SOME FACTORS INFLUENCING RESPIRATION IN AMPHIOXUS

by

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INTRODUCTION

Studies of amphioxus have contributed much to our knowledge of embryology yet very little is known of the physiology or life history of this phylogenetically significant animal. Twenty-two species in the genus Branchiostoma have been described so far and are distributed throughout much of the tropical and temperate zones. Branchiostoma is a protochordate and belongs to the Class Leptocardii of the Subphylum Cephalochordata.

The systematic position of the amphioxus reported in this thesis is at the present time uncertain. I first collected specimens of this animal in April of 1958 at Bahia la Cholla, Sonora, Mexico, where the genus has not before been reported to occur. This locality is on the northeastern coast of the Gulf of California, about 200 miles southwest of Tucson, Arizona, and about 100 miles northwest of the farthest point in the Gulf at which amphioxus previously has been recorded. Carl L. Hubbs, Professor of Biology at Scripps Institution of Oceanography, has examined nineteen of these specimens preserved in seventy percent alcohol. It is his opinion that the amphioxus found in the Gulf may now represent a new form. In his correspondence he has suggested, "For the time being, I think it would be best for you to refer to your specimens as Branchiostoma sp. near californiense". Branchiostoma californiense Andrews is the species that is found along the coast of California, Baja California and presumably extends

into the Gulf (Ricketts and Calvin, 1952).

The problem investigated here is not one of systematics, but rather one of oxygen metabolism and of some of the factors that seem to influence its rate. Varying concentrations of salinities, temperature changes, and body weight have been considered. With reference to earlier works on the ecology of two different species (Chin, 1941; Webb and Hill, 1958) I have tried to show a correlation between the substrate at Bahia la Cholla and the non-uniform distribution of amphioxus found there.

I am indebted to Dr. John V. Slater for his continuous encouragement and valuable suggestions during this study; to Kieth Justice for his expert advice concerning electrical matters and for the assistance which he offered in the construction of certain parts of the physiological equipment used in these experiments; to Dr. Albert R. Mead for his assistance in obtaining equipment necessary for this study; to Dr. W. J. McConnell for the use of his standardized Winkler reagents; and to Dr. E. L. Cockrum for his helpful criticisms in the writing of this thesis. Special thanks go to John Tremor and other members of the Department of Zoology at the University of Arizona who helped collect specimens on different occasions.

METHODS AND TECHNIQUES

The Winkler method was used in all experiments involving a measurement of dissolved oxygen in sample solutions. This method was first described by L.W. Winkler (1934). It involves the formation of manganous compounds which are oxidized to manganic ions by the dissolved oxygen in the water sample, and which subsequently release iodine quantitatively from potassium iodide when the sample is acidified. The iodine is then titrated with a standardized sodium thiosulfate solution.

Samples of 125 milliliters (ml.) were tested in pairs; one sample contained an animal, the other, the blank, did not. This procedure provided for an accurate analysis of the oxygen used by the animal in its metabolism. The reagents used in all titrations were standardized by Dr. W.J. McConnell, of the Cooperative Wildlife Research Unit at the University of Arizona, to permit a faster calculation of results as follows:

Number of ml. Normal/64 sodium thiosulfate times a factor
value of 0.985 equals milligrams of oxygen/liter (Anon., 1948).

Samples were incubated for two hours at specific temperatures and salt concentrations before the Winkler reagents were added. At the end of this period the animal was carefully removed and first one ml. of manganous sulfate and then one ml. of potassium iodide were

added to the sample. The sampling jar was then shaken for thirty seconds producing a flocculent precipitate. When this precipitate had settled half way, one ml. of concentrated sulfuric acid was added. The solution was then ready to be titrated. The amount of oxygen used by the animal was calculated as milligrams of oxygen/liter/hour/gram wet weight of the animal.

It was necessary in all of these experiments to use artificial sea water. This eliminated measurements of excess oxygen metabolism that would have been obtained from normal sea water containing oxygen consuming plankton. It also provided for a standardized procedure for all experiments. The formula (page 5) used in the preparation of a solution approximating the composition of normal sea water is a modification of Brujewicz (Sverdrup et al., 1946). It was adjusted to yield a solution of 19 ‰ chlorinity.¹ For higher salinities² the formula was doubled or tripled. Lower salinities were prepared by appropriate dilutions with distilled water.

Temperature control was maintained by means of a water bath. The heating element was chromium plated and of the knife type; 250 watts and 115 volts. An electrical mercury thermo-regulator was used to

1. Chlorinity is defined as the total amount of chlorine, bromine, and iodine in grams contained in one kilogram of sea water, assuming that the bromine and iodine had been replaced by chlorine. ‰ is used to mean parts per thousand (Sverdrup et al., 1946).

2. Salinity equals 0.03 plus 1.805 times chlorinity.

Formula for artificial sea water modified from
Brujewics (Sverdrup et al., 1946).

Salt	g/kg
NaCl	26.601
MgCl ₂	2.447
MgSO ₄	3.305
CaCl ₂	1.141
KCl	0.725
NaHCO ₃	<u>0.202</u>
Total	34.421
Distilled water to one liter	

obtain specific temperatures. This piece of apparatus works on the same principle as a thermometer. Two platinum electrodes are connected in such a way as to permit an electrical current to complete circuit when the mercury column reaches a certain designated level. The setting factor for this temperature is valid to 0.5 of a degree. Connected to the thermo-regulator and heater is a battery supplied relay. This portion of the equipment was homemade and is illustrated by the schematic diagram on page 6.

All tests were made in glass-stoppered reagent bottles containing 125 ml. samples. Before experiments the animals were maintained at room temperature (approximately 24°C) in a plastic-lined box containing aerated artificial sea water. All injured animals were removed the first few days after collection to insure consistency of results. Animals were selected at random without discrimination as to size. At the end of each experiment the animal involved was measured

Schematic diagram for electrical heating unit.
(Designed by K.E. Justice.)

Ry₁: Sigma 4F sensitive relay.

Sw₁: Function switch (for open on temperature fall or open on temperature rise; shown in the latter position).

Sw₂: Heater unit on-off switch (single pull, single throw).

Sw_{3A,B}: Main switch (double pull, single throw).

Ne: Pilot lamps.

Batt.: Two six volt lantern cells in series (Burgess F₄BP).

and blotted several times with paper toweling before it was weighed.

This weight was then recorded as the wet weight.

A portion of this thesis deals with the tolerance of the amphioxus to different salinities in artificial sea water. Single specimens of amphioxus were placed in petri dishes containing 25 ml. of artificial sea water of varying salinities and held at room temperature. Cessation of movement of the velar tentacles (fig. 1) was used as the criterion for death. A bright flash of light or vibrations from the tapping on the dish with a pencil served as the stimuli. After being exposed to abnormal concentrations of salinity for six hours, some of the animals were returned to artificial sea water to determine their capacity to recover. If the animal returned from an opaque to the normal translucent appearance and if it responded rapidly to stimuli, it was considered to have recovered.

Since this amphioxus is not found everywhere in the bay at Bahia la Cholla, a sample of the substrate from its habitat was collected and its composition was determined according to particle size. For this study the soil was first weighed and then passed through a series of strainers. The copper screen mesh of the strainers varied in size from 5 squares per inch to 300 squares per inch.

fig. 1

Anterior portion of amphioxus (Drawing modified from Willey, 1894, p. 18).

RESULTS

The animals used in these experiments were collected from Bahia la Cholla on February 23, 1959. One week was permitted for adjustment of the animals to laboratory conditions before experiments were started. Tables I, II, III, and IV represent a compilation of data collected for the month of March 1959, with respect to oxygen metabolism and certain variables that seem to influence its rate (i.e., salinity, temperature, and body weight). Adult forms only were used. The tables have been arranged according to salinities and include date, temperature, weight, length, ml. of sodium thiosulfate used and the calculated milligrams of oxygen/liter/hour/gram (indicated in the tables as $\text{Mg O}_2/\text{l/hr/gm}$) for each test.

Graphs I, II, III, and IV represent an interpretation of the corresponding tables. In these graphs temperature has been plotted against oxygen metabolism. An average of the plots at a specific temperature is indicated by a horizontal line. These averages have then been connected by a continuous line to show the overall effects of temperature on the rate of oxygen metabolism.

Graph V is intended to show the effects of salinity on oxygen metabolism at 30°C. This graph represents data from all four tables. Horizontal lines again represent the averages.

TABLE I

Data representing oxygen metabolism for adult amphioxus at different temperatures; salinity constant at 20 ‰.

Date	Temp. (°C)	Weight (gm)	Length (mm)	Na ₂ S ₂ O ₃ ·5H ₂ O (ml)	Mg. O ₂ /l/hr/gm
March 6	10	Blank	Blank	5.55	
" "	10	0.0543	35	5.47	0.73
" "	30	Blank	Blank	5.60	
" "	30	0.0736	37	5.40	1.34
" "	10	Blank	Blank	5.50	
" "	10	0.0487	33	5.42	0.82
" "	35	Blank	Blank	5.40	
" "	35	0.0420	32	5.32	0.94
March 10	20	Blank	Blank	5.50	
" "	20	0.0543	35	5.36	1.27
" "	30	Blank	Blank	6.10	
" "	30	0.0306	29	6.00	1.64
" "	35	Blank	Blank	5.92	
" "	35	0.0550	35	5.82	0.90
March 19	30	Blank	Blank	6.50	
" "	30	0.0286	26	6.40	1.72
" "	20	Blank	Blank	6.55	
" "	20	0.0487	33	6.42	1.31

TABLE II

Data representing oxygen metabolism for adult amphioxus at different temperatures; salinity constant at 30 ‰.

Date	Temp. (°C)	Weight (gm)	Length (mm)	Na ₂ S ₂ O ₃ ·5H ₂ O (ml)	Mg O ₂ /l/hr/gm
March 6	10	Blank	Blank	5.05	
" "	10	0.0500	34	4.80	2.46
" "	30	Blank	Blank	5.30	
" "	30	0.0543	35	4.95	3.45
" "	10	Blank	Blank	5.20	
" "	10	0.0389	31	5.00	2.53
" "	35	Blank	Blank	5.15	
" "	35	0.0496	34	4.90	2.49
March 10	20	Blank	Blank	5.53	
" "	20	0.0563	35	5.20	2.95
" "	30	Blank	Blank	5.45	
" "	30	0.1750	50	4.30	3.24
" "	35	Blank	Blank	5.74	
" "	35	0.0400	31	5.52	2.71
March 19	30	Blank	Blank	6.20	
" "	30	0.0310	29	5.95	3.86
" "	20	Blank	Blank	6.46	
" "	20	0.0199	24	6.40	2.97

TABLE III

Data representing oxygen metabolism for adult amphioxus at different temperatures; salinity constant at 40 ‰.

Date	Temp. (°C)	Weight (gm)	Length (mm)	Na ₂ S ₂ O ₃ ·5H ₂ O (ml)	Mg O ₂ /l/hr/gm
March 6	10	Blank	Blank	4.50	
" "	10	0.0620	36	4.30	1.59
" "	30	Blank	Blank	4.55	
" "	30	0.1109	41	4.40	1.78
" "	10	Blank	Blank	4.85	
" "	10	0.0533	34	4.67	1.66
" "	35	Blank	Blank	4.82	
" "	35	0.0718	37	4.57	1.71
March 10	20	Blank	Blank	4.87	
" "	20	0.0341	30	4.75	1.73
" "	30	Blank	Blank	5.31	
" "	30	0.0370	30	5.15	2.08
" "	35	Blank	Blank	5.65	
" "	35	0.0350	30	5.51	1.92
March 19	30	Blank	Blank	5.95	
" "	30	0.0238	25	5.85	2.16
" "	20	Blank	Blank	6.15	
" "	20	0.0280	25	6.04	1.90

TABLE IV

Data representing oxygen metabolism for adult amphioxus at different temperatures; salinity constant at 50 ‰.

Date	Temp. (°C)	Weight (gm)	Length (mm)	Na ₂ S ₂ O ₃ ·5H ₂ O (ml)	Mg O ₂ /l/hr/gm
March 6	10	Blank	Blank	3.97	
" "	10	0.0733	37	3.85	0.81
" "	30	Blank	Blank	4.20	
" "	30	0.0770	38	4.00	1.30
" "	10	Blank	Blank	4.65	
" "	10	0.0628	36	4.53	0.92
" "	35	Blank	Blank	4.65	
" "	35	0.0408	31	4.56	1.08
March 10	20	Blank	Blank	4.45	
" "	20	0.0325	29	4.35	1.52
" "	30	Blank	Blank	5.25	
" "	30	0.0742	37	5.05	1.33
" "	35	Blank	Blank	5.95	
" "	35	0.0342	30	5.86	1.28
March 19	20	Blank	Blank	5.97	
" "	20	0.0608	36	5.80	1.37

GRAPH I

Temperature effects on oxygen metabolism; salinity
constant at 20 ‰

GRAPH II

Temperature effects on oxygen metabolism; Salinity
constant at 30 ‰

GRAPH III

Temperature effects on oxygen metabolism; salinity
constant at 40 ‰

GRAPH IV

Temperature effects on oxygen metabolism; salinity
constant at 50 ‰

GRAPH V

Salinity effects on oxygen metabolism; temperature
constant at 30 °C

GRAPH VI

Effects of body weight on oxygen metabolism; temperature constant
at 30°C and salinity constant at 30‰

Graph VI is an interpretation of body weight plotted against oxygen metabolism. This animal showed the greatest utilization of oxygen at 30 ‰ salinity and 30°C. Keeping these two conditions constant, the effects of body weight on the rate of oxygen metabolism were then considered.

Tolerances to different salinity concentration were studied by using four animals at each concentration at room temperature (approximately 24°C). Petri dishes containing 25ml. of artificial sea water and one animal per dish were observed with a binocular dissecting scope at varying intervals. When the velar tentacles no longer responded to stimuli, the animal was considered dead. The time of death was then recorded (Table V) as a measure of its tolerance at that salinity. Fifteen salinities ranging from 0 ‰ to 70 ‰ and by intervals of 5 ‰ were used for this determination. Half of the test animals from the 20, 55, and 60 ‰ solutions were removed after six hours and returned to artificial sea water. Their capacity to recover from abnormal salinities was determined and then recorded in the table on salinity tolerance.

Samples of sand in which the amphioxus were found were brought into the laboratory. Subsamples of one thousand grams were weighed before being passed through a series of strainers varying in size by their number of squares per inch. The sand collected by each strainer was then weighed and its percentile calculated from the original sample. These percentiles are shown in Table VI.

TABLE V

Salinity tolerance of Branchiostoma cf. californiense Andrews;
room temperature approximately 24°C.

<u>Salinity</u>	<u>Average duration of tolerance</u>
0 ‰	1½ minutes
5 "	3 "
10 "	8 "
15 "	45 "
20*	15 hours
25 "	still living after 10 days
30 "	" " " " "
35 "	" " " " "
40 "	" " " " "
45 "	" " " " "
50 "	" " " " "
55*	79 hours
60*	10 "
65 "	3 "
70 "	45 minutes

* Animals returned to artificial sea water.

20 ‰	still living after 10 days
55 "	" " " " "
60 "	slow death in 15 hours

TABLE VI

Particle size of sand taken from the natural habitat
of amphioxus at Bahia La Cholla.

<u>Size of strainer</u>	<u>Grams of sand retained</u>	<u>Percent of sample</u>
5 squares/inch	4.7	0.47
8 " "	4.3	0.43
13 " "	10.7	1.07
25 " "	39.9	3.99
40 " "	218.0	21.80
60 " "	615.0	61.50
120 " "	91.0	9.10
300 " "	16.4	1.64

DISCUSSION

Amphioxus living at Bahia la Cholla are subject to frequent and at times, drastic changes in temperature and salinity. Air temperatures over 100°C during the summer months and near freezing during the winter months are not uncommon for this area. In order to survive therefore, these animals must have a wide range of adaptation.

Normal sea water has approximately 34.421 ‰ salinity (Sverdrup et al., 1946). At Bahia la Cholla water is completely removed to the Gulf twice daily with each low tide. Warm temperatures in this area increase the evaporation, which in turn results in a higher salt concentration. There is no immediate source of fresh water near this area to counteract this rise in salt concentration. The salinity taken at the habitat of these amphioxus (see map) on February 23, 1959, was 37.63 ‰. It should be understood that this figure probably would not agree with one taken during the summer months when temperatures become extremely high and evaporation is at its peak.

Graphs I, II, and III show similar results for the effects of temperature on oxygen metabolism. Oxygen metabolism was found to increase with temperature from 10°C to 30°C at each of these salinities. Table IV indicates a rise in oxygen metabolism with temperature from 10°C to 20°C. At this salinity, however, higher temperatures seem to be less desirable for this animal. Of the four salt concentrations considered,

30 ‰ seemed to be favored by this animal. Activity was greatest and response to stimuli was rapid. The body was also translucent. At this concentration and at 30°C the greatest amount of oxygen metabolism was recorded (Graph V); the least amount was at 10°C and 20 ‰ salinity. If the greatest amount of oxygen metabolism is correlated with optimal conditions for this animal, then it is assumed from these observations that this species of amphioxus is best adapted to salinities approximating normal sea water and temperatures near 30°C. These are laboratory results, of course, and they may not hold true in the natural environment.

Although larval forms were not available for these experiments, the adults used did vary enough in size to indicate an inverse relationship between body weight and that of oxygen metabolism. Graph VI represents the body weights of three animals plotted against their oxygen metabolism at a salinity and temperature which seemed to be the optimum. Under these conditions differences in oxygen metabolism appear to be correlated with body size. The smallest animal with a weight of 0.0319 grams used 0.62 mg O_2 /1/hr/gm more than one weighing 0.1750 grams. It is understood that three animals may not represent an adequate sample, however, data accumulated by other investigators (Edwards and Irving, 1943) on studies of this kind support the findings in this experiment.

The period of time for which amphioxus were able to tolerate different salt concentrations is shown in Table V. Death occurs in a matter of minutes at concentrations below 15 ‰ salinity. Just below 20 ‰ and just above 60 ‰ solutions death was slow. After six hours in the 20 ‰ solution, half of the animals were returned

to normal salt concentrations. Recovery was slow, but obvious. The animals returned from their opaque appearance to the more normal translucent one and also, became more active. Animals from the 60 ‰ solutions were unable to recover. However, at a lower concentration of 55 ‰, recovery was complete and followed the same pattern as that observed at 20 ‰ salinity. It can be concluded from these observations that this species of amphioxus tolerates a wide range of salinities, and that the threshold values for the adult are approximately 20 ‰ and 55 ‰.

Some of the characteristics observed in this study on salt tolerance deserve mention at this time.

1. At concentrations of 35 ‰ and below, the buccal cirri (fig. 1) remain open, whereas at higher concentrations they close tightly over the buccal cavity.
2. At higher salt concentrations the animals tend to bend ventro-medially. Chin (1941) also observed this phenomenon and attributed it to a greater osmotic pressure change in the gonadal region than in the muscular back.
3. At abnormal salinities the animals became opaque compared to the translucent appearance of those kept at normal concentrations.
4. Temperature changes affected activity. The amphioxus were most active at 30°C and least active at 10°C.

The distribution of Branchiostoma cf. californiense Andrews in Bahia la Cholla is not uniform (see map). Salt concentration and temperature are very important in this distribution, but other factors must be considered. Amphioxus live in a sandy type of substrate

of characteristic composition (Chin, 1941; Webb and Hill, 1958). Chin (1941) points out that mud as a substrate is harder than sand and amphioxus cannot burrow in it; also, too fine a sand interferes with its respiration. The map of this area shows the distribution of amphioxus at Bahia la Cholla so far as it has been investigated. Table VI gives an analysis for the particle size of the sand where amphioxus is found. The sand found farther inside the bay is very fine and therefore is undesirable as a habitat for this animal.

KNOWN DISTRIBUTION OF AMPHIOXUS AT BAHIA LA CHOLLA
AREA (Map modified from Hertlein and Emerson, 1956, p.160)

SUMMARY

The fact that the protochordate Branchiostoma cf. californiense Andrew can live at temperatures above 30°C and at salinities above that of normal sea water no doubt accounts for the survival of this species in the subtropical waters of Bahia la Cholla, Sonora, Mexico.

Oxygen metabolism was determined by a modification of the Winkler method. Artificial sea water was used in all experiments to insure more precise and accurate results than could have been obtained from the use of natural sea water containing oxygen consuming plankton. Temperature, salt concentration, and body size were considered as variables influencing the rate of oxygen metabolism. From the experimental observations it was concluded that 30°C and 30 ‰ salinity approximate optimum laboratory conditions for this animal. Larvae were not available for these experiments, so the interpretation of data should only be considered valid for the adult form of this species.

This species is best adapted for the higher salt concentrations. The tolerance range for salt concentration extends from 20 ‰ to 55 ‰. The exact temperature range was not established. However, oxygen metabolism increased with temperature from 10°C to 30°C at all salt concentrations except 50 ‰. At this concentration the highest oxygen metabolism recorded was reached at 20°C.

It has been suggested that the bottom deposits found at Bahia

la Cholla must be considered as one of the governing factors in the non-uniform distribution of amphioxus. An analysis of the substrate from where these animals were collected was compared with those recorded from China by Chin (1941), and from Africa by Webb and Hill (1958). As was reported of the China and African species of Branchiostoma, sand with a particle size of 1/60 inch seems to be the favored substrate composition in which these animals live.

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