), it was noticed that a large number of them, including their envelopes, appeared to collapse. Initially it was thought that this was due either to the osmotic influence of the culture medium or possibly to temperature. However, after experimenting with several concentrations of Holtfreter's solution, artificial pond water (containing NaCl, KCl,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and  $\text{N}_2\text{HCO}_3$ ), and charcoal-filtered water, no definite conclusions could be drawn. It was ultimately decided to use filtered water in the remaining experiments, because it was assumed to be closer to the natural environment in the properties it possessed.

Average temperatures of 9.6° C. and 13.8° C. appeared to have no adverse effect on the eggs. However, none survived above 13.8° C. Temperatures below 9.6° C. were not tried.

Experiments were repeated in several cases, using the same culture medium at the same temperature. Similar results were never obtained, in terms of egg survival.

Table 1 shows the number of females injected with pituitary

Table 1. Ovulation and Fertilization Data.

Animal No.	Date Collected	Pituitary Treatment	Date Fertilized	Total No. of Eggs	No. and % Fertilized
1	Nov. 11	2 R. p.	Jan. 17	80	20(25%)
2	Nov. 11	2 R. p.	Jan. 17	64	0
3	Nov. 4	2 R. p.	Jan. 17	71	19(26.7%)
4	Nov. 24	2 R. p.	Jan. 23	47	10(21.3%)
5	Nov. 25	2 R. p.	Jan. 23	0	0
6	Nov. 25	2 R. p.	Jan. 23	61	17(27.9%)
7	Nov. 22	2 R. p.	Jan. 29	20	5(25%)
	Nov. 22	2 R. p.	Jan. 30	42	17(40.5%)
	Nov. 22	2 R. p.	Jan. 31	25	4(16%)
8	Nov. 22	2 R. p.	Jan. 31	82	35(42.7%)
9	Nov. 25	2 R. p.	Feb. 11	86	24(27.9%)
10	Nov. 25	2 R. p.	Mar. 5	79	28(35.4%)
11	Nov. 24	2 R. p.	Mar. 5	28	2(7.14%)
12	Nov. 25	2 R. p.	--	0	0
13	Nov. 25	2 R. p.	--	0	0
14	Nov. 24	2 R. p.	Mar. 5	267	40(14.9%)
15	Nov. 10	2 R. p.	Mar. 16	65	15(23.1%)
16	Nov. 10	2 R. p.	Mar. 16	59	14(23.73%)
	Nov. 10	2 R. p.	Mar. 16	21	7(33.33%)
17	Nov. 24	2 R. p.	--	0	0
18	Nov. 24	2 R. p.	--	0	0
19	Nov. 22	2 R. p.	Mar. 16	26	8(30.75%)
20	Nov. 4	2 R. p.	Mar. 16	123	30(24.69%)
	Nov. 4	2 R. p.	Mar. 16	17	5(29.4%)
21	Nov. 4	2 R. p.	Mar. 17	221	39(17.65%)
22	Nov. 25	2 R. p.	Apr. 1	9	0
23	Nov. 25	4 R. p. + 2 <u>Taricha</u> (Apr. 6-May 12)	--	0	0
24	Nov. 25	3 R. p. + 2 <u>Taricha</u> (Apr. 6-May 12)	--	0	0
25	Nov. 25	3 R. p. + 2 <u>Taricha</u> (Apr. 6-May 12)	--	0	0
26	Nov. 25	4 R. p. + 2 <u>Taricha</u> (Apr. 6-May 12)	--	0	0
27	Nov. 10	4 R. p. (Apr. 19-27)	--	0	0
28	Nov. 24	3 R. p. (Apr. 23-27)	--	0	0
29	Nov. 25	3 R. p. (Apr. 23-27)	--	0	0

glands, the date the animals were collected, the number and type of pituitaries each was given, the date the eggs were fertilized (if any were obtained), the total number of eggs obtained from each female, and the percentage which were fertilized. The table shows rather definitely that pituitary injections had no effect upon those animals which were injected five to six months after being collected. After this length of time, the animals evidently resorb their eggs. Because of the variance in the physiological states of the females, the number of eggs obtained varied considerably and few conclusions are possible.

Of the eggs which were fertilized, only those which were fertilized on January 23 and March 17 lived past the early cleavage stages.

## DESCRIPTION OF STAGES

Although the developmental stages of Ambystoma macrodactylum embryos are very similar in appearance to those drawn by R. G. Harrison (15) for Ambystoma punctatum, there are variations which occur, especially in the stages just prior to hatching.

Figures 1, 2 and 3 illustrate the external features of Ambystoma macrodactylum from fertilization to hatching (stage 40). All of the intervening stages are not represented; however, the stages drawn are intended to aid the reader in tracing the sequence of development.

Stages 1-39 comprise the embryonic period and stages 40-55, which include the development of the hind limb, comprise the larval stages. A brief description of each stage is given and the variations of this species from the development of Ambystoma punctatum are included.

Stage 1: The fertilized egg rotates so that the animal or pigmented hemisphere is uppermost. The eggs when "stripped" from the female are deposited as a mass in most cases, but occasionally are deposited in what appears to be a double row of two strings joined by a common gelatinous mass. The egg has two distinct envelopes. The eggs were not measured in the living state, but Stebbins (28, p. 37) measured "wild" eggs as follows: 12-17 mm. to outer surface of outer capsule; 6-7 mm. to outer surface of inner envelope; ovum,

2.5 mm. in diameter. In the present study, when measured after being preserved in formalin, the ovum averaged 1.5 mm. in diameter, and the outer envelope 10.5 mm.

Stage 2: First cleavage, or two cell stage.

Stage 3: Second cleavage, or four cell stage.

Stage 4: Third cleavage, or eight cell stage.

Stage 5: Fourth cleavage, or 16 cell stage.

Stage 6: 32 cell stage.

Stage 7-8: 64 cell stage through early blastula.

Stage 9: Middle blastula.

Stage 10: Early gastrula; involution of dorsal lip of blastopore.

Stage 11: Middle gastrula; blastopore large.

Stage 12: Late gastrula; yolk plug stage.

Stage 13: Blastopore closed to slit.

Stage 14-15: Early neural plate.

Stage 16: Later neural plate.

Stage 17: Late neural folds.

Stage 18-19: Neural folds fusing.

Stage 20: Neural tube just formed.

Stage 21: Optic vesicle appears.

Stage 22: Beginning of cephalization; somites begin to appear.

Stage 23: Pronephros appears as a small outpouching to median-dorsal area of embryo.

Stage 24: Mandibular arch appears.

Stage 25: More somites become visible.

Stage 26: Early tail bud stage; gills beginning to form and otocyst appears as a small dark spot anterior and dorsal to gill region.

Stage 27-28: Further development of the gills and tail bud, with the earliest appearance of the mouth and anus. In A. macrodactylum, the embryos appear thinner than are Harrison's drawings (15) of A. punctatum.

Stage 29-30: The nose is becoming differentiated and tail bud is increasing in length.

Stage 31: Tail fin appears.

Stage 32-33: Further development of tail bud.

Stage 34: First appearance of balancers.

Stage 35: Gills are beginning to differentiate into three distinct pairs. Small black melanophores beginning to appear.

Stage 36: Forelimb buds are beginning to appear. Balancers and gills are longer. Melanophores definitely visible, concentrated along tail fin and dorsal part of head, but beginning to invade tail fin.

Stage 37: Mouth is clearly visible, melanophores beginning to encroach on the gills, along approximately one-half the length of them.

Stage 38: Gill filaments begin to appear as small bumps on the

gills; melanophores well developed, forming two distinct bands on either side of the dorsal fin; melanophores scattered along sides and dorsal fin, which is about 1 mm. high at this point.

Stage 39: Gill filaments become more numerous but there are not as many on A. macrodactylum as there are in Harrison's (15) drawings of A. punctatum; melanophores well developed on head, except for a clear area dorsal and posterior to each eye. Dorsal-lateral ground color is a light yellow-tan. Many small yellow chromatophores are evident, especially on the tail fin, and a few encroaching on the eye. The eye appears non-functional and dull. Ventrally, the embryos are much lighter, probably due to the yolk. There is considerable variation in the amount of pigmentation among individuals. In A. macrodactylum, the posterial portion of the digestive tract and the anal region is strongly differentiated in this stage, whereas in A. punctatum this occurs in stage 40.

The rate of development at two temperatures up to stage 39 is compared in Figures 5 and 6.

Stage 40: This is the hatching stage and is characterized by increased numbers of gill filaments, flattening of the fore limb bud, and greater development of the balancers, which serve to keep the larvae in an upright position when resting.

Stage 41: Much the same as stage 40 except for an indentation in the fore limb bud which signifies the beginning of digits. It was

during this stage that a few of the larvae were observed to snap at Daphnia placed in the container, although it is doubtful that they were actually feeding.

Stage 42: Characterized by a deepening of the indentation in the fore limb. Three to four filaments are present on the posterior most gill.

Stage 43-44: Deepening of indentation in fore limb; presence of feces in containers indicates feeding is taking place. There are 10-12 filaments per gill. Melanophores and yellow chromatophores appear to be in the same epidermal layer. The eye appears to be covered with bright iridophores. The ventral surface of the animal has no pigment, but dorsally there are two bands of melanophores, one on either side of the fin, which extend from the head region back to the end of the tail. Lateral surfaces are sparsely speckled with melanophores. Size of melanophores varies and there are minor differences in pattern. Balancers are beginning to disappear.

Stage 45: There are two toes and a bud on the fore limbs. Balancers disappear completely during this stage. All animals have begun feeding.

Stage 46: Three toes present on forelimbs.

The following stages cover the development of the hind limb until five toes are present. The stage numbers assigned are 47-55 in order to be continuous with Harrison's (15) 46 stages.



Stage 47: Hind limb bud from first appearance to 1 mm. in length. The fourth toe on the fore limb begins to appear during this stage. During this stage the variation in pigmentation among animals appears to decrease and they begin to appear more uniform. This condition is probably due to the fact that they have been raised in similar environments of featureless white trays.

Stage 48: Hind limb bud is 1-2 mm. long. There is a uniform sprinkling of small melanophores over the dorsal surface of the head and a more concentrated sprinkling along either side of the fin. Very small melanophores are sparsely distributed over the lateral surfaces. Around the dorsal and ventral edges of the fin are larger, more spreading melanophores. The fin also has a liberal sprinkling of large yellow lipophores.

Stage 49: Hind limb bud has widened at end and a slight indentation has formed.

Stage 50: Two toes<sup>2</sup> are present on hind limb. Blood circulation in the limb is now visible and melanophores are beginning to invade it.

Stage 51: Three toes present on hind limb. 24-26 gill filaments are present on middle gill. The gill filaments are flattened laterally and the direction of blood flow is through the ventral

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<sup>2</sup>"Buds" are those protrusions which were not long enough to be measured. "Toes" are those which were measurable.

capillary to the end of the filament and back to the main gill arch via the dorsal capillary.

Stage 52: Three toes and a bud present on hind limb. Silver iridophores are scattered along lateral surfaces and are more concentrated on dorsal surfaces of gills.

Stage 53: Four toes present on hind limb.

Stage 54: Four toes and a bud present on hind limb.

Stage 55: Five toes present on hind limb.

The order of development of the toes of the hind limb, in order of appearance, is: 1 (the ventral-most toe when limbs are adressed to side of larva), 2, 3, 4, and 5 (the dorsal-most).

Table 2 shows the mean of measurements taken from stage 40 to stage 55. Measurements of head width, tail depth, and snout-vent length were not taken for stages 40-41 owing to the small size of the individuals and difficulties encountered in anesthetizing them. It should be noticed that the ratio between head width and tail depth measurements at the stages measured appear to be very close to a 1:1 ratio. This is even more noticeable when examining the measurements of the individual animals (not shown).

Table 3 shows the change in the number of animals which were at each stage from 40 to 55 and the progression as the number of days from fertilization increases.

Table 4 shows the progression in the average length of the hind

Table 2. Measurements Obtained for Each Stage of Development for Stages 40-55.

Stage	Head Width in mm.		Total Length in mm.		Tail Depth in mm.		Snout-vent Length in mm.		No. of Measurements
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	
40			9.5-11.0	10.00					10
41			10.0-14.0	12.50					10
42	2.0-2.5	2.25	10.5-14.0	12.74	2.0-3.0	2.43	6.5-8.0	7.33	17
43	2.5-3.0	2.64	12.0-14.0	13.17	2.0-3.0	2.50	7.0-8.0	7.38	24
44	2.5-3.0	2.83	12.5-15.0	13.96	2.0-3.0	2.67	7.0-8.5	7.81	35
45	2.5-3.5	2.89	12.5-16.5	14.30	2.0-3.5	2.74	7.5-9.5	8.48	27
46	3.0-3.5	3.28	14.0-18.0	16.28	3.0-3.5	3.16	9.0-10.0	9.47	16
47	3.0-4.0	3.72	16.0-22.5	19.20	3.0-4.5	4.34	9.0-12.5	10.96	48
48	4.0-5.0	4.45	20.0-25.5	22.60	4.0-5.0	4.48	11.5-14.5	13.04	24
49	4.5-5.0	4.71	23.0-27.0	25.57	4.0-5.5	4.90	13.0-15.0	14.50	15
50	5.0-5.5	5.11	26.0-30.0	27.56	4.5-5.5	5.00	15.0-16.5	15.31	8
51	5.0-6.0	5.13	27.0-32.5	29.55	5.0-6.0	5.30	15.0-18.0	16.58	31
52	5.0-6.0	5.61	30.5-37.5	33.46	5.0-6.5	5.89	16.5-19.5	18.84	28
53	5.5-6.0	5.8	31.5-39.0	34.45	5.5-6.5	5.90	18.0-20.0	19.40	10
54	5.5-6.5	5.93	31.5-42.5	36.97	5.5-6.5	6.15	19.5-22.5	20.90	34
55	5.5-6.5	6.00	31.5-43.0	37.13	5.5-7.0	6.20	20.0-22.6	21.50	10



Table 4. Average Growth of the Hind Limb as the Number of Days From Hatching Increases.

Days Since Hatching	Hind Limb Bud	1st toe	2nd toe	3rd toe	4th toe	5th toe
48	b(25%)					
50	b(46%)					
52	b(91%)					
54	b(91%)					
56	b(91%)					
58	.75mm					
60	.93					
62	1.15					
64	1.30					
66	1.32	.04mm	.02mm	b(10%)		
68	1.69	.28	.25	.04mm		
70	1.97	.29	.48	.10		
72	2.17	.29	.57	.17	b(10%)	
74	2.50	.40	.78	.39	b(10%)	
76	2.94	.44	.82	.56	.06mm	b(10%)
78	3.32	.52	.95	.74	.18	.01mm
80	3.63	.53	1.01	.85	.20	.02
82	4.14	.53	1.13	1.03	.34	.02
84	4.25	.54	1.17	1.05	.40	.07
86	4.54	.55	1.18	1.24	.56	.10
88	4.73	.56	1.25	1.24	.68	.15
90	5.14	.57	1.30	1.32	.71	.18
93	5.39	.58	1.32	1.48	.92	.33

\* b% = % of animals showing beginnings of a hind limb bud.

limb and each of the five toes as the number of days from hatching increases.

The number of animals at hatching was 39 and samples were drawn from this total for measurements. However, due to the high mortality, by stage 46 there were only 12 animals alive. From stage 46 on all animals were measured at each measuring period.

Figure 1. Embryonic Stages 1, 2, 3, 8, 13, 14, 18, 20, 23

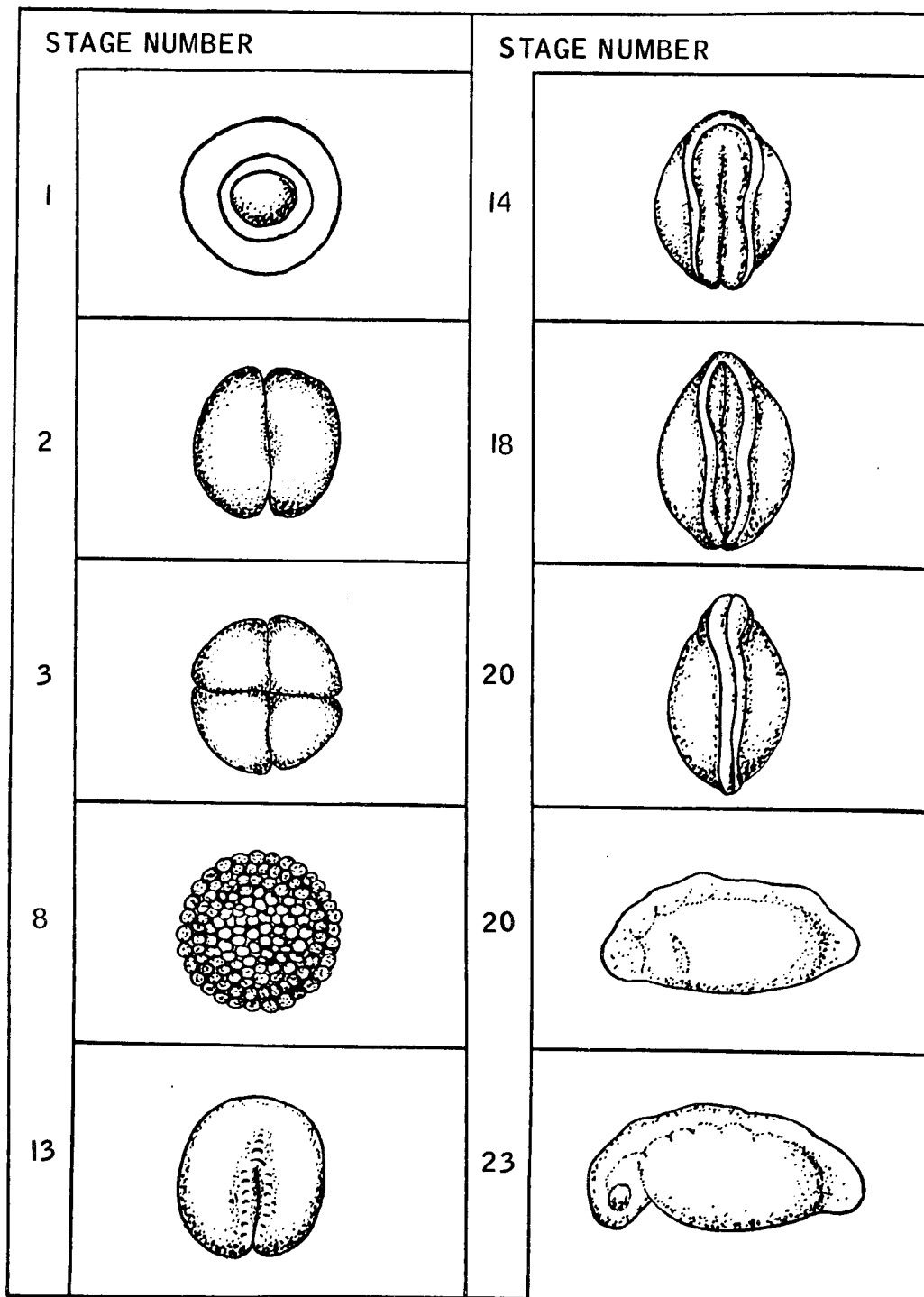


Figure 2. Embryonic Stages 27, 31, 32, 34, 35, 37

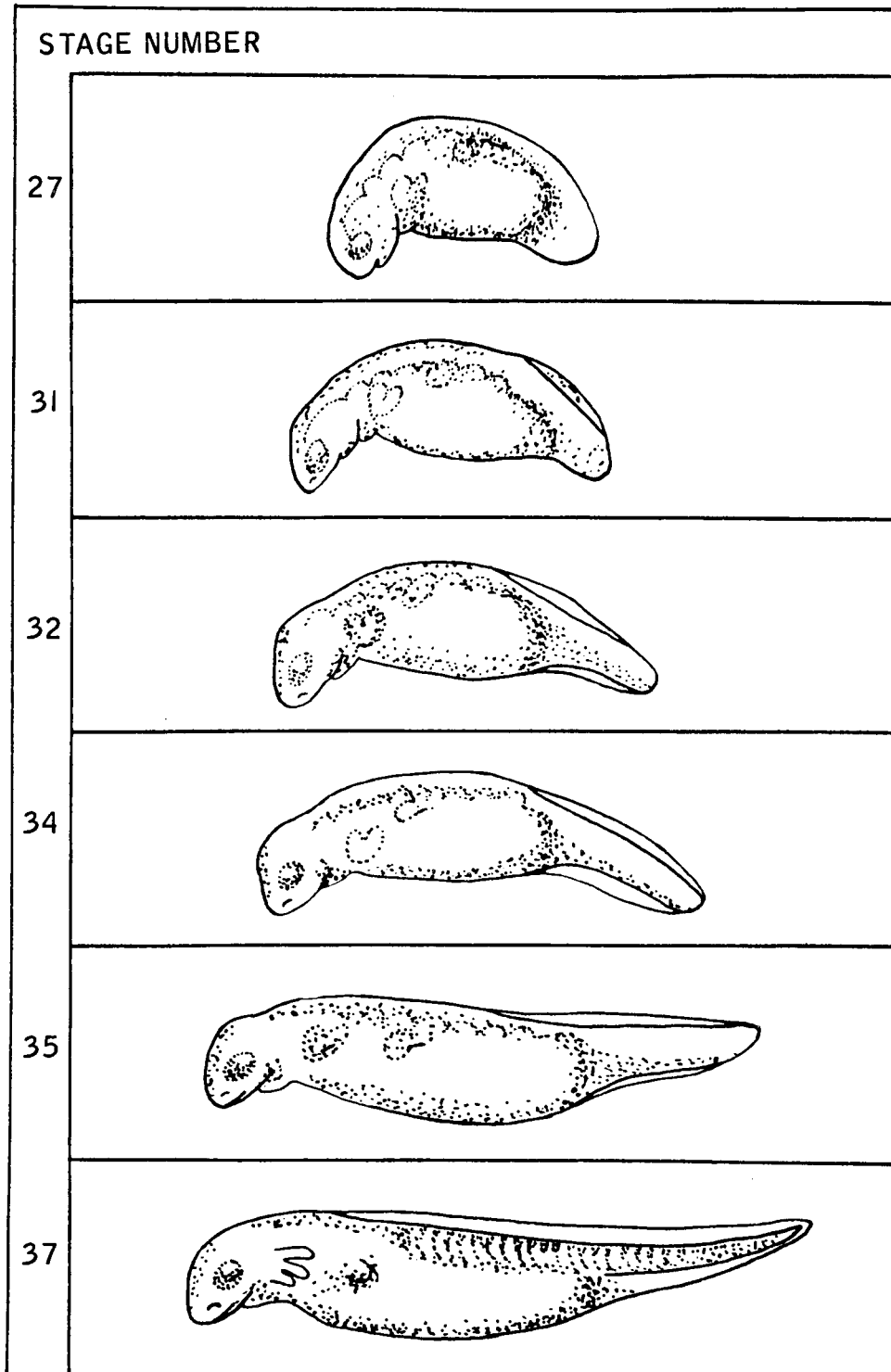




Figure 3. Embryonic Stages 39, 40, 41, 42

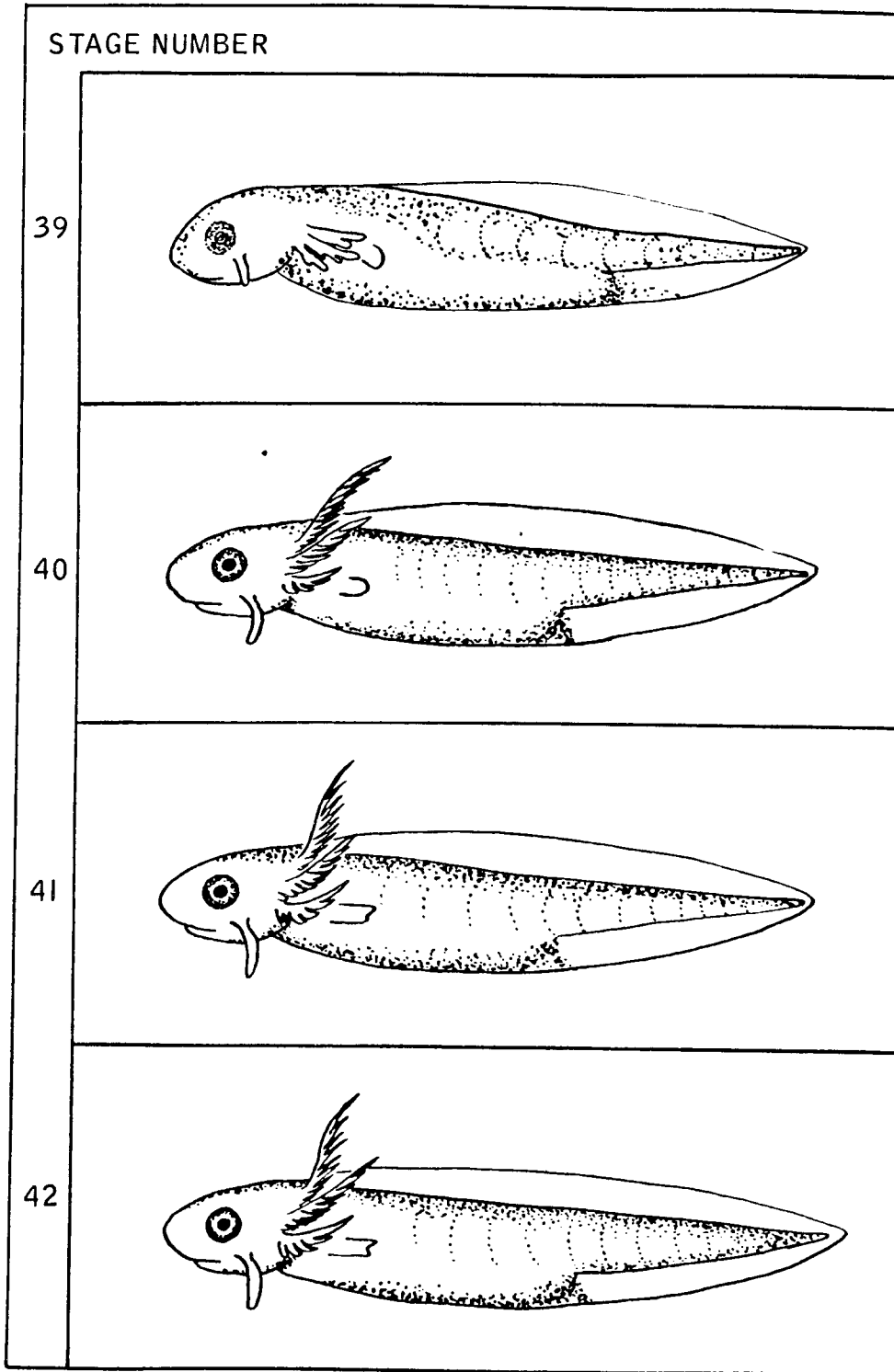
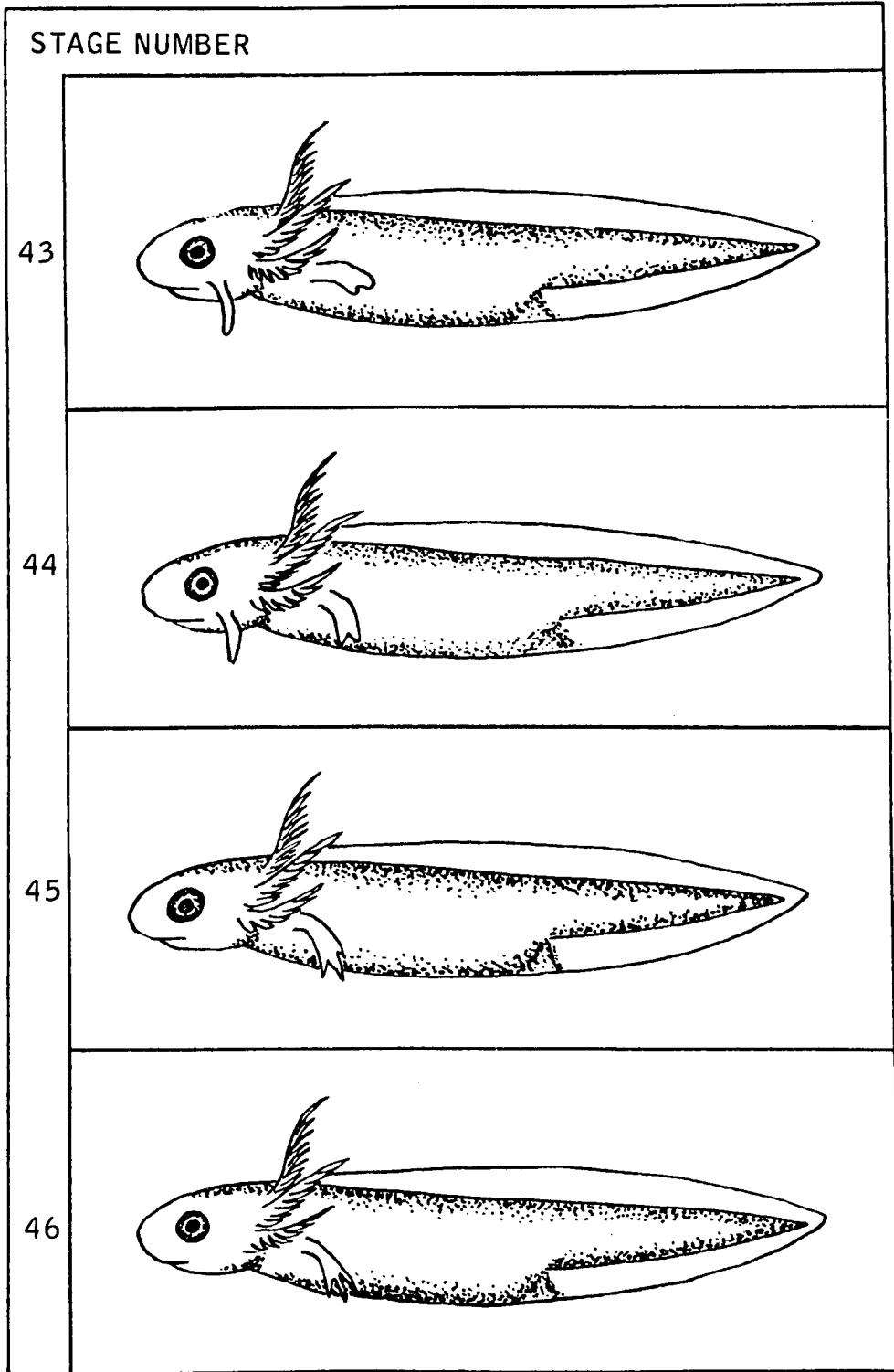


Figure 4. Embryonic Stages 43, 44, 45, 46



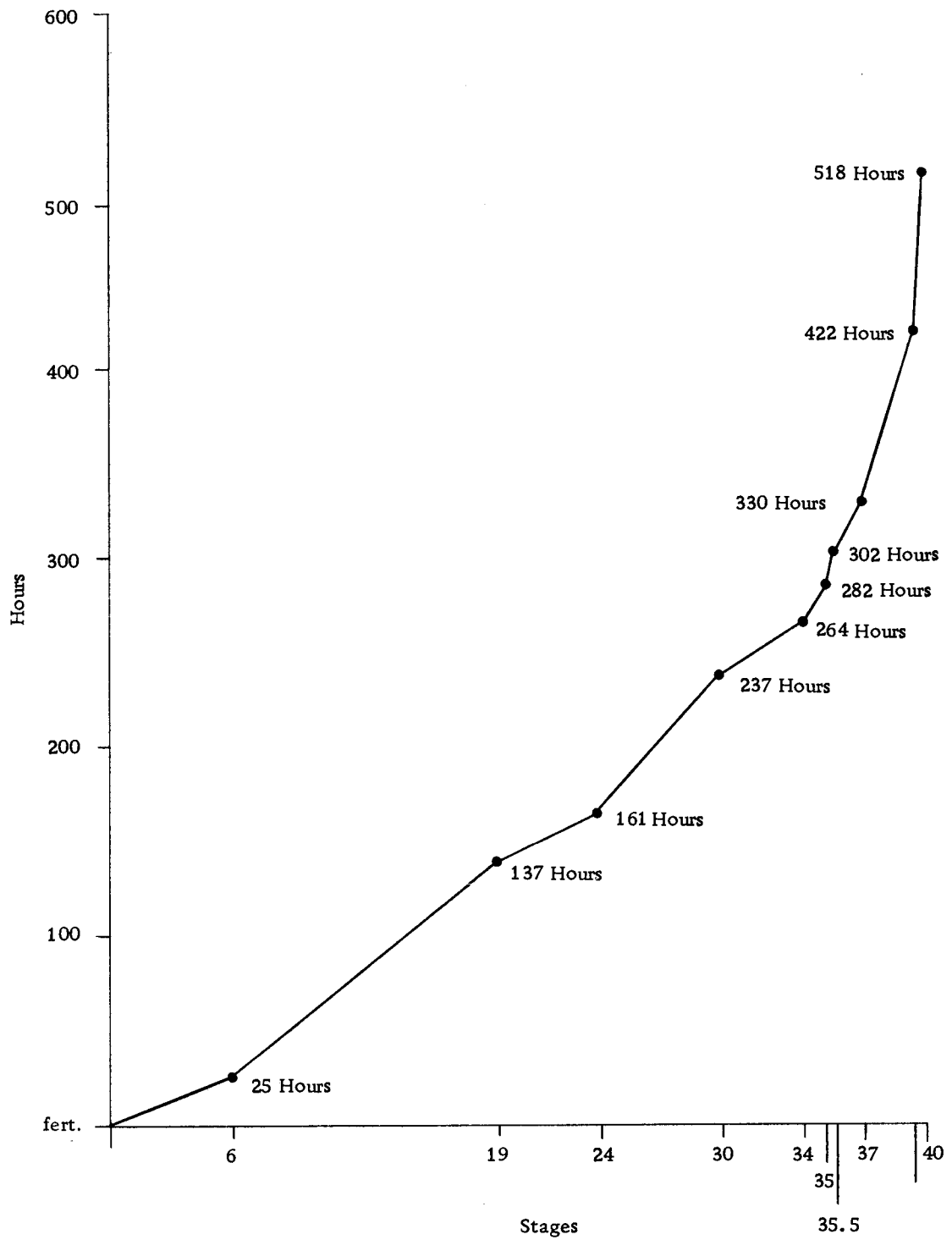


Figure 5. Developmental Rate as Hours from Fertilization Increase Stages 1-40, 13.8°C. (More complete information is given in Appendix A.)

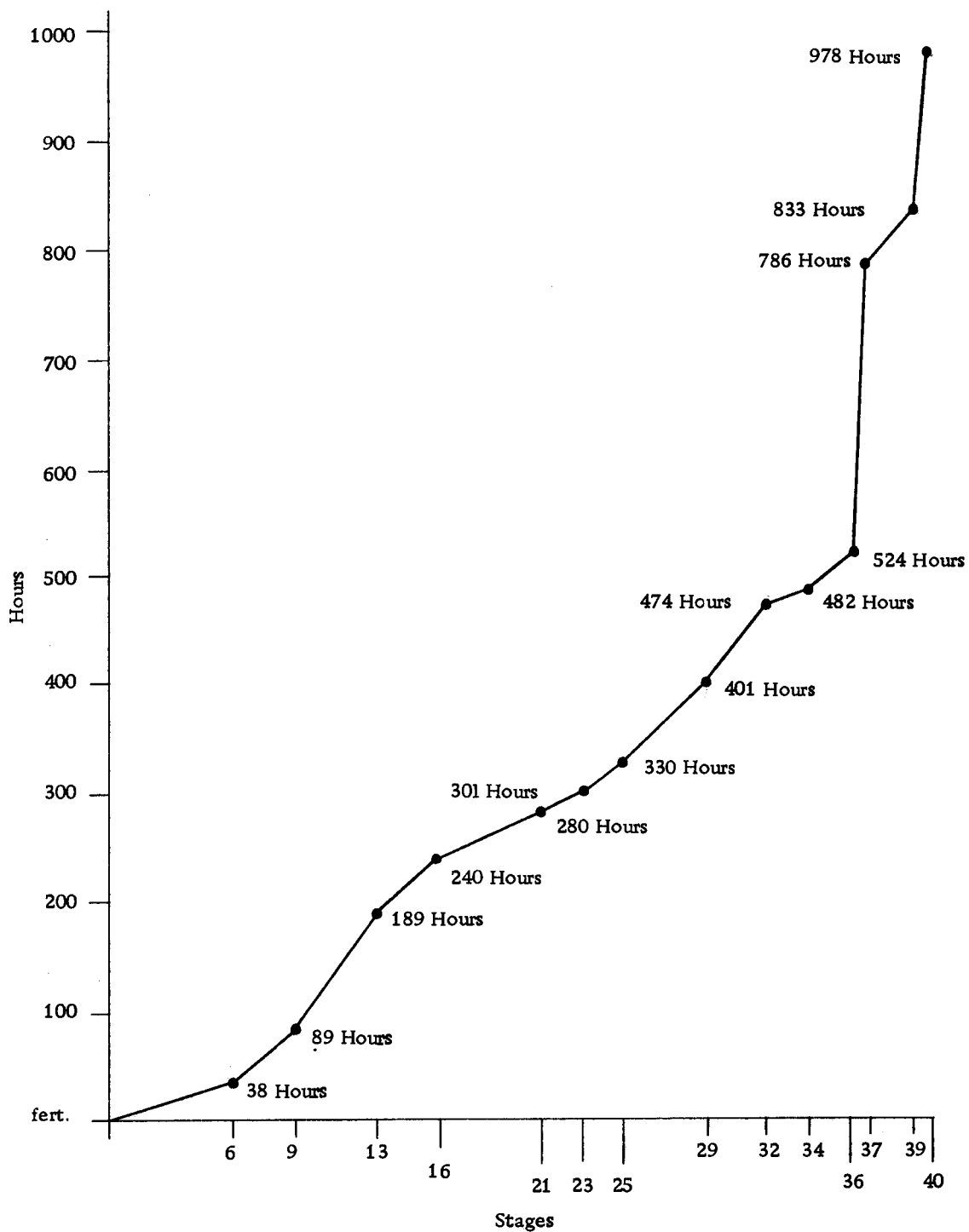


Figure 6. Developmental Rate as Hours from Fertilization Increase Stages 1-40, 9.6° C. (More complete information is given in Appendix B.)

## DISCUSSION

Embryonic Development

Table 5 is a chart from Moore (20) showing the rate of early development in four species of Ambystoma at a temperature of 19.9° C. Studies of A. macrodactylum at comparable temperatures (18° C. and 21° C.) have been made by other workers (24) and I have included their findings in the chart. Also included in Table 5 are the results from the present study, comparing the rate of development at 13.8° C. and at 9.6° C.

Moore (20) claims that "those species which lay their eggs early in the season when it is cold, and are more northern in their distribution, develop more rapidly than those which spawn later, and which may be considered southern species." The species in Table 5 are listed in order from the highest rate of development to the lowest: first is A. tigrinum; second, A. jeffersonianum; third, A. maculatum; and fourth, A. opacum which has the lowest.

At comparable temperatures, A. macrodactylum appears to be very similar in developmental rate to A. maculatum. If Moore's (20) generality is to hold, then A. macrodactylum is a more southern species and breeds later in the season. However, the opposite is true. The range of A. macrodactylum extends well into British

Table 5. Comparison of Early Developmental Rates in Hours for Five Species of Ambystoma.

Stage	<u>A. tigrinum(20)</u>	<u>A. jefferson-</u> <u>ianum(20)</u>	<u>A. maculatum(20)</u>	<u>A. opacum(20)</u>	<u>A. macrodactylum(24)</u>		<u>A. macrodactylum</u>	
	19,9°C.	19,9°C.	19,9°C.	19,9°C.	21°C.	18°C.	13,8°C.	9,6°C.
2			0	0				
7			14				(St. 6)25	50
8			16	20		26.5		64
9			31-36		25.5			89
10		0	42	40				133
11			49.5	50		50		
12		4-9	53-55.5	60	49.5			160
13	0-10	11	63-64	80				189
14	13	21	72-74			75		
15		24	77.5	85				231
16		26						240
17			82					
19		28	88		73.5		137	
21		31		110		95.25		280
23	23							301
24				130			161	307
26	37	47	122	140	(St. 27)94.75	124		354
29	48							401
30							237	
31			146					

Columbia and the breeding season is as early as January 19 in western Washington (18), and begins in November or December in western Oregon (personal communication with R. M. Storm).

A. macrodactylum appears to follow the general pattern of development for ambystomatid salamanders. An example of an exception is the development of A. tigrinum, which although at stage 40 with respect to other morphological features, is still without fore-limb buds (15).

#### Larval Development

Larval development is considered to be the period from hatching through the development of the hind limb. Brandon (6) has found that larvae generally hatch in the laboratory at a smaller size than is usual in the field. A. macrodactylum raised in the laboratory averaged 10.0 mm. at hatching. This compares to 13 mm. for A. jeffersonianum, 12-13 mm. for A. maculatum, and 14 mm. for A. tigrinum, all collected from the field (4, p. 98, 123, 164). A. opacum, which lays its eggs on land, hatches at an average length of 18.35 mm. depending upon the length of time between egg-laying and the heavy rains which cause hatching to take place (4, p. 144).

Although the larvae raised in the laboratory during this study all hatched at stage 40, Dr. R. M. Storm (31) has collected eggs in the field which hatched at stage 39.

A chart constructed by Brandon (6) which compares the larvae of five species of *Ambystoma* is shown in Table 6. This chart shows the development of the posterior and anterior limbs, the presence or absence of balancers, and throat pigmentation as the total length of the larvae increases. This writer has added *Ambystoma macrodactylum* to the chart so that it may be compared to the other species. As the chart indicates, *A. macrodactylum* reaches a state of full development with respect to limb development at a lesser total length than do *A. opacum* and *A. jeffersonianum* but at a greater length than do *A. maculatum* and *A. texanum*.

Orton (22) tentatively refers to a larva as *A. cingulatum* with a total length of 37.0 mm. and having five distinct toes on rear legs. She compared this larva to *A. talpoideum* and *A. texanum* larvae as follows: *A. talpoideum*, total length 35.0 mm., three toes on rear legs and fourth and fifth toes are indicated; *A. texanum*, total length 50.0 mm., five well developed toes on rear legs. Volpe and Shoop (33) say that at 37 or more mm. digits of fore and hind limbs of *A. talpoideum* are well developed.

As was previously mentioned, the order of appearance of the toes of the hind limb in *A. macrodactylum* was found to be as follows: 1 (the ventral-most when limbs are adpressed to side of larva), 2, 3, 4, and 5. In anurans, however, the developmental pattern differs. Toes 4 and 5 appear to differentiate first, followed by 3, 2, and 1 in



Table 6. Comparison of Larval Developmental Characteristics as Length Increases in Six Species of *Ambystoma*.

Total Length in mm.	Species	Balancers	Anterior Limbs	Posterior Limbs	Throat Pigment
13	A. j.	+	small limb buds	none	-
	A. t. t.	-	very small limb buds	none	-
	A. m.	+	2 toe buds	small limb buds	-
	A. o.	+	2 toe buds	none	+
	A. t.	+	2 toe buds	none	+
	*A. m.	+	2 very small toe buds	none	-
14	A. j.	+	limb buds	none	-
	A. t. t.	-	small limb buds	none	-
	A. m.	+	2 toes	small limb buds	-
	A. o.	+	2 toe buds	none	+
	A. t.	+	2 toes	none	+
	*A. m.	+	2 toes	none	-
15-16	A. j.	+	2 toe buds	none	-
	A. t. t.	-	limb buds	none	-
	A. m.	-	3 toes	2 toe buds	-
	A. o.	+	2-3 toes	none	+
	A. t.	+	2 toes. 1 bud	limb buds	+
	*A. m.	-	2 toes. 1 bud	very small limb buds	-
17-18	A. j.	-	2 toes. 1 bud	small limb buds	-
	A. t. t.	-	2 toe buds	none	-
	A. m.	-	3 toes. 1 bud	3 toes	-
	A. o.	+	3 toes. 1 bud	small limb bud	+
	A. t.	-	3 toes	limb buds	+
	*A. m.	-	3 toes. 1 bud	small limb buds	-
19-20	A. j.	-	3 toes	limb buds	-
	A. t. t.	-	2 toe buds	none	-
	A. m.	-	3 toes. 1 bud	3 toes. 1 bud	-
	A. o.	-	4 toes	limb buds	+
	A. t.	-	3 toes	2 toe buds	+
	*A. m.	-	4 toes	small limb buds	-
22-23	A. j.	-	3 toes. 1 bud	2 toe buds	-
	A. t. t.	-	2 toes. 1 bud	none	-
	A. m.	-	4 toes	3 toes. 1 bud	-
	A. o.	-	4 toes	limb buds	+
	A. t.	-	3 toes. 1 bud	2 toes	+
	*A. m.	-	4 toes	2 small toe buds	-
25-26	A. j.	-	3-4 toes	3 toe buds	-
	A. t. t.	-	2 toes. 1 bud	none	-
	A. m.	-	4 toes	4 toes	-
	A. o.	-	4 toes	2 toe buds	+
	A. t.	-	4 toes	3 toes	+
	*A. m.	-	4 toes	2 small toes	-

Table 6. (Continued)

Total Length in mm.	Species	Balancers	Anterior Limbs	Posterior Limbs	Throat Pigment
28-30	A. j.	-	4 toes	2 toes. 2 buds	-
	A. t. t.	-	3 toes. 1 bud	3 small toe buds	-
	A. m.	-	4 toes	4 toes. 1 bud	-
	A. o.	-	4 toes	3 toe buds	+
	A. t.	-	4 toes	4 toes	+
	*A. m.	-	4 toes	3 small toes	-
33-35	A. j.	-	4 toes	3 toes. 1 bud	-
	A. t. t.	-	4 toes	not examined	-
	A. m.	-	4 toes	5 toes	-
	A. o.	-	4 toes	3 toes. 1 bud	+
	A. t.	-	4 toes	5 toes	+
	*A. m.	-	4 toes	4 toes	-
38-40	A. j.	-	4 toes	4 toes	-
	A. t. t.	-	4 toes	not examined	-
	A. m.	-	4 toes	5 toes	-
	A. o.	-	4 toes	4 toes. 1 bud	+
	A. t.	-	4 toes	5 toes	+
	*A. m.	-	4 toes	5 toes	-
42-45	A. j.	-	4 toes	5 toes	-
	A. t. t.	-	4 toes	not examined	-
	A. m.	-	4 toes	5 toes	-
	A. o.	-	4 toes	5 toes	+
	A. t.	-	4 toes	5 toes	+
	*A. m.	-	4 toes	5 toes	-

\* Present study.

that order (13).

Metamorphosis in A. macrodactylum apparently takes place later or at a larger size than in other species of Ambystoma. A. macrodactylum transform at 60-80 mm. in length at elevations below 5,500 feet and at 70-90 mm. at higher elevations (17), whereas A. jeffersonianum transforms at 45 mm., A. maculatum at 35 mm., and A. opacum at 37-50 mm., and A. texanum at 37-41 mm. (6).

Gills are often studied in ambystomatid salamanders, mainly because they are such a conspicuous part of the animal. Valentine and Dennis (32) state that all larval ambystomatids have four gill slits. In Ambystoma the gill rakers or filaments are erect, conical and often sharply pointed. They have found as few as six rakers on the third arch of A. laterale and A. cingulatum, and they report an observation by F. R. Gehbach that A. tigrinum has as many as 23 rakers on the anterior face of the third arch. This writer is of the opinion that significant comparisons between species cannot be made on the basis of the number of gill rakers present, unless the stage of development is stated rather precisely. A. macrodactylum begins to develop rakers on the gills during stages 38 and 39 and the number increases until at stage 55 (43 mm.) the larvae have 21-22 filaments on the anterior face of the third arch. This process probably goes on until the time of transformation into the adult.

In fully mature A. macrodactylum larvae, the relative lengths

of the toes on the posterior limbs starting with the shortest are 1-5-2-4-3 (30, p. 86-87). In the present study, the average relative lengths of the toes were 5-1-4-2-3 starting with the shortest. However, in individuals 42-43 mm. in total length the relative lengths of these toes was the same as those mentioned by Storm (30, p. 86-87). Of the larvae inhabiting the same ponds as A. macrodactylum, A. gracile rear toes are: toes 1 and 5 and toes 2 and 4 almost equal with 3 being the longest. Taricha granulosa: rear toes are also 1-5-2-4-3 in order from the shortest to the longest (30, p. 74).

#### Pigmentation

The pigment changes in A. macrodactylum are described periodically in a previous section as development progresses. Drager and Blount (10) state that the secretion of intermedin, the melanophore-expanding hormone, occurs in A. maculatum at stages 38 and 39 which corresponds to the time of change in the melanophores from small black spots to larger, more spreading units. In A. macrodactylum, this change in melanophore appearance was observed to take place somewhat earlier, about stage 36-37.

The pigmentation of the "mature" larvae as raised in the laboratory is as follows: ground color is a tan-yellow dorsally with uniform small, branched melanophores scattered liberally over the head and extending posteriorly along both sides of the dorsal fin,

concentrating in irregular patches which are connected by thin lines of melanophores. This "patchy" pattern continued the length of the fin. The sides have sparsely distributed melanophores and an occasional "patch" of concentration. These are separated from the more dorsal pigmentation by a relatively clear area of tan-yellow. There are small flecks and patches of silvery iridophores scattered along the lateral surfaces and also along the dorsal sides of the gills. The center is a whitish color, devoid of either the dorsal yellowish ground color or melanophores. The iris of the eye appears to be covered with silver iridophores. Melanophores are present sparsely on gills and limbs. The tail fin is translucent with scattered grayish and yellowish pigment cells.

When viewed without the microscope from above, the larvae presents a dirty yellow appearance with occasional dark areas dorso-laterally.

Dr. R. M. Storm, describing 45-60 mm. larvae of A. macrodactylum in his thesis (30, p. 86) indicates that in larvae obtained from the field the general coloration is "dark greenish-brown, obscurely mottled with dark brown and black." This difference is apparently due to the presence of more and larger melanophores, although the general pigment pattern is the same.

The difference in coloration is almost certainly due to the white background of the trays in which the larvae in this study were

kept. This phenomenon has been noted by other workers. Lantz (19) has found that larvae of A. opacum raised on a white background become a brownish-white almost without markings and a uniform dense black if kept on a black background. Bishop (4, p. 98), in describing the larvae of A. jeffersonianum has found that a light background causes a lightening of the ground color of the larvae.

Larvae of A. macrodactylum in this study at stage 45 were observed to have larger melanophores than those larvae at much later stages. This would seem to indicate that the longer the period in the white trays, the smaller the melanophores become.

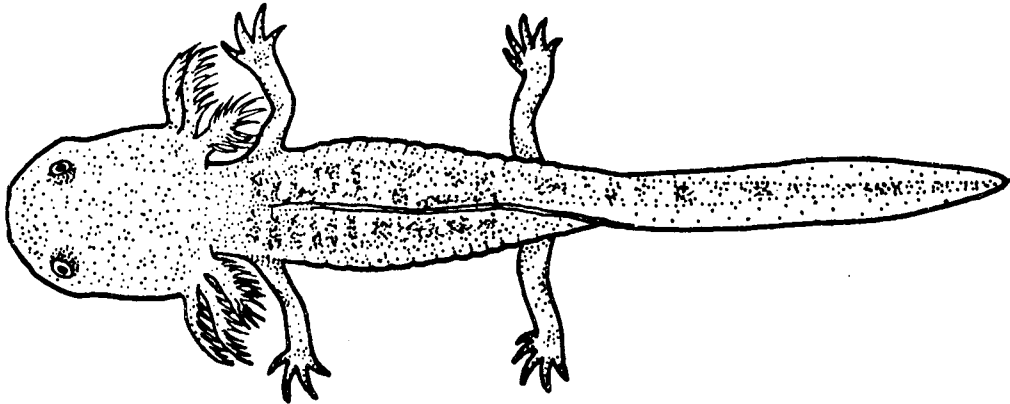
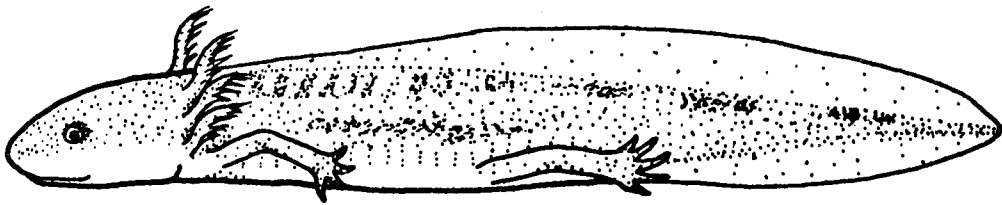
In comparing the coloration of A. macrodactylum larvae with those in Benton County with which they might be confused, Storm (30, p. 86) says that A. macrodactylum are darker than either A. gracile (lighter and more conspicuously marked) and Taricha granulosa (reddish brown with light markings).

Bishop (4, p. 82-155) presents good descriptions and coloration patterns for the larvae of several eastern Ambystoma species.

#### Larval Feeding

In Ambystoma punctatum, Hamburger (14, p. 21) states that the beginning of feeding occurs in stages 45-46. However, in A. macrodactylum all larvae were definitely feeding by stage 45. This was determined by the presence of feces in the containers. Even as

Figure 7. Side and dorsal views of fully developed larvae.



early as stage 41, larvae were observed to snap at passing Daphnia, although this was probably due to some other reason than a feeding response.

As stated previously, the developing larvae were fed mainly Daphnia and lesser amounts of small annelids (Enchytraeus). They appeared to develop satisfactorily with this diet. However, the literature indicates that several workers have found fault with both of these food items. Reinhardt (25) states that the feeding of Enchytraeus to Ambystoma and Pleurodeles larvae causes the posterior limbs to become crippled and the water balance to be disturbed. However, addition of Tubifex or calf-liver to the diet favors normal development, and evidently provides needed minerals or vitamins not found in a diet composed entirely of Enchytraeus.

On the other hand, Dorris (9) showed that Ambystoma larvae, fed only on Enchytraeus, had low mortality rates when fed moderately (three times weekly). When fed maximally, rapid growth was achieved but all the larvae died before metamorphosis.

Daphnia or similar small crustaceans appear to be the best general food type for salamander larvae. Apparently it is difficult if not impossible to overfeed the larvae on this diet. Stewart (29) has found that in the marbled salamander (A. opacum) abundant food hastens metamorphosis and results in a greater length at metamorphosis. A stomach analysis of these larvae revealed that the



most common food items were cladocera, ostracods, and copepods. Dr. Robert M. Storm (31) has also found that young larvae collected from the field feed heavily on cladocera and copepods without apparent ill effects. On the other hand, Moulton (21) advocates the use of newly hatched Artemia, or brine shrimp, in feeding Ambystoma larvae. He says that the Daphnia exoskeleton may tear the tissues of the young larvae. Patch (23) indicates that beef liver is favorable for growth and development of larvae, but is deficient in minerals.

Some A. macrodactylum larvae which were kept in the same container exhibited aggressive tendencies. Slits and notches in the fin were observed, and one individual was without the posterior quarter of his tail. Bragg (5) observed some evidence of cannibalism in A. texanum and states that the larvae may attack toad tadpoles.

Another aid to development, although not used in the present study, is the use of biologically conditioned water. Shaw (27) found that a substantial increase in growth in A. maculatum was obtained when using filtered mussel-conditioned water.

Dushane and Hutchison (11) state that embryos of Ambystoma maculatum have different growth rates depending on geographical location. Embryos from New Jersey and Pennsylvania grew at more rapid rate and were significantly larger than embryos from Illinois and Michigan, both groups having been raised under the same

conditions. The range of A. macrodactylum is quite extensive, including most of British Columbia, all of Washington, the northwest portion of Montana, all of Oregon except the southeast corner of the state, most of Idaho except for the southwest portion and extending into northern California (28, p. 82). Thus a wide environmental variation is encountered and it is quite likely that size range and rate of development are affected by geographical location of this species. Indeed, Stebbins (28, p. 37) and Kezer and Farner (17) state that larvae at high elevations apparently do not metamorphose until the second year. Storm (30, p. 87) indicates that in lower elevations around Corvallis larvae transform during the first year. This observation is reinforced by the fact that A. macrodactylum often breed in semi-permanent ponds.

There are, at present, no identification keys for salamander larvae which are useful over wide geographic areas such as continents. Keys by Brandon (7) and Bishop (4, p. 13-16) are regional keys, intended for use in particular areas (states). Brandon (6) has found that morphological development appears to be a function of size, not age, and bases his key (7) to a large extent upon this observation besides including information on pigmentation. At the present time this appears to be the only really workable system. It is hoped that eventually more of these regional keys will be constructed and then combined, thus making one key which would be useful over a wide area.

## SUMMARY AND CONCLUSIONS

The embryonic and larval stages of the long-toed salamander, Ambystoma macrodactylum are described from fertilization through the development of five toes on the posterior limbs. New stage numbers are employed in the description of the hind limb.

The eggs used in the study were obtained by the injection of pituitary glands into mature female salamanders in order to stimulate them to ovulate. Fair numbers of eggs are secured in this manner if the salamanders are injected within four months after being collected. However, if injected four and one-half to five months after collection, they are found to be non-productive.

The embryonic development rate of Ambystoma macrodactylum appears to be slower than northern forms such as A. tigrinum, A. jeffersonianum, and A. maculatum but faster than A. opacum, a more southern form. However, the range of A. macrodactylum extends farther north than any of the others.

A comparison of larval development as length increases shows that A. macrodactylum reaches a state of full development with respect to limb development at a lesser total length than do A. opacum and A. jeffersonianum but at a greater length than does A. maculatum.

Pigmentation in laboratory-reared larvae is found to be lighter in color than that exhibited by A. macrodactylum larvae collected in

the field. However, the same general pattern remains constant.

The use of the number of gill rakers in determining the species of a larval ambystomatid is found to be unreliable unless the stage of the larva is known.

The correlation of body dimensions at various stages of development to geographic distribution and to other species for identification purposes is not feasible until similar information for other locations and other species is obtained.

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## APPENDICES

## APPENDIX A

Stage No.	Hours Elapsed Since Previous Stage	Total Hours Since Fertilization
fert.	--	--
6	25	25
19	112	137
24	24	161
30	76	237
34	27	264
35	18	282
35.5	20	302
37	28	330
39.5	92	422
40	96	518

## APPENDIX B

Stage No.	Hours Elapsed Since Previous Stage	Total Hours Since Fertilization
fert.	--	--
6	38	38
7	12	50
8	14	64
9	25	89
10.5	44	133
12	27	160
12.5	23	183
13	6	189
15	42	231
16	9	240
20.5	15	255
21	25	280
22	8	288
23	13	301
24	6	307
25	23	330
26	24	354
28	7	361
29	40	401
32	73	474
34	8	482
35.5	14	496
36	28	524
37	262	786
38	27	813
39	20	833
40	145	978