THE UNIVERSITY OF MANITOBA

ADAPTIVE RESPONSES TO HYPOXIA IN THE FATHEAD MINNOW *PIMEPHALES PROMELAS*: BUOYANCY CONTROL AND UPTAKE OF AQUATIC AND AERIAL O_2

BY

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

WINNIPEG, MANITOBA

April, 1980

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ABSTRACT

Fathead minnows (*Pimephales promelas*) were exposed to hypoxia to determine changes in swimbladder gas composition, buoyancy, standard volume and internal pressure. Percentages of CO_2, O_2 and N_2 in the swimbladder did not change over 24 h in normoxic water. Evidence for gulping of air into the swimbladder, combined with uptake of O_2 during the first 12 h of exposure to hypoxia was indicated by: 1) increases in buoyancy and standard volume at the dissolved O_2 level of 0.5 ppm and 2) uptake of 31% of metabolic O_2 requirement directly from air (respirometer experiment). Declines in buoyancy, standard volume and percent swimbladder O_2 after 12 h of exposure to hypoxic water (0.5 ppm) suggested that gulping of air could no longer be sustained at previous levels. Uptake of aerial O_2 appeared to be a temporary and limited means of supplementing aquatic respiration during hypoxia. A model depicting the sequence of hypothesized and observed events leading to uptake of aerial O_2 and subsequent decline with prolonged exposure to hypoxia ($\leq$ 1.0 ppm) was constructed.

ACKNOWLEDGEMENTS

Sincere thanks to my supervisor Dr. Gee, for his patience and advise during the course of this study. To my committee members: Dr. Dandy, Dr. Rauch and Dr. Halvorson, my gratitude for their comments and suggestions in preparing this manuscript. This work was financially supported by the Natural Sciences and Engineering Research Council of Canada.

TABLE OF CONTENTS

PAGE

ABSTRACT i

ACKNOWLEDGEMENTS ii

LIST OF FIGURES iv

INTRODUCTION 1

MATERIAL AND METHODS 4

Changes in swimbladder composition over 24 h 4

Reaction to hypoxia 5
Changes in swimbladder gas composition and buoyancy 6
Changes in buoyancy, standard volume and internal pressure 7
O_2 uptake from air and water 8
statistical analysis 13

RESULTS 14

Changes in swimbladder composition over 24 h 14

Reaction to hypoxia 14
Changes in swimbladder gas composition and buoyancy 15
Changes in buoyancy, standard volume and internal pressure 17
O_2 uptake from air and water 21

DISCUSSION 24

Changes in swimbladder gas composition over 24 h.. 24

Reaction to hypoxia 25
Changes in swimbladder gas composition and buoyancy 25
Changes in buoyancy, standard volume and internal pressure 27
O_2 uptake from air and water 30

LITERATURE CITED 35

APPENDIX 38

LIST OF FIGURES

FIGURE		PAGE
1	Respirometer flask depicting: A, air phase; M, magnetic stirring bar; P, oxygen probe; S, septum; GS, glass syringe and W, water phase	10
2	Mean percent CO_2, O_2 and N_2 in the swimbladder of fish with access to the surface (solid line) and without access to the surface (dashed line) during exposure to hypoxic water at the level of 0.5 ppm. Vertical bars represent 95% confidence limits on the means	16
3	Effects of exposure to hypoxic water (hypoxia level 0.5 ppm) on buoyancy in fish with and without access to the surface. Vertical bars represent 95% confidence limits on the means	18
4	Frequency of opercular beats (upper curves) and dissolved O_2 (lower curves) for fish with access to the surface (solid lines) and without access to the surface (dashed lines) at the hypoxia level of 0.5 ppm. Vertical bars represent 95% confidence limits on the means. Limits less than 5 beats $\cdot$ min^{-1} were not represented	19
5	Relationships between dissolved O_2 and frequency of opercular beats on exposure to three levels of hypoxic water with access (A, B, C) and one level of hypoxic water without access (D). Changes in buoyancy ($mL \cdot g^{-1}$), internal pressure (kPa) and standard volume ($mL \cdot g^{-1}$) appear for each treatment (E, F, G, H). Vertical bars represent 95% confidence limits on the means	20

LIST OF FIGURES (continued)

FIGURE		PAGE
6	Frequency of opercular beats with declining levels of dissolved 0_2 (respirometer study). Vertical bars represent 95% confidence limits on the means	22
7	Model depicting sequence of hypothesized and observed events during exposure to hypoxic water ($\leq$ 1.0 ppm) in the fathead minnow. Arrows represent percent increases ↑ and decreases ↓ ; AB, absorbed swimbladder gas and EX, expired swimbladder gas	33

INTRODUCTION

The ability to exploit residual and alternative sources of O_2 in hypoxic water is of critical survival value to fishes inhabiting shallow ponds and streams. Many species possess morphological, physiological and behavioural adaptations for reducing O_2 demand and utilizing low levels of dissolved O_2 in a water column. When this source of O_2 becomes unavailable, fishes may either move to the surface and breathe air or utilize dissolved O_2 found in the upper 1-2 mm of the surface water. Considerable literature is available on the air-breathing habits of fishes but information is scant on aquatic surface respiration. Although described by Carter and Beadle (1931) it was found only recently to occur commonly amongst tropical and temperate species (Lewis 1970; Gee *et al*. 1978; Graham *et al*. 1978; Kramer and Meghan 1980).

Fishes adopting this mode of respiration rise to the surface and irrigate their gills with the relatively O_2 rich surface water. Activity is reduced to minimize metabolic O_2 demand and precise hydrostatic control is required to reduce metabolic costs associated with surface skimming (Gee *et al*. 1978). However, a reduction in swimbladder O_2 during asphyxia has been noted in a number of species (Black 1946; Jones and Marshall 1953), which could disrupt buoyancy

control during aquatic surface respiration if not replaced by other gases. The ability to resolve such a conflict by gulping air is possible in physostome fishes. Graham *et al*. (1978) suggested that such a response was an important step in the sequence of events leading to the evolution of aerial respiration, although nothing is known of the changes in buoyancy and composition of swimbladder gases that occur during the initial stages of hypoxia when fish commence aquatic surface respiration. The purpose of this research is to analyze such changes in the fathead minnow, *Pimephales promelas*.

The fathead minnow is a physostome found in weedy ponds and streams in central North America. Depending on the amount and state of vegetation, hypoxia may be encountered on a nocturnal or extended basis. Aquatic surface respiration has been demonstrated in the fathead minnow, although it possesses no apparent morphological adaptations to do so (Gee *et al*. 1978). The ability to use its swimbladder as an accessory respiratory organ is unknown. However, fathead minnows have been shown to secrete, reabsorb and gulp in response to changes in the velocity of normoxic water (Stewart 1980). Secretion and gulping were employed to increase buoyancy in response to decreased water velocity, while reabsorption led to a reduction in buoyancy when water velocity increased.

The specific objectives of this study were to describe changes in buoyancy and swimbladder gas composition that occurred in the fathead minnow during hypoxia and to assess the swimbladder for a possible respiratory function during low levels of dissolved 0_2. Three separate lines of investigation were undertaken:

1) proportions of gas present in the swimbladder were monitored over 24 h in fish held in normoxic water,

2) changes in swimbladder gas composition, buoyancy, standard volume and internal pressure were measured during exposure to hypoxia and

3) uptake of aquatic and aerial 0_2 were measured during self induced hypoxia in a respirometer study.

MATERIALS AND METHODS

Fathead minnows were collected from the Red River drainage basin, Manitoba, in September 1978 and October 1979. Fish were held at 10 $\pm$ 2°C under the 12L:12D photoperiod and fed daily. Prior to each experiment fish were acclimated for at least 7 d to 20 $\pm$ 2°C. Daily increments of 1°C were employed to reach the desired temperature.

Changes in swimbladder gas composition over 24 h

To determine if changes in the gas composition of the swimbladder over 24 h would offer an advantage to fish entering hypoxia during a particular period of the day, 48 fish (total length 4.0 - 7.0 cm) were sampled at random every 30 min from a larger group held in a (45 x 90 x 45 cm) aquarium. Light period extended from 0600 h to 1800 h and dark period from 1800 h to 0600 h. At night fish were sampled under red light illumination.

To sample swimbladder gases, fish were anesthetized in MS222, dissected to expose the swimbladder, the ductus *communicans* and pneumatic duct immediately tied off, and two gas samples were withdrawn (25 - 100 µL): one from each of the anterior and posterior lobes using 100 µL gas tight Hamilton syringes (Hamilton, Inc., Reno, Nevado). The needle

of the first syringe was inserted into a septum cap to prevent leakage, while the second sample was analyzed using a Carle Basic Gas Chromatograph 8700 (Carle, California), modified for respiratory gases (one Poropack QST 50/80 mesh and one molecular seive 5A separation column). Processing of the first sample immediately followed the second. Peaks for CO_2, O_2 (actually O_2 plus Ar) and N_2 were resolved on a strip chart recorder as each fraction passed the thermal conductivity detector in the gas chromatograph. Peak areas were calculated using the formula: peak area = maximum peak height · width of the peak at half the peak height. The instrument was calibrated using a known gas mixture of 9.59% CO_2, 50.98% O_2 and Ar (inseparable) and 39.4% N_2.

Reaction to hypoxia

As it was impossible to measure gas composition, buoyancy and buoyancy dependent variables on one fish, two experiments were completed to describe the reaction of fathead minnows to hypoxia. In the first, changes in swimbladder gas composition and buoyancy were described whereas in the second changes in buoyancy, standard volume and internal pressures of swimbladder gases were analyzed. In all experiments the number of opercular beats · min^{-1} was counted as an index of the stress induced by hypoxia. In hypoxia experiments 5 fish were measured for opercular beat frequency at each sampling time,

whereas 4 fish were measured for opercular beat frequency in the uptake of aerial and aquatic O_2 experiment at each sampling time. This was measured by taking the reciprocal of elapsed seconds per 60 beats.

Changes in swimbladder gas composition and buoyancy.

Fish with and without access to the surface were examined to determine if changes in swimbladder gas composition and buoyancy occurred during exposure to hypoxia. Fish (total length 4.0 - 7.0 cm) were placed individually in plastic cups 24 h in advance of the experiment. Cups providing access to the surface were positioned with tops 1 cm above the surface, cups denying access to the surface were screened at the top and suspended 5 cm below the surface. Batches of 8 fish each were sampled from both treatments at 0 h (in normoxic water) and 12, 36 and 60 h after hypoxia was reached (0.5 ppm). Cups were 66 mm deep, bottoms 90 mm in diameter, tops 55 mm in diameter, covered with a screen (non-access only) and two screened openings 20 mm in diameter on opposite sides. A series of moveable glass rod supports placed across the tank regulated the height of the cups, determining access or non-access to the surface.

Hypoxia was generated and maintained by regulating the flow rate of N_2 bubbled into the water through an airstone. Although N_2 effectively reduced dissolved O_2 concentrations, identical and consistent profiles were difficult to achieve.

Dissolved O_2 did not fluctuate during the 60 h of exposure to hypoxia beyond the values observed at each successive sampling time of 12, 36 and 60 h. Variations were within the limits of ascertaining responses made by fathead minnows during hypoxia. In view of the objectives a more elaborate method of stripping oxygen from the water was not adopted. Dissolved O_2 was determined using a YSI oxygen meter (Model 57). A continuous record of dissolved O_2 was obtained by means of a strip chart recorder connected to the YSI oxygen meter.

Gas samples were withdrawn and analyzed as above. Buoyancy was determined after Gee (1970) (Appendix 1) by dividing the volume of the swimbladder by the weight of the gas free fish in water. All weights were recorded to $\pm$ 0.001 g.

Volume of the swimbladder was determined by the following formula:

$$\frac{\text{wt fish in water} - \text{wt gas free fish in water}}{1.000\ \text{g} \cdot \text{mL}^{-1}\ (\text{water density } 4^{\circ}\text{C})}$$

(Appendix 1)

Changes in buoyancy, standard volume and internal pressure.

To determine if changes in buoyancy, standard volume and internal pressure occurred following exposure to hypoxic water, fish were examined at three levels of hypoxia with

access to the surface: 1.5, 1.0 and 0.5 ppm and at one level of hypoxia without access to the surface: 1.5 ppm. Cups, holding time before the experiment, sampling times, number of fish, size of fish and buoyancy measurements were as above. Volume of free swimbladder gas at atmospheric pressure and 20°C was determined by releasing swimbladder gas under water into a collecting funnel, suspended from an under-the-balance hook. Volume of gas released was calculated by the following formula:

$$\frac{\text{wt funnel without gas} - \text{wt funnel with gas}}{0.9982\ \text{g} \cdot \text{mL}^{-1}\ (\text{density of water } 20^{\circ}\text{C})}$$

(Appendix 1)

All weights were measured to ± 0.001 g. To determine standard volume of swimbladder gas, volume of gas released from the swimbladder was corrected to 101.3 kPa and divided by the weight of the gas free fish in water. Internal pressure was determined by dividing the volume of free swimbladder gas at atmospheric pressure by the volume of the intact swimbladder gas at atmospheric pressure, after substituting a water density of 1.000 g · mL^{-1} in the above formula. The theory and principles behind these measurements are presented in Appendix 1.

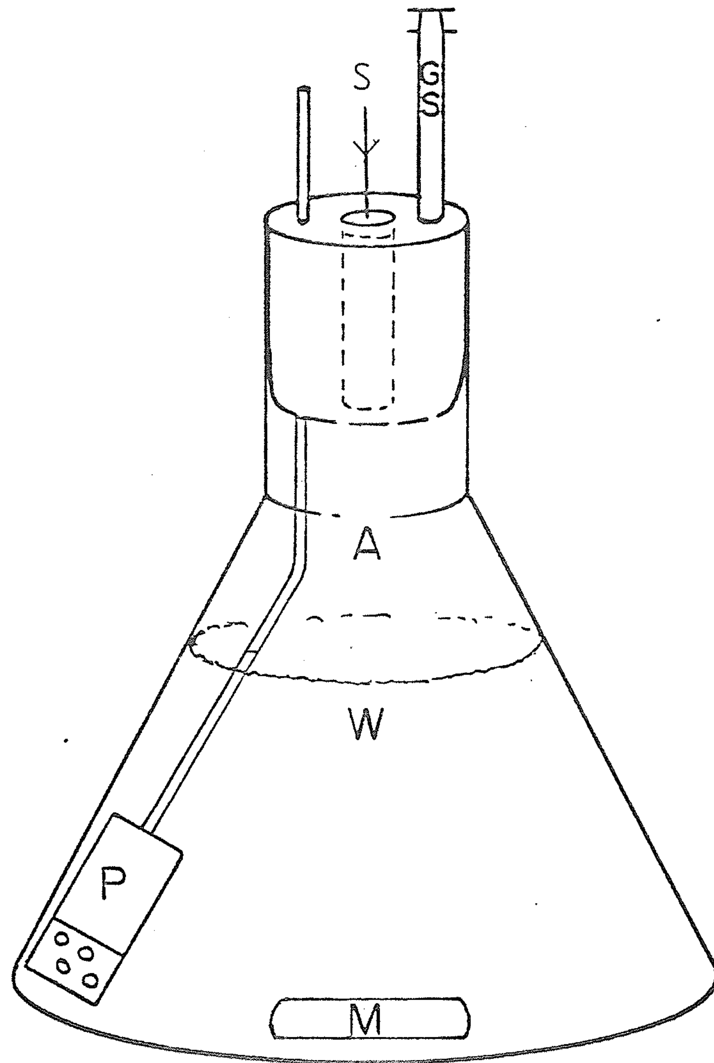
O_2 uptake from air and water

To determine if fathead minnows were able to take up O_2 directly from the air using the swimbladder as an accessory

respiratory organ, four fish were placed in a closed respirometer and decreases in aerial and aquatic 0_2 were monitored during self induced hypoxia. Utilization of aerial 0_2 would be demonstrated if aerial 0_2 declined faster than its diffusion rate into the water.

A 500 mL narrow mouth flask was used as a respirometer chamber. Fish were placed into the flask 24 h prior to the start of the experiment with a magnetic stirring bar in operation and an overflow of fresh water ($20 \pm 1^{\circ}C$). During this period the flask with fish was placed in a $20 \pm 1^{\circ}C$ water bath. The cable of the oxygen probe was cemented into a rubber stopper and the rubber stopper was fitted with a septum cap through which gas samples were withdrawn using a gas tight Hamilton syringe. The needle of a 1.0 cc glass syringe was driven through the rubber stopper to permit compensation for pressure differences upon inserting the rubber stopper into the flask and upon withdrawal of gas samples from the flask during the experiment. After 24 h the flask was submerged just below the water surface in a large aquarium (water, $20 \pm 1^{\circ}C$) and inverted, to retain 150 ± 0.5 mL of injected atmospheric air. The oxygen probe was then placed in the flask and the rubber stopper was inserted into the mouth of the flask and slowly tightened. The assembled apparatus was righted (Fig. 1), removed from the tank and the glass syringe filled with atmospheric air was attached to the needle.

Figure 1. Respirometer flask depicting: A, air phase; M, magnetic stirring bar; P, oxygen probe; S, septum; GS, glass syringe and W, water phase.


S
GS
A
W
P
M

The apparatus was placed back into the 20 $\pm$ 1°C water bath in which the water level reached the top of the rubber stopper but did not flood the septum cap area, thereby preventing contamination of gas samples with water. The magnetic stirrer was engaged and O_2 decrease in the air was determined using gas chromatograph samples drawn periodically through the septum, while O_2 decrease in the water was monitored using the YSI oxygen meter and strip chart recorder.

To determine milligrams of O_2 absorbed from the air within the flask it was assumed that the volume of N_2 in the air phase remained constant during the experiment. Since a known volume of air was injected, the volume of N_2 could be determined and the following equality applied:

$$\frac{\%\ O_2}{\%\ N_2} = \frac{\text{volume } O_2}{\text{volume } N_2} \text{ (constant term)}$$

solving for volume O_2

$$\text{volume } O_2 = \frac{\text{volume } N_2 \cdot \%\ O_2}{\%\ N_2}$$

Milliliters were converted to milligrams by solving for n (number of moles) in the equation PV = nRT (gas equation) for 20°C and atmospheric pressure.

O_2 utilized by the probe was determined by measuring O_2 uptake from a sealed vessel of water without air and under constant circulation.

Diffision rate of 0_2 from atmospheric air into hypoxic water (0.9 ppm) was determined by using the same volume of water and air as found in the respiration experiment but without fish. The level of dissolved 0_2 was lowered to a concentration of 0.3 ppm by bubbling N_2 into the water. The air phase in the flask was then continually flushed with atmospheric air, while the water was under circulation by the magnetic stirrer to allow for diffusion of excessive N_2 out of the water phase. When dissolved 0_2 increased to 0.9 ppm, the vessel was sealed the magnetic stirrer operated at the same speed as in the first experiment and 0_2 uptake into the water measured by using the YSI oxygen meter and strip chart recorder.

Two estimates of metabolic rate were obtained: the first based on 0_2 loss from the water in the first 10 min of sealing the flask (this will be an underestimation due to diffusion from air into water) and a second more accurate estimate based on 0_2 loss from the gas phase and water phase after a hypoxia level of less than 1.0 ppm was reached.

STATISTICAL ANALYSIS

All means were constructed with 95% confidence limits. Differences between means were judged to be biologically significant if a clear separation (non-overlap) of 95% confidence limits was observed. Regression analysis (linear) was used to determine the various rates of oxygen uptake in the respirometer experiment and was evaluated at the alpha level of 0.05.

RESULTS

Changes in swimbladder gas composition over 24 h

Three gases, CO_2, O_2 and N_2 accounted for 98 to 100% of the sampled volumes. Mean concentrations over 24 h adequately predicted swimbladder gas composition for an intermediate time interval, no cycles were observed, nor were differences found between swimbladder lobes. Mean percentages (n = 45) and 95% confidence limits were as follows:

Gas	CO_2	O_2	N_2
Anterior lobe	1.49 (± 0.10)	10.03 (± 0.96)	89.37 (± 0.98)
Posterior lobe	1.56 (± 0.20)	10.37 (± 1.04)	89.11 (± 1.03)

Compared to atmospheric air CO_2 and N_2 values were above and O_2 values below percent concentrations.

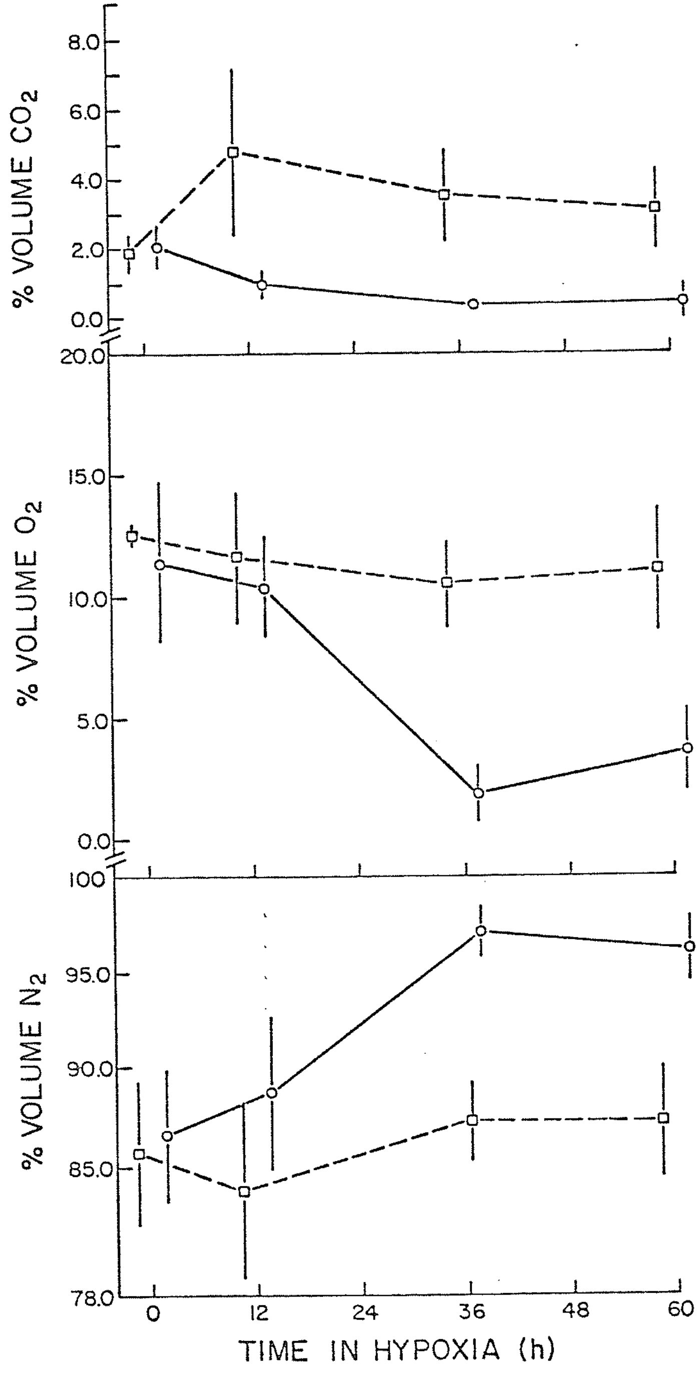
Reaction to hypoxia

Fathead minnows remained in the lower half of the cups until dissolved O_2 declined to 1.5 ppm, fish then rose to the surface or screens but immediately returned to deeper water. This behavior persisted in access fish throughout exposure to the hypoxia level of 1.5 ppm, whereas non-access fish remained pressed against the screens after 12 h at this same concentration of dissolved O_2.

At 1.0 ppm and lower fish with access remained just below the surface and swam around the perimeter of the cups. Non-access fish remained at the screens. Air bubbles were occasionally taken into the buccal cavity by access fish for approximately 10 seconds but this behavior was infrequent. Bubble retention appeared to be associated with accidental intake of existing bubbles at the surface. Otherwise fish did not demonstrate a gulping behavior at the surface that could be clearly sequenced and identified. However spitting of gas was observed in fish considered not to have taken an air bubble into the buccal cavity at the water surface.

Changes in swimbladder gas composition and buoyancy. In normoxic and hypoxic water, anterior and posterior lobes of the swimbladder were described by a common store of CO_2,O_2 and N_2 and were combined within each treatment (Fig. 2). The percentages of CO_2 and O_2 declined in fish with access to the surface (hypoxia level 0.5 ppm) and were complemented by an increase in N_2 (Fig. 2). Fish without access to the surface (hypoxia level 0.5 ppm) demonstrated no change in swimbladder gas composition during the 60 h of exposure to hypoxic water but compared to access fish after 12 h, CO_2 and O_2 were higher and N_2 was lower (Fig. 2).

Figure 2. Mean percent CO_2, O_2 and N_2 in the swimbladder of fish with access to the surface (solid line) and without access to the surface (dashed line) during exposure to hypoxic water at the level of 0.5 ppm. Vertical bars represent 95% confidence limits on the means.



% VOLUME CO2
8.0
6.0
4.0
2.0
0.0
% VOLUME O2
20.0
15.0
10.0
5.0
0.0
% VOLUME N2
100
95.0
90.0
85.0
78.0
0
12
24
36
48
60
TIME IN HYPOXIA (h)

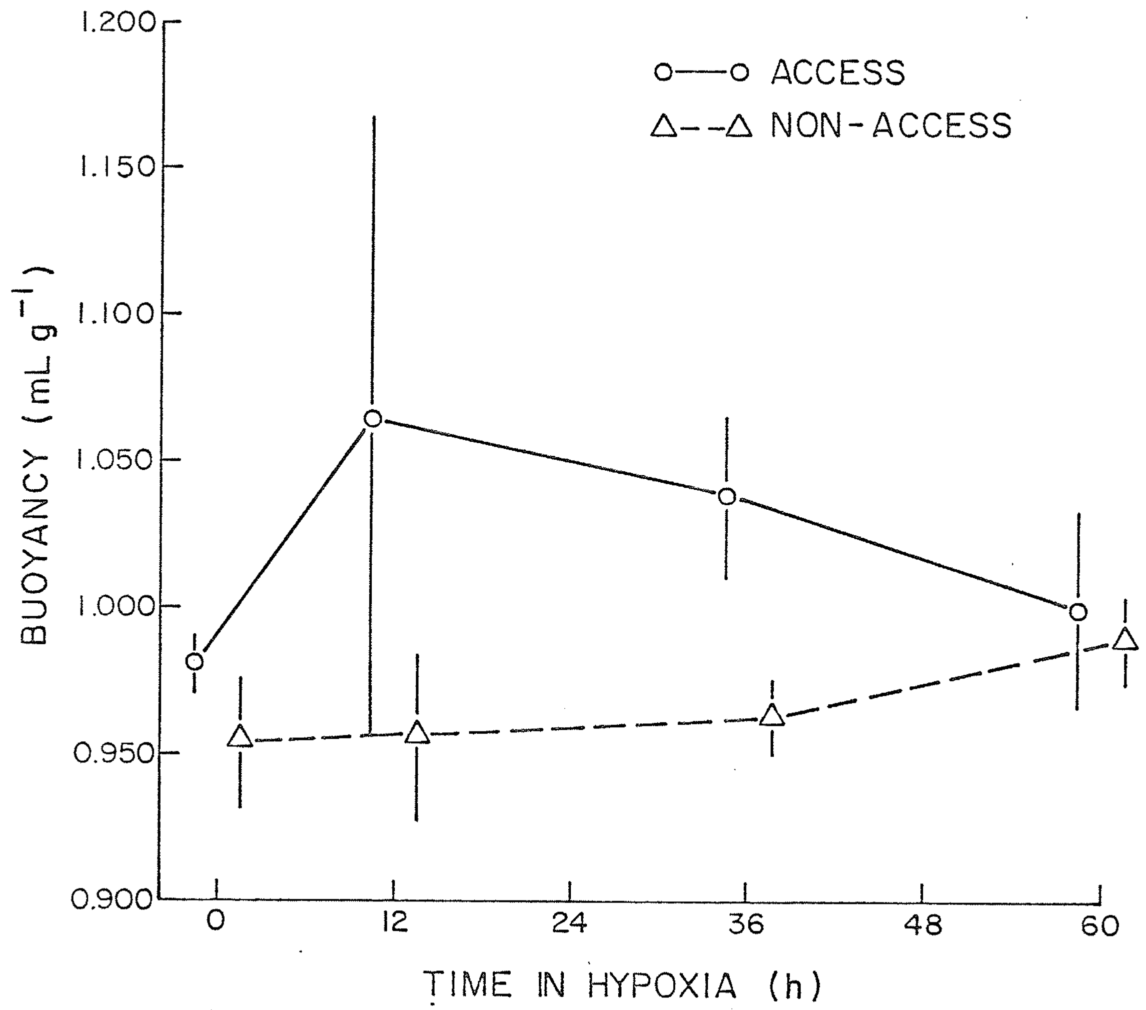
In fish with access to the surface buoyancy increased at 12 h but not to a degree considered significant in this study; however, a large increase in variance was observed at 12 h (Fig. 3). Fish without access to the surface did not demonstrate a change in buoyancy during the 60 h of exposure to hypoxic water; nor did variance increase at 12 h as observed in fish with access to the surface.

Opercular beats $\cdot$ min $^{-1}$ responded predictably with dissolved O_2 concentrations (Fig. 4). Access fish maintained an elevated frequency of opercular beats during consistently low concentrations of dissolved O_2 and non-accessfish decreased the frequency of opercular beats with increasing concentrations of dissolved O_2.

Changes in buoyancy, standard volume and internal pressure. The frequency of opercular beats (Fig. 5 B-D) was observed as above. Fish with access to the surface at the hypoxia level of 1.5 ppm (Fig. 5 A) did not follow this pattern and were able to decrease the frequency of opercular beats without the associated increases in dissolved O_2.

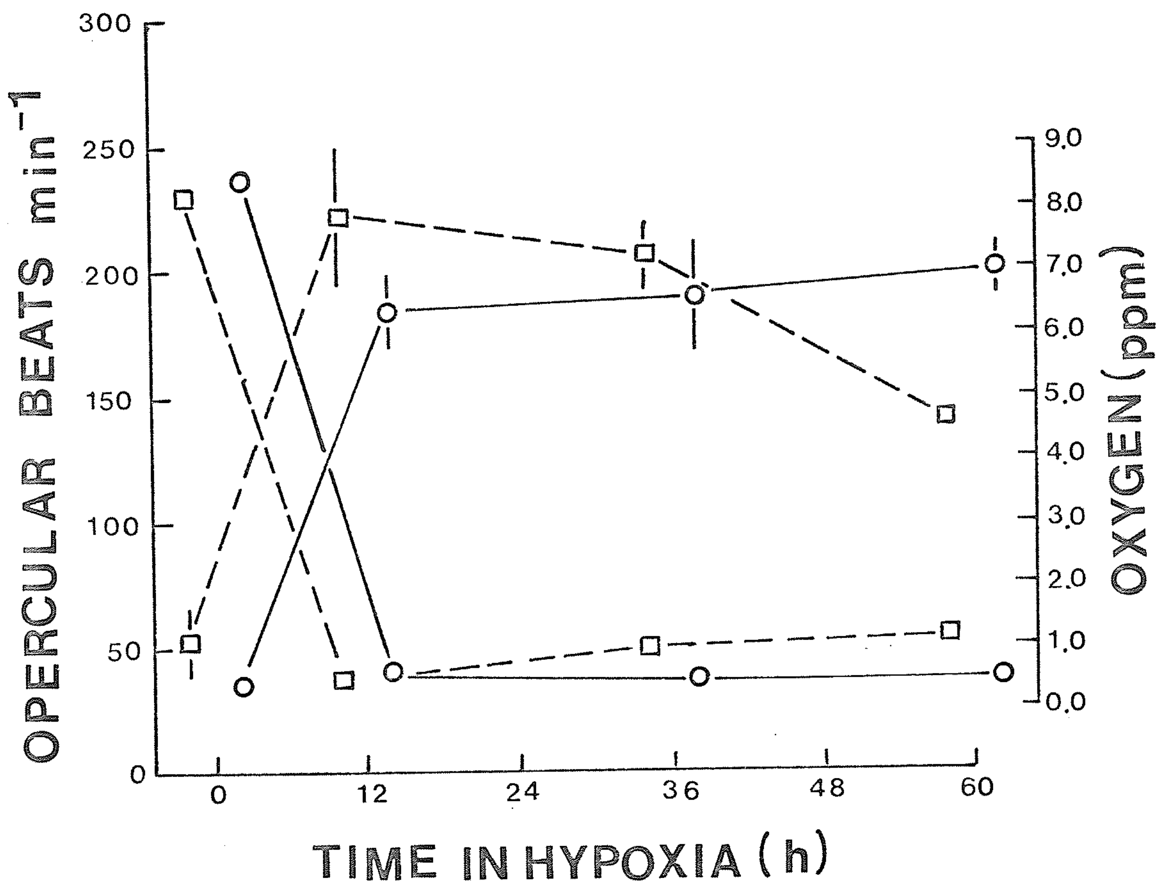
Fish without and with access to the surface at the hypoxia level of 1.5 ppm demonstrated no change in

Figure 3. Effects of exposure to hypoxic water (hypoxia level 0.5 ppm) on buoyancy in fish with and without access to the surface. Vertical bars represent 95% confidence limits on the means.



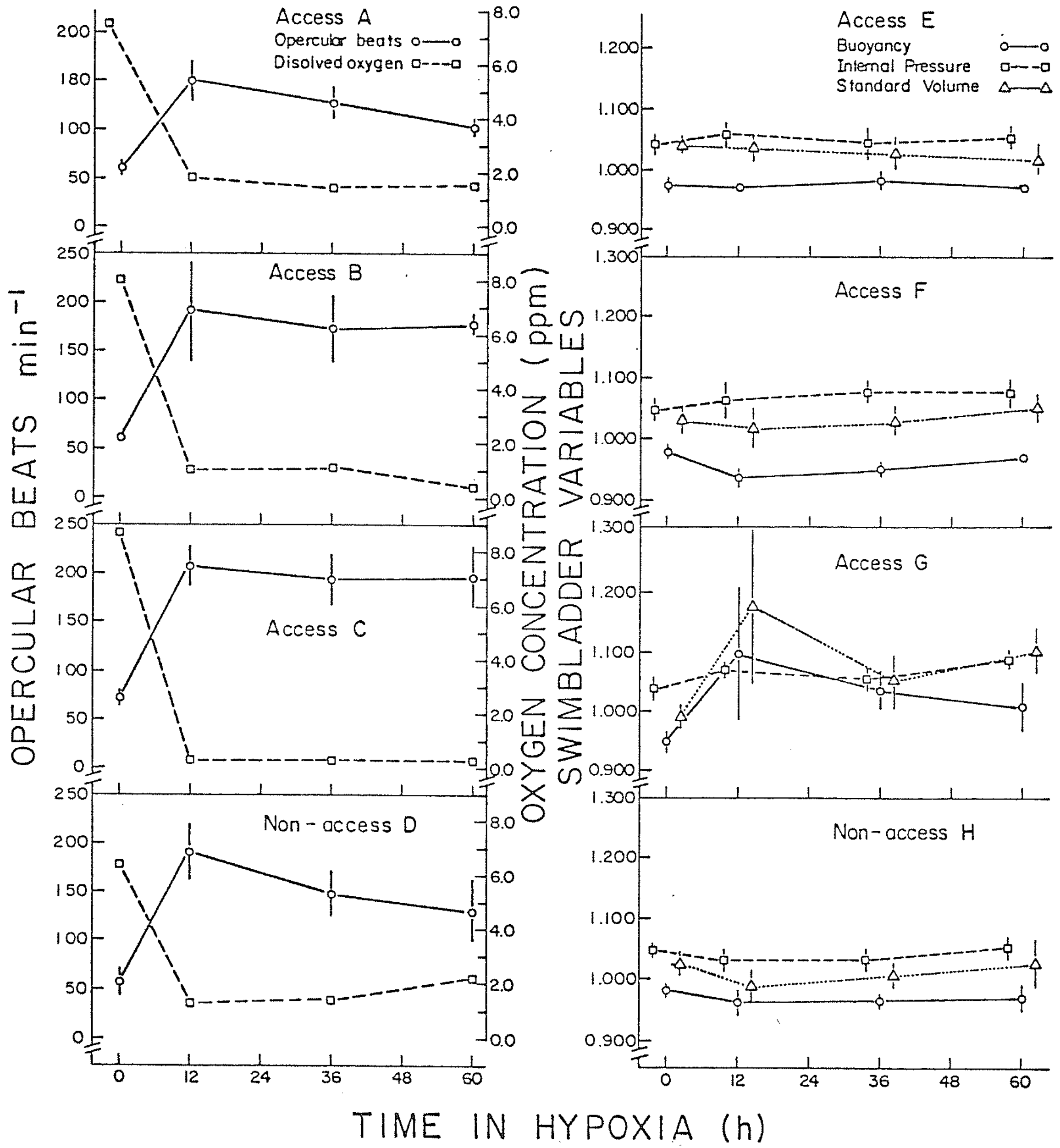
1.200
1.150
1.100
1.050
1.000
0.950
0.900
BUOYANCY (mL g^{-1})
o—o ACCESS
△--△ NON-ACCESS
0
12
24
36
48
60
TIME IN HYPOXIA (h)

Figure 4. Frequency of opercular beats (upper curves) and dissolved O_2 (lower curves) for fish with access to the surface (solid lines) and without access to the surface (dashed lines) at the hypoxia level of 0.5 ppm. Vertical bars represent 95% confidence limits on the means. Limits less than 5 beats · min^{-1} were not represented.



OPERCULAR BEATS min^{-1}
300
250
200
150
100
50
0
0
12
24
36
48
60
TIME IN HYPOXIA (h)
OXYGEN (ppm)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0

Figure 5. Relationships between dissolved O_2 and frequency of opercular beats on exposure to three levels of hypoxic water with access (A, B, C) and one level of hypoxic water without access (D). Changes in buoyancy (mL · g^{-1}), internal pressure (kPa) and standard volume (mL · g^{-1}) appear for each treatment (E, F, G, H). Vertical bars represent 95% confidence limits on the means.



Access A
Opercular beats
Disolved oxygen
Access B
Access C
Non - access D
Access E
Buoyancy
Internal Pressure
Standard Volume
Access F
Access G
Non-access H
OPERCULAR BEATS min⁻¹
OXYGEN CONCENTRATION (ppm)
SWIMBLADDER VARIABLES
TIME IN HYPOXIA (h)