SOME PHYSIOLOGICAL RESPONSES TO ANOXIA IN THE
GREAT PLAINS TOAD, BUFO COGNATUS

by

DEDE ARMENTROUT, B.S. in Ed.

A THESIS

IN

ZOOLOGY

Submitted to the Graduate Faculty
of Texas Tech University in
Partial Fulfillment of
the Requirements for
the Degree of

MASTER OF SCIENCE

Approved

December, 1969

55
3
969
No. 252
p. 2

ACKNOWLEDGMENTS

I am indebted to Dr. Francis L. Rose for his encouragement and help in the direction of this work. In addition, special acknowledgment is due Mr. Frank W. Judd and Mrs. Alvin G. Flury for their many hours of help in the collection of specimens. I am also grateful to the other members of my committee, Doctors Robert L. Packard, John S. Mecham, Chester M. Rowell, Jr., and Michael K. Rylander, for their advice and helpful criticism.

CONTENTS

ACKNOWLEDGMENTS	11
LIST OF FIGURES	1v
I. INTRODUCTION	1
II. METHODS AND PROCEDURES	4
III. RESULTS	8
IV. SUMMARY AND CONCLUSIONS	25
BIBLIOGRAPHY	27

LIST OF FIGURES

Figure	Page
1. Heartrate during Anoxia	10
2. Representative Heartrate of Female <u>Bufo cognatus</u> at Selected Intervals during Induced Anoxia	12
3. Arrhythmicity in Heartrate of Female <u>Bufo cognatus</u> at Selected Intervals during Anoxia	14
4. Hepatic Glycogen Depletion during Anoxia	17
5. Blood Sugar Elevation during Anoxia	20
6. Relationship between Blood Sugar Concentrations and Size in Control Animals	22
7. Lactic Acid Elevation during Anoxia	24

CHAPTER I

INTRODUCTION

Vertebrates are occasionally subjected to prolonged periods of low oxygen tension requiring physiological adjustments. Diving vertebrates, which may experience extreme oxygen stress, accommodate by reduction of metabolism (Jackson and Schmidt-Nielsen, 1955), cardiovascular adjustments (Murdaugh and Jackson, 1962; White and Ross, 1966), bradycardia and, in some instances, anaerobiosis (Anderson, 1961; Robin, et al., 1964). In addition, diving vertebrates may exhibit one or more of the following: oxygen debt, increased tissue tolerance to elevated lactic acid levels, conversion of at least some acid to less lethal compounds, and excretion of some or all acid (Jones, 1967).

Krogh (1941) suggested that a metabolic shift to anaerobic respiration occurred in frogs during anoxia. Although Aubert and Bohr believed that frogs were capable of sustained anaerobiosis (Jones, 1967), Poczopka's studies on amphibian respiration (1959) failed to support their contention. While Leivestad's data on submergence with toads (1960) revealed a 20% reduction in metabolism, he found no evidence that toads utilized anaerobic pathways since no oxygen debt was detected. In addition, Leivestad reported only slight increases in lactic acid levels following

emergence. These small increments may be attributed to the release of accumulated lactic acid in the temporarily occluded skeletal muscles, and do not necessarily reflect anaerobic respiration. Jones and Shelton (1964) suggested that Rana temporaria experienced both a reduction of metabolism and occurrence of an oxygen debt during submergence. Jones (1967) measured oxygen consumption in several anurans during submergence and found that they generally exhibit reduced oxygen metabolism. Although he detected increased oxygen consumption upon emergence, he had no evidence to indicate that the increase was due to an oxygen debt rather than to the increased activity of the animal. Rose and Drotman (1967) demonstrated that anaerobic pathways were utilized in Rana pipiens. They found that frogs poisoned with iodo-acetate to inhibit anaerobic glycolysis succumbed to anoxia after only 20 minutes. Non-poisoned frogs recovered after two hours in anoxia.

The ability of Rana pipiens to utilize anaerobiosis during periods of oxygen stress makes questionable the suggestion (Leivestad, 1960) that such a mechanism is lacking in toads. Furthermore, the possibility of a shift to anaerobic metabolism in toads is not precluded by Leivestad's work, because he measured only oxygen consumption. An oxygen debt repaid very slowly could not be detected by his techniques, nor could any excretion of lactic acid be determined. If toads do shift to anaerobic respiration,

as Krogh (1941) has proposed, several physiological responses should be detectable during experimental anoxia: reduction of glycogen stores with concomittant increases in blood sugar and lactic acid. In addition, one might expect to observe other general anuran responses common to situations of oxygen stress: bradycardia, cutaneous vasodialation and vasoconstriction in skeletal muscles. The controversy as to the import of anaerobic glycolysis in amphibia (Jones, 1967) and the paucity of information concerning general physiological responses to anoxia by bufonids motivated this study.

CHAPTER II

METHODS AND PROCEDURES

The Great Plains toad, Bufo cognatus, was collected in Lubbock County, Texas, during the summer of 1968. Only individuals measuring between 60 mm and 90 mm from the tip of the snout to the tip of the urostyle were used. Determinations of blood sugar, blood lactic acid and liver glycogen levels were made on animals which had remained in captivity less than forty-eight hours. In most cases animals were collected at night, and determinations were made the following morning. Prior to subjecting the toads to anoxia they were maintained for one hour in a 15-gallon aquarium containing one inch of water. Sex and length were recorded for each animal.

Anoxic Chambers

Anoxia was induced by placing the toads in one gallon jars which had been previously flushed with nitrogen. A constant flow of nitrogen was maintained to prevent accumulation of expired carbon dioxide. Upon removal from the chambers the toads were double-pithed. Inhalation of air was prevented by squeezing the animal.

Heartrate

Heartrates were recorded on an E & M Physiograph. Two pin electrodes were attached laterally, just behind either forelimb and a ground electrode was pinned to the animal's dorsum in the anal region. The animal was placed in a styrofoam box, 11.5 cm square, and allowed to acclimate for one hour. A subsequent recording of its heartrate was considered the control value. Nitrogen was then flushed into the box and a constant flow was maintained to prevent the accumulation of expired carbon dioxide. Heart-rate was recorded at five-minute intervals for 80 minutes. The toads' intermittant muscular activity occasionally interfered with recordings at the desired intervals.

Liver Glycogen Determinations

Approximately one gram of liver tissue was excised from each of 29 freshly killed animals. After it was blotted with a paper towel to remove excess blood and body fluid, the tissue was placed in a preweighed centrifuge tube containing 3 ml of 30% potassium hydroxide, and weighed. Glycogen concentration per gram was determined by the phenol-sulfuric acid method of Montgomery (1957).

Blood Sugar Determinations

A ventral incision was made to expose the heart. The cardiac membranes were incised, and the heart was pulled through a small hole in a paper towel which was placed over the incision. The paper towel absorbed body fluids, preventing their entrance into the collection tube. Blood was released by puncturing the tip of the ventricle. One ml of blood was collected in a calibrated centrifuge tube which had been previously treated with anti-coagulant (4% sodium fluoride and 4% potassium oxalate). Five ml of distilled water were added and the mixture was placed immediately on a Vortex shaker to hemolyze the blood cells. From a protein-free solution of the blood obtained by the zinc sulfate-barium hydroxide method (Somogyi, 1945), blood sugar concentration was determined using the Somogyi-Nelson procedure (Somogyi, 1945).

Blood Lactic Acid Determinations

Blood was obtained by ventricle puncture as in the glucose determinations. One ml of blood was collected in a calibrated centrifuge tube containing 1.0 ml of 6% perchloric acid. The mixture was shaken and centrifuged until the supernatant was clear (usually about 30 minutes). Lactic acid concentration was determined enzymatically (Sigma Chemical Co., 1965). Lactic dehydrogenase was used

to catalyze the oxidation of L (+) lactic acid to pyruvic acid with the subsequent reduction of beta-DPN to beta-DPNH. The samples were incubated at 37°C for 90 minutes. Optical densities of the resulting solutions were measured against a blank at 340 mμ on a Beckman DB Spectrophotometer.

CHAPTER III

RESULTS

Heartrate and Heartbeat

Heartrate decreased from a mean of 64 beats per minute (42-79) before exposure to anoxia to 22 beats per minute (14-33) after five minutes in anoxia. The rate continued to decrease, leveling off after 20 minutes to 10 beats per minute (Figures 1 and 2). In addition the heartbeat became sporadic and arrhythmic in some individuals (Figure 3). Although the toads remained quiescent after a few minutes in anoxia, some sporadic muscular activity was noted as much as 30 minutes later. When this activity occurred at one of the designated five-minute intervals, heartrate for that individual at that interval was not recorded. Two of the nine animals failed to show a heartbeat after only 20 minutes in anoxia. They appeared unconscious, but revived upon exposure to air. Heartrate could be recorded for only three toads after 55 minutes in anoxia. All toads recovered within two hours after removal from the anoxic chamber, even though one individual had been allowed to remain in anoxia for 190 minutes. This animal exhibited a heartrate of 10 beats per minute after the 190 minutes. Although it lived for two days subsequent to the experiment,

Fig. 1.--Heartrate during Anoxia

The mean (horizontal line), range (verticle line), and two standard errors (open rectangle) are included. Numbers above the verticle lines indicate the sample sizes.

Fig. 2.--Representative Heartrate of Female Bufo cognatus
at Selected Intervals during Induced Anoxia.

Fig. 3.--Arhythmicity in Heartrate of Female Bufo cognatus
at Selected Intervals during Induced Anoxia.

control

5 min.

20 min.

35 min.

it was never able to exhibit the "righting action" (Ray, 1958), suggesting damage to the central nervous system.

During the collection of blood for sugar and lactic acid determinations, it was observed that the contractions of an anoxic animal's heart were much more intense and prolonged than those of controls.

Circulatory Shunts

Blood flow in the anoxic specimens was evidently regulated to some extent by vasoconstriction and vasodilation. Vessels supplying and draining skeletal muscles and viscera appeared constricted, and blood flow from such vessels, when cut, was virtually non-existent. Cutaneous vessels were dilated, giving the anoxic animal a pinkish tint, particularly on the venter where melanin cells are less numerous.

Hepatic Glycogen

Individuals varied greatly with regard to hepatic glycogen concentration (Figure 4). Values in eleven controls ranged from 40-130 mg of glycogen per gm of tissue. Nonetheless, significant depletion of hepatic glycogen was obvious: the values dropped from a mean of 80 mg/gm tissue in the controls, to a mean of 30 (0-70) mg/gm tissue in animals subjected to anoxia for 60 minutes. A 50%

Fig. 4.--Hepatic Glycogen Depletion during Anoxia

reduction in mean glycogen level was evident after 40 minutes in anoxia.

Blood Sugar

Blood sugar levels showed a dramatic elevation after the animals had been subjected to anoxia (Figure 5). Values increased from a mean of 45 (26-75) mg % of blood in the controls to a mean of 101 (40-174) mg % after 20 minutes in anoxia. After 60 minutes in anoxia, animals averaged 144 (91-194) mg %.

Blood sugar concentrations in the controls varied with the length of the toad. The longer animals had less blood sugar per 100 ml of blood (Figure 6). No relationship between size and blood sugar level was obvious in the animals subjected to anoxia. Nonetheless, to eliminate as much bias as possible, only toads within the size range of the controls were used.

Lactic Acid Determinations

Blood lactic acid levels rose sharply from a mean of 28.8 (12.9-38.9) mg % in the controls to a mean of 82.6 (77.4-86.0) mg % (Figure 7) after 20 minutes in anoxia. Levels rose gradually to a mean of 88.2 mg % after 40 minutes, 90.3 mg % after 60 minutes, and 95.8 mg % after 80 minutes in anoxia. The increase after the initial 20 minutes was not significant.

Fig. 5.--Blood Sugar Elevation during Anoxia

Fig. 6.--Relationship between Blood Sugar Concentrations
and Size in Control Animals.

Fig. 7.--Lactic Acid Elevation during Anoxia.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Some physiological responses of Bufo cognatus to anoxia include depletion of liver glycogen stores with subsequent increases in blood sugar and lactic acid levels. These changes are accompanied by marked bradycardia and, in some cases, arrhythmicity of heartbeat. Cutaneous vasoconstriction and skeletal muscle vasodilation were also observed.

Individual variation in response to anoxia was striking throughout all the experiments. For example, a tendency for blood sugar to vary inversely as the size (snout-urostyle length) of the animal was obvious in controls, but not apparent in anoxic animals. Possibly this trend was masked by the quantitative individuality of responses to anoxia. Individual variation was great in heartrate before and during anoxia, particularly in the first 15 minutes of exposure to anoxic conditions.

The cardiovascular adjustments apparently serve to maintain most of the blood in the area of the heart and brain, where available oxygen can be most advantageously used (Popzopko, 1959). In addition, restriction of blood flow from muscles and viscera would prevent or postpone the build-up of lethal levels of lactic acid in the

vicinity of the heart and brain. Localization of blood increases the blood volume in the heart, causing distension of the cardiac muscles. The intense, prolonged heart contractions of anoxic individuals probably reflect Starling's law: increased blood volume in a vertebrate heart results in increased strength of the beat (Prosser and Brown, 1950). Bradycardia in anoxic individuals is believed to be a reflex due to vagal stimulation (Prosser and Brown, 1950).

Liver glycogen depletion and blood glucose increases during anoxia suggest anaerobic glycogenolysis. In addition, accumulation of lactic acid in the blood of an anoxic animal suggests the anaerobic utilization of glucose. The increase in blood lactic acid during anoxia, which incidentally, was not found by Leivestad (1960), supports Krogh's suggestion (1941) that a shift to anaerobic metabolism does occur in anoxic anurans. A shift to anaerobiosis, especially when accompanied by cardiovascular adjustments and a general lowering of metabolic rate, would be adaptive to anurans encountering oxygen stress while diving or burrowing.

BIBLIOGRAPHY

- Anderson, H. T. 1961. Physiological adjustments to prolonged diving in the American alligator, Alligator mississippiensis. Acta Physiol. Scand. 53: 23-45.
- Jackson, D. C. and K. Schmidt-Nielsen. 1965. Heat production during diving in the fresh water turtle, Pseudemys scripta. J. Cell Physiol. 67: 225-232.
- Jones, D. R. 1967. Oxygen consumption and heart rate of several species of anuran amphibia during submergence. Comp. Biochem. Physiol. 20: 691-707.
- Jones, D. R. and G. Shelton. 1964. Factors influencing submergence and the heart rate in the frog. J. Exp. Biol. 41: 417-431.
- Krogh, A. 1941. Comparative Physiology of Respiratory Mechanisms. University of Pennsylvania Press, Philadelphia.
- Leivestad, H. 1960. The effect of prolonged submersion on the metabolism and the heart rate in the toad. Arbok. Univ. Bergen 5: 1-15.
- Montgomery, R. 1957. Determination of glycogen. Arch. Biochem. Biophys. 67: 378-386.
- Murdaugh, H. V. and J. E. Jackson. 1962. Heart rate and blood lactic acid concentration during experimental diving of water snakes. Am. J. Physiol. 202: 1163-1165.
- Poczopko, P. 1959. Changes in blood circulation in Rana esculenta L. while diving. Zoologica Pol. 10: 29-43.
- Prosser, C. L. and F. A. Brown, Jr. 1950. Comparative Animal Physiology. W. B. Saunders Company, Philadelphia. Ch. 7.
- Robin, E. D., J. W. Vester, H. V. Murdaugh, and J. E. Millen. 1964. Prolonged anaerobiosis in a vertebrate: anaerobic metabolism in the freshwater turtle. J. Cell. and Comp. Physiol. 63: 287-297.

- Rose, F. L. and R. B. Drotman. 1967. Anaerobiosis in a frog, Rana pipiens. J. Exp. Zool., 166(3): 427-432.
- Sigma chemical company. 1965. The determination of L (+) lactic acid in blood, plasma, or other fluids. Technical bulletin # 825-UV.
- Somogyi, M. 1945. Determination of blood sugar. J. Biol. Chem. 160: 69-73.
- White, F. N. and G. Ross. 1965. Circulatory changes during experimental diving in the turtle. Am. J. Physiol. 211(1): 15-18.