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THE ORAL AND BRANCHIAL MOVEMENTS OF DEVELOPING ANURAN EMBRYOS AND LARVAE

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INTRODUCTION

During the last part of the nineteenth century, studies on the development of behavior in amphibians were discontinued. These studies were re-established by Coghill, who worked extensively with *Ambystoma*. (See his 1929 review for a summary of this work.) The researches of Coghill were confined primarily to the swimming, walking and feeding mechanisms in *Ambystoma*.

In *Ambystoma*, there is a progressive antero-posterior recruitment of the axial musculature in the development of the swimming responses. As mouth, gill and limb musculature develops, it too is recruited to take part in the total reaction of the animal. The local reflexes are individuated from the total pattern only after this generalized activity can be elicited.

Coghill's studies attracted the attention of many investigators and led to an ever increasing amount of literature on behavior problems. See Hooker (1936) and Carmichael (1946) for reviews of the literature.

Among the workers directing their efforts toward the solution of behavior problems was Youngstrom (1938), who studied the development of swimming movements, spontaneous eye movements, and the onset of local reflexes in the fore and hind limbs of Anurans.

In Anurans, a previously uninvestigated behavior pattern is established in late embryonic life. This is the development of mouth movements and the establishment of a regular respiratory rhythm. It is with the onset of local mouth reflexes and the establishment of a rhythmic ventilatory rate that this paper concerns itself.

The only reference to the respiratory movement reflex is that of Youngstrom (1938), who notes that in *Pseudacris triseriata* "the gill rhythm is established by the 6 millimeter stage".

Anurans lend themselves well to studies of this nature because of their accessibility and their size, which is small enough to require little laboratory space, yet large enough to permit convenient manipulation for study. As the embryos are nourished by their own yolk, the constancy of a food supply presents no problem.

MATERIALS AND METHODS

Animals observed in this study were embryos of *Rana pipiens*, *Pseudacris nigrita triseriata* and *Bufo americanus*. Larval forms of *Rana pipiens* only were observed.

Several groups of *Rana* eggs were used. The first group of eggs was obtained by induced ovulation and artificial fertilization using Rugh's method (1934). Later, additional embryos of *Rana* plus those of *Pseudacris* were secured from ponds in the Iowa City area. *Bufo* specimens were supplied by Mr. Chih Ye Chang.

Groups of approximately 30 eggs each were placed in finger bowls about one-half full of water. The animals were allowed to develop at room temperature.

Upon attaining a morphological stage of development suitable for this study, each embryo was transferred to a dish lined with paraffin containing 2-3 cc. of water, just enough to cover the specimen. The paraffin contained a shallow groove which helped to hold the animal in place. The embryo was then placed on its dorsal surface, with the ventral surface exposed. With the animal in this position, identification of its morphological stage of development was made possible, and the responses to tactile stimulation of the oral region were readily observed.

The animals were stimulated with a human hair sealed into a glass tube by paraffin. Because of the variability of the responses to oral stimulation, the side of the hair was drawn over the oral region of the animal several times in order to elicit the most advanced response of which the embryo was capable.

The embryo was then transferred to a glass caster dish containing about 10 cc. of water through which air had been bubbling for several hours. Following the transfer, the animal was left undisturbed for several minutes. The branchial region was then observed for evidences of respiratory movements. When these movements were present, they were timed to two-tenths of a second with the use of a Meylan stop watch. The time required for twenty respiratory movements in a quiescent embryo was recorded, and the number of respiratory movements per minute was calculated.

The temperature of the water in which the animals were placed for observation of their respiratory rhythms varied between 23° - 27° C. during the course of these experiments.

Serial sections of some animals in representative stages were prepared. Staining of these sections was done with Delafield's haemotoxylin and eosin.

Morphological stages

The morphological staging followed in this experiment was that propounded by Shumway (1940) for *Rana pipiens* embryos. Stage 24 was subdivided into quarter intervals (24 1/4, 24 1/2 and 24 3/4) according to the extent of closure of the operculum. The method used for stage identification of sectioned material was that of Shumway (1942) for *Rana pipiens*. The staging of larval forms of *Rana pipiens* followed that of Taylor and Kollros (1946).

Behavior stages

The stages of response to tactile stimulation are primarily adapted from those described by Coghill (1929) for *Ambystoma*. No attempt was made to differentiate between the S response and early and late swimmers. All were classified as giving the S type response.

Stage C - The coil stage. The embryo forms a half-circle with its body in a direction toward the side of stimulation, as in the early flexure stage.

Stage RC - The reversed coil stage. The embryo forms a half-circle as in the preceding stage, but then reverses the half-circle.

Stage S - The repeated reversed coil stage. The embryo forms several rapid reversed coils in succession, giving the appearance of an S shape to the body. Included in this stage are the early and late swimmers.

Stage G - The external gills are depressed and the mouth of the embryo twitches.

RESULTS

Onset of mouth movements

Embryos of *Rana, Bufo* and *Pseudacris* were tested for responses to tactile stimulation of the oral region. The results are given in Tables I, II and III. These tables indicate that, for all forms studied, movements of the mouth are established in morphological stage 23.

Establishment of a regular respiratory rate

Respiration by an internal gill system is instituted only after the embryo has established mouth movements. The first indication of respiratory movements in the *Rana pipiens* embryo occurs in late stage 23. These movements consist of spasmodic gulps and are evidenced particularly after prolonged swimming. These movements are highly irregular in rate and were observed in only a few animals. In animals exhibiting these spasmodic movements, the number of gulps between prolonged pauses ranged from 3 to 14.

With the beginnings of opercular closure on the right side of the embryo (stage 24 1/4), the animal initiates rhythmic respiratory movements.

The influence of temperature on the rate of respiratory movements was determined in a series of timings of *Rana* embryos in stages 24 and 25. The results are presented in Graph I. With but three exceptions in twenty-six cases, the higher temperature was accompanied by the higher respiratory rate. The Q_{10} or respiratory movement activity for *Rana* ranged between 0.9 and 1.5.

TABLE I

The number of *Rana pipiens* embryos showing different types of responses to tactile stimulation of the oral region

Behavioral Stage Morph. Stage	No Response	C	RC	S	G
20	2	1	3	7	
21	1		3	9	
22			1	11	
23				5	29
24					35

TABLE II

The number of *Bufo americanus* embryos showing different types of responses to tactile stimulation of the oral region

Behavioral Stage Morph. Stage	No Response	C	RC	S	G
20			3	9	
21			3	12	
22				11	1
23				1	22
24					31

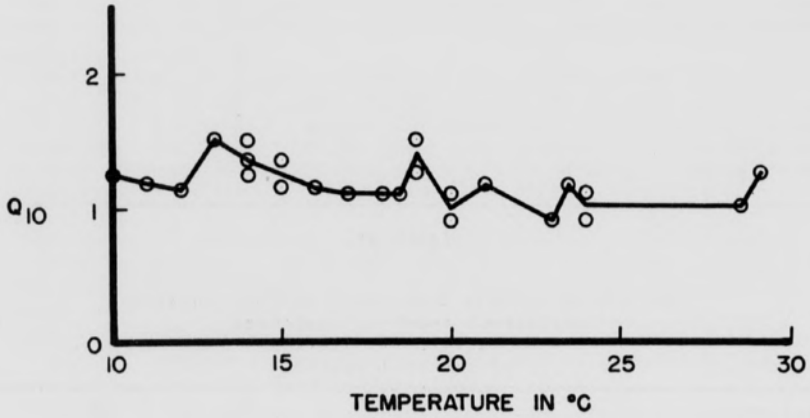
TABLE III

The number of *Pseudacris nigrita triseriata* embryos showing different types of responses to tactile stim- region

Behavioral Stage Morph. Stage	No Response	C	RC	S	G
20				14	
21				11	
22				16	
23				2	20
24					29

GRAPH I

THE EFFECT OF TEMPERATURE ON THE RATE OF RESPIRATORY MOVEMENTS OF RANA IN MORPHOLOGICAL STAGES 24 TO 25.



The rates of breathing movements for *Rana* in morphological stages 24 1/4, 24 1/2, 24 3/4 and 25, in the 23° - 27° C. temperature range, are presented in Graph 2. This graph shows that there is a progressively faster rate of respiratory movements in stages 24 1/4 to 24 3/4. The number of ventilatory movements per minute decreases in stage 25. The differences between successive intervals are all significant. (See Table IV for significance of differences.)

Of the various forms studied, *Bufo* is the first to establish respiratory rhythm, the onset taking place usually in stage 23. Regularity of breathing movements does not appear in *Pseudacris* until late stage 25. Graphs 3 and 4 give the number of breathing movements per minute for *Bufo* and *Pseudacris*. From Graph 3, the respiratory movement rate for *Bufo* appears to increase with growth from stages 23 to 25, although the only significant difference is that between stages 24 1/2 and 24 3/4. (See Table IV for significant differences.)

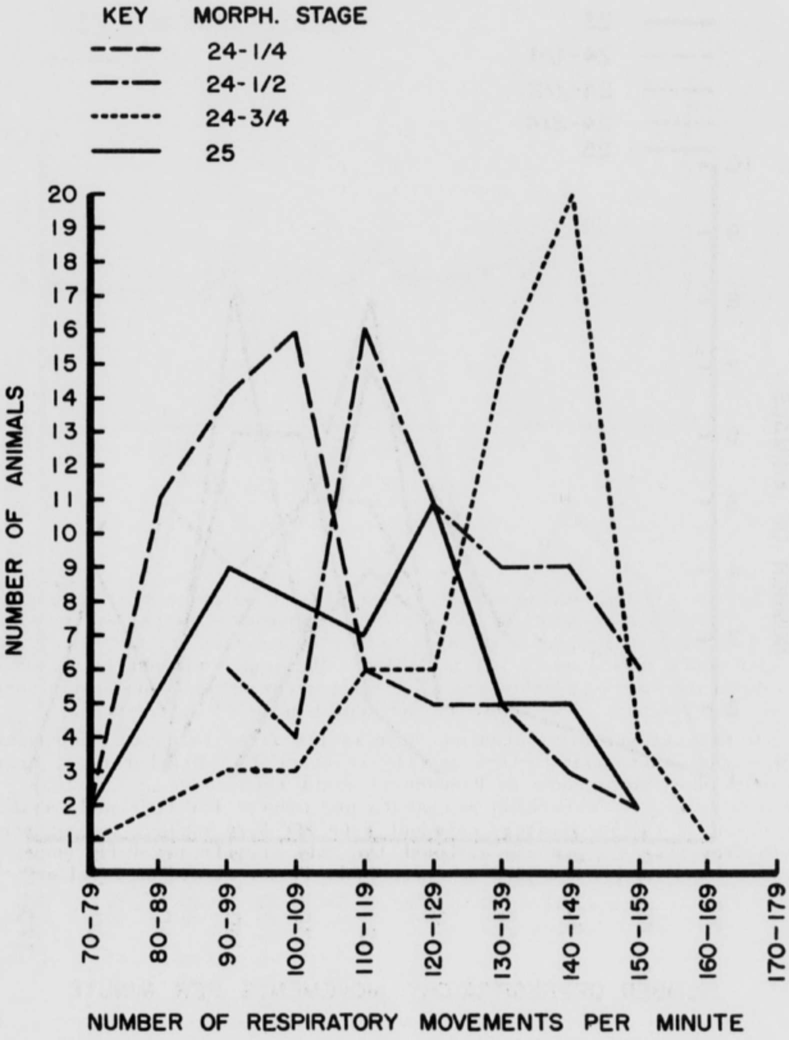
Graph 4 indicates that the number of ventilatory movements per minute for *Pseudacris* ranges between 70 and 169.

A comparison of the average rates of breathing movements of *Rana*, *Bufo* and *Pseudacris* and the significance of the differences is presented in Table IV. *Bufo*, besides initiating respiratory rhythm at an earlier morphological stage than *Rana* or *Pseudacris*, also has significantly faster rates of ventilatory movements than *Rana* in stages 24 1/4 and 25 and *Pseudacris* in stage 25. The difference between rates of *Rana* and *Pseudacris* in stage 25 is not significant.

Rana larvae (stages I to IV), as seen in Graph 5, show a range of 108 to 121 respiratory movements per minute in these stages.

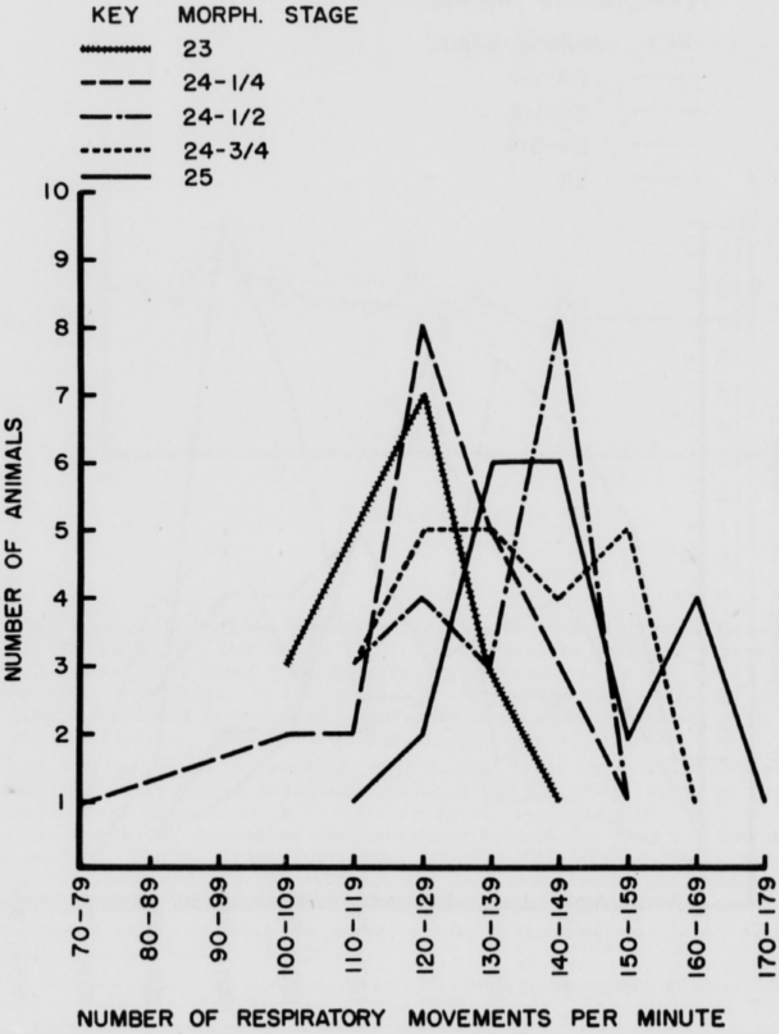
GRAPH 2

THE NUMBER OF RESPIRATORY MOVEMENTS PER MINUTE FOR RANA EMBRYOS IN A 23°-27° C. TEMPERATURE RANGE.



GRAPH 3

THE NUMBER OF RESPIRATORY MOVEMENTS PER MINUTE FOR BUFO AMERICANUS EMBRYOS IN A 23° - 27° C. TEMPERATURE RANGE.



GRAPH 4

THE NUMBER OF RESPIRATORY MOVEMENTS PER MINUTE FOR PSEUDACRIS NIGRITA TRISERIATA EMBRYOS IN A 23°–27° C. TEMPERATURE RANGE.

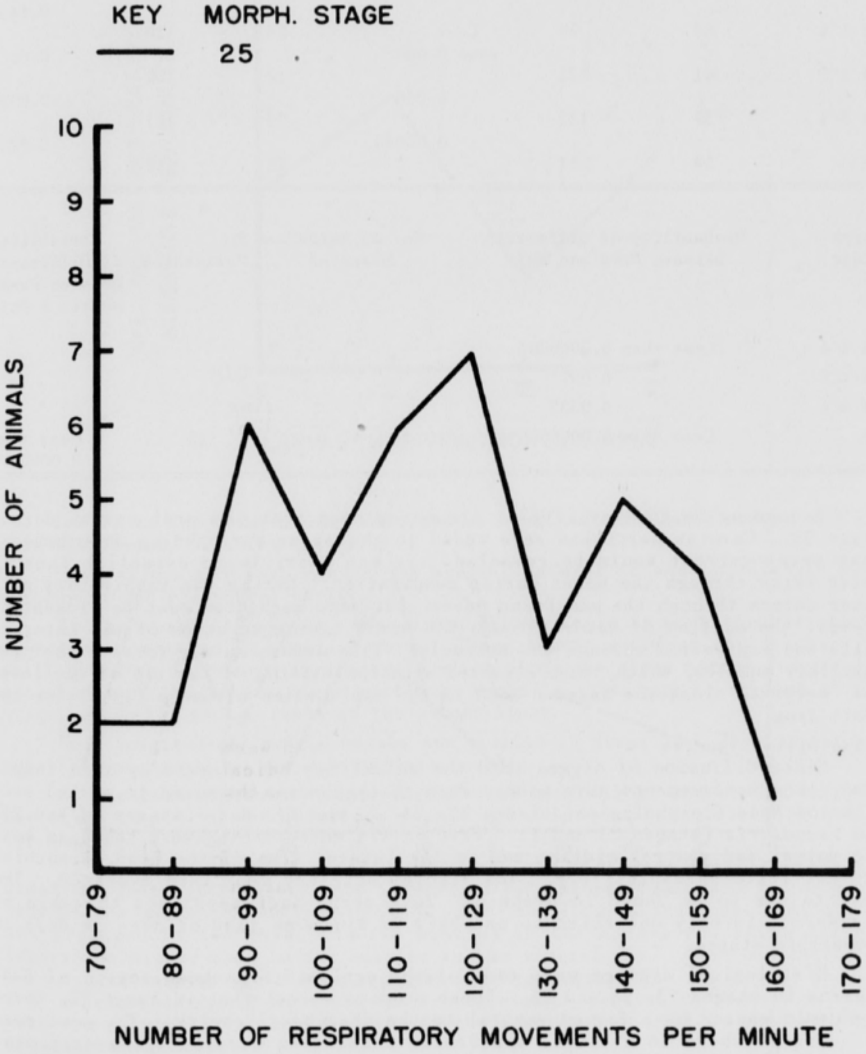


TABLE IV

The average number of breathing movements per minute for *Rana*, *Bufo* and *Pseudacris* in the 23° - 27° C. temperature range and the significance of the differences of these means

Morph. Stage	No. of Animals Observed	*Rana*	Probability	No. of Animals Observed	*Bufo*	Probability
23				19	126	
						0.6456
24 1/4	64	99	Less than 0.006	24	128	
						0.66
24 1/2	61	122		19	130	
			0.026			0.0086
24 3/4	59	133		23	141	
			0.00034			0.8258
25	54	111		22	142	

Morph. Stage	Probability of difference between *Rana* and *Bufo*	No. of Animals Observed	*Pseudacris*	Probability of Difference Between *Pseudacris* & *Bufo*
24 1/4	Less than 0.0000006			
24 1/2	0.097			
24 3/4	0.0332			
25	Less than 0.0000006	40	113	Less than 0.0000006

According to Shumway (1947), the nares open into the oral cavity during stage 23. Carmine particles were added to the water surrounding an embryo so that water current would be revealed. It was possible to establish that no water exits through the nares during respiration. During the inspiratory act, water enters through the mouth and nares, but some mechanism must be present to prevent the outflow of water through the nares during aeration of the internal gills and expiration through the spiracle. This mechanism may involve the premaxillary muscles, which, when elevated with the raising of the tip of the lower jaw, serve to close the nares. Such is the explanation of Gaupp (1896) for the adult frog.

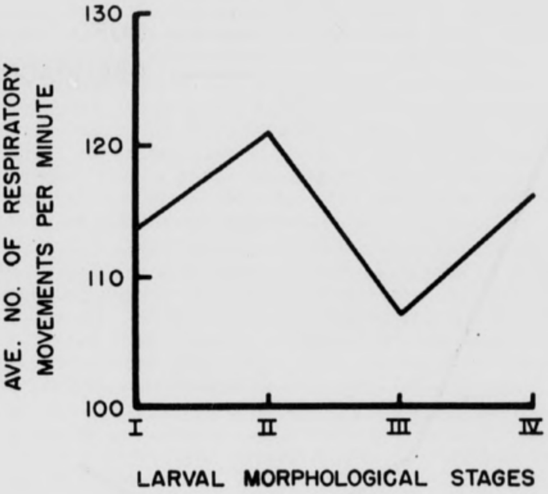
Histological studies

Since diffusion of oxygen into the animal may be affected by skin thickness, skin measurements were made. Skin thickness was measured in serial sections of *Rana* (morphological stages 23, 24, 25 and I), *Bufo* (stages 24 and 25) and *Pseudacris* (stages 23 and I). Five or six measurements were taken in both the dorsal and ventral midline and in the lateral area of the head, branchial and gut regions respectively. The figures obtained were then averaged. The results are to be found in Graph 6. This graph indicates that the skin of *Pseudacris* is only one-third to one-half as thick as that of *Rana* or *Bufo* in comparable stages.

Histological studies were made of the oral and branchial region of *Rana pipiens* in stages 23, 24 and 25. These studies reveal that in stage 23, three premuscle masses have formed ventral to the pharyngeal cavity. The most conspicuous of these is a thick, apparently interwoven mass of transverse myoblasts. This will become the submaxillary muscle which serves to raise the floor of the buccal cavity. Fibrillae may be seen and the nuclei of the fibers exhibit various stages of mitoses.

GRAPH 5

THE AVERAGE NUMBER OF RESPIRATORY MOVEMENTS PER MINUTE FOR RANA PIPIENS LARVAE AT 25° C. (THE AVERAGE GIVEN FOR EACH STAGE IS BASED ON THE VENTILATORY RATE OF TEN ANIMALS IN THAT STAGE.)



Dorsal to the submaxillary muscle, two longitudinal muscle masses are visible with ovoid, dividing nuclei.

Several precartilage masses are present lateral to and immediately under the buccal and pharyngeal cavity.

In the stage 24 embryos, the submaxillary muscle is much thinner and the fibers are arranged in parallel rows. These fibers manifest striations. The still dividing nuclei are beginning to align along the sides of the fibers. The attachment of the submaxillary muscle to Meckel's cartilage is evident at this stage, as is the median raphe of the submaxillary.

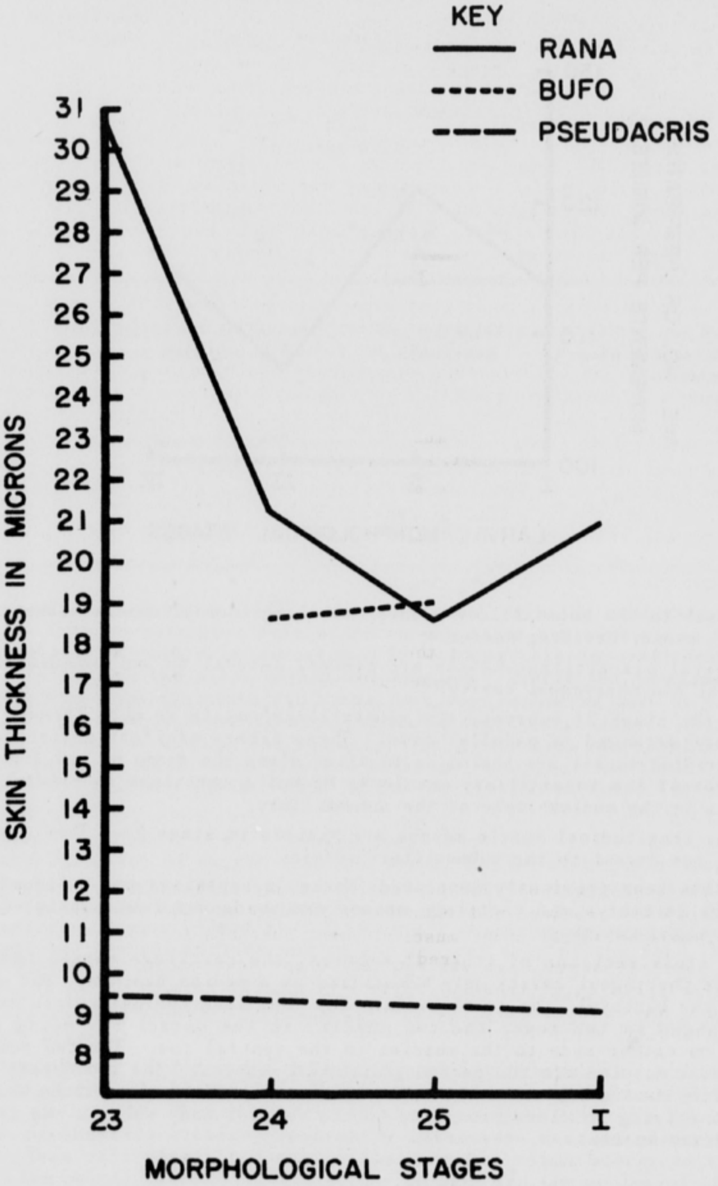
Four longitudinal muscle masses are visible in stage 24. They appear in a parallel row dorsal to the submaxillary muscle.

As has been previously mentioned, Meckel's cartilage may be identified in the stage 24 embryo and cartilage masses are evidenced immediately ventral to the pharyngeal cavity.

In cross sections of stage 25 embryos, the cartilage masses immediately under the pharyngeal cavity were identified as a median basihyal, and on either side of the basihyal, the ceratohyals. The four longitudinal muscle masses are now arranged in two rows, the two muscles in the dorsal row being slightly lateral on either side to the muscles in the ventral row. The two ventral and more medial muscles are the paired geniohyoid muscles. The two dorsal and lateral muscle masses represent the split hyoglossus. Ventral to these four muscle masses and lying in close proximity to the ventral body wall is the transverse submaxillary muscle.

GRAPH 6

THE AVERAGE THICKNESS OF THE SKIN FOR RANA, BUFO AND PSEUDACRIS.



DISCUSSION

This problem, which is an amplification of the work of Coghill, reveals that embryos of *Rana*, *Bufo* and *Pseudacris* show a sequence of developing behavior patterns for the oral region similar to that found by Coghill in *Ambystoma*. However, Coghill also demonstrated that *Ambystoma*, a carnivorous feeder, showed a feeding or snapping reaction. *Rana*, *Bufo* and *Pseudacris*, herbivorous or omnivorous feeders as larvae, do not show this sort of response until after metamorphosis.

In all forms included in this study, the onset of a local mouth response to tactile stimulation occurs in stage 23. It is at this stage that cross sections of *Rana* reveal the presence of fibrillae in the myoblasts of the buccal region. Hooker (1911) noted that in the normal frog embryo, fibrillae must be present for contraction of skelatal muscle to take place.

Water is allowed entry into the buccal cavity through the mouth and nares only when the mouth is opened. Some mechanism, perhaps the premaxillary muscles, causes occlusion of the nares when the mouth is closed. When shut, the mouth and nares, in conjunction with the floor of the buccal cavity, appear to function somewhat like a pump, forcing water into the internal gill chamber. Therefore, respiration by an internal gill mechanism does not take place until after the mouth is capable of movement.

Bufo, in most cases, initiates regular respiratory movements in late stage 23. In *Rana*, ventilatory rhythm is first evidenced in early stage 24 embryos, while the onset of regular breathing movements is delayed in *Pseudacris* embryos until late stage 25. Therefore, spontaneous mouth movements occur at a later developmental stage than do mouth movements in response to tactile stimulation. Hooker (1911) similarly found that contractility in response to stimulation precedes spontaneous contractility.

The fact that *Pseudacris* does not initiate rhythmic respiratory movements until late stage 25 is perhaps explainable by its small size and thin skin. It is possible that a sufficient oxygen supply is obtained in *Pseudacris* by diffusion through its body wall. Thus, the need for a special respiratory mechanism may not arise in *Pseudacris* until a later morphological stage than in the larger and thicker skinned *Rana* and *Bufo* embryos.

The size of the animal is a factor because of the relationship of surface area to mass. When the animal increases in size, the surface increases only as the square, the mass as the cube. There is, then, a relative decrease in the area through which diffusion may take place.

In *Rana*, which shows a significant increase in breathing movement rate up to stage 25, the surface-mass relationship may also be a factor, for the skin is thicker in stage 23 than in any following stage observed.

Very little difference is evidenced in the respiratory movement rate of *Rana* in the change from embryonic to larval life.

SUMMARY

1. Egg clutches of *Rana pipiens*, *Bufo americanus* and *Pseudacris nigrita triseriata* were procured and allowed to develop at room temperature until attaining a morphological stage of development suitable for this study.

2. Tactile stimulation was applied to the oral region of these embryos. A sequence of developing behavior patterns, similar to that found by Coghill in *Ambystoma*, was demonstrated for all forms studied. In almost every case, the onset of a local mouth reflex was established in stage 23.

3. Histological studies of *Rana* reveal the presence of fibrillae in the myoblasts of the oral and branchial region in stage 23. Cross-striations are present at stage 24.

4. In every case, the onset of regular respiratory movements, which involves spontaneous mouth movements, was delayed until after the establishment of a mouth response to tactile stimulation.

5. *Bufo*, the largest form studied, exhibits rhythmic ventilatory movements in late stage 23 with but few exceptions. Regular breathing movements are not

seen in *Rana* until early stage 24, and do not occur in *Pseudacris* until late stage 25. The comparatively late establishment of regular breathing movements in *Pseudacris* may be attributed to its smaller size and thinner skin.

6. In *Rana* embryos, with the exception of stage 25, an increase in age is accompanied by a significant increase in the average number of respiratory movements per minute. In *Bufo*, the average ventilatory rate appears to increase with age, but the differences are not significant.

7. *Bufo*, the first to institute ventilatory rhythm, has a significantly higher average rate of respiratory movements per minute than does *Rana* or *Pseudacris* in stage 25. There is no significant difference in the average ventilatory movement rate per minute between *Rana* and *Pseudacris* in stage 25.

8. No marked difference is evidenced in the respiratory movement rate of *Rana* in the change from embryonic to larval life.

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