This is to certify that the

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PATHOGENESIS OF GAS BUBBLE TRAUMA
IN RAINBOW TROUT (Salmo gairdneri)

presented by

Joao Paciano Machado

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Major professor

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PATHOGENESIS OF GAS BUBBLE TRAUMA IN RAINBOW TROUT (*Salmo gairdneri*)

By

Joao Paciano Machado

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ABSTRACT

Pathogenesis of Gas Bubble Trauma in Rainbow Trout (Salmo gairdneri)

By

Joao Paciano Machado

The sudden and usually fatal appearance of bubbles within the body of fish is known as gas bubble trauma (GBT). For over 80 years GBT has been associated with excessive pressure of atmospheric gases in water but knowledge of the pathogenesis of the disease has remained incomplete. This laboratory investigation was undertaken to test the hypothesis that the first nucleation of gas emboli is associated with accelerated unloading of oxygen from hemoglobin into gas supersaturated blood. Three temporally and methodologically separate investigations determined (1) the early-phase gas emboli association with specific tissue/organ type, (2) the effect of carbon monoxide (CO) induced hemoglobin dysfunction on the development of GBT and (3) the association of altered blood and red cell pH with

the onset of the disease.

A detailed histologic study revealed the pathogenesis of the early stages of gas emboli formation. Fish were sampled during GBT convulsions and preserved by a rapid fixation method. Lesions were found only in tissues associated with high metabolic requirements or with acid secreting glands. A lesion found in every treated fish but never in controls was gas displacement of the blood from the afferent arteriole within the gill filaments. Following the development of small gas emboli in the acidic environment of retinal chorio-capillaries, a progressive development of exophthalmia was detected.

High mortality resulted from a chronic exposure of fish to water containing graded levels of atmospheric gases from 114 to 118% N_2 and 103 to 110% O_2 supersaturation. Fish held in N_2 varying between 103 and 117% and O_2 from 50 to 94% saturation experienced insignificant mortality. The effect of carbon monoxide (CO) induced hemoglobin dysfunction on the development of GBT was studied at 130% total dissolved gas saturation (TDGS). A prior exposure to carbon monoxide which converted 80% of the hemoglobin to carboxyhemoglobin (COHb) achieved a significantly prolonged survival time, but the cumulative mortality was insignificantly reduced. The respiratory rate of both control and CO-exposed fish slowed greatly when supersaturation was imposed. Carbon monoxide treated fish

maintained a relatively higher respiratory rate for a time period which was similar to their prolonged survival time. It was concluded that the initial formation of gas emboli preceding fatal GBT was delayed as a result of a CO-inhibition of hemoglobin function.

The association of altered blood and red cell pH with the onset of the disease was examined during supersaturation exposure. A detailed investigation of selected hematological parameters revealed that the GBT convulsive stage is preceded by a red blood cell pH drop below 7.00 which is the optimal pH range for Root effect hemoglobins.

To Teresa with love

ACKNOWLEDGEMENTS

I would like to express my appreciation to my major advisor, Dr. Donald L. Garling Jr., for his advice and assistance thoughout my graduate program. My deepest thanks go to my research director, Dr. Thomas G. Bell. I am indebted to him for providing direction, encouragement and moral support. He shared his technical expertise, suggesting new experimental approaches and discussing the interpretation of my results.

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Finally, I would like to express my sincerest thanks to my mother and father for their encouragement and support.

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INTRODUCTION

Gas bubble trauma (GBT), gas bubble disease, is a pathological condition caused by environmental factors that may occur whenever fish are exposed to water supersaturated with atmospheric gases. Marsh and Gorham (1905), biologists at the Fisheries Aquarium at Woods Hole, Massachusetts, were the first to record incidence of GBT in fishes. Many studies have since been published which deal with gas embolism, especially in the late 1960's, when high fish mortalities were associated with hydroelectric projects in the northwest United States.

Problems with GBT in fish culture operations are typically associated with water from wells which have had air introduced by the pumping process or from springs that have become supersaturated with gases. In a body of water, changes in water flow, temperature or biologic homeostasis may result in total dissolved gas pressure (TDGP) over 100%, a supersaturation condition often resulting in GBT. Commonly, in spring run-off flooding, cascading river water plunges over hydraulic-fill dams deep into the post-barrage pool and the pressure change results in elevation of TDGP.

High mortality has occurred yearly in some Michigan

State fish hatcheries. For example in the newly constructed Harrietta State Fish Hatchery, personnel reported a 92% mortality in rainbow trout (Salmo gairdneri) juveniles in 1981. In 1982, over two thirds of the steelhead (Salmo gairdneri) fry and fingerlings died at Wolf Lake Hatchery, and in 1983 losses of steelhead and brown trout (Salmo trutta) were as high as 70%. Pendill's Creek National Fish Hatchery lost 1.3 million lake trout (Salvelinus namaycush) in 1983. The Marquette Fish Hatchery lost all of their two million lake trout fingerlings in 1984.

Because little is known about the cause and effect relationship of the gas supersaturation condition and the physico-chemical factors involved in bubble nucleation, it is important to investigate the pathogenesis of gas embolism in fish affected by GBT. A clearer understanding of this problem as it occurs in hatcheries might lead to better design for new hatcheries and other water related construction projects.

Fish with GBT, both in hatcheries and in the natural watershed, have a similar pathologic presentation. The formation of microbubbles in the blood of fish exposed to supersaturated water is responsible for gas emboli which block the circulation and cause death by circulatory failure (Harvey 1974).

Reports describing the symptoms observed in trout

affected by the disease included loss of equilibrium, abnormal buoyancy and aimless swimming as the convulsive stage commenced (Stroud et al. 1975; Bell et al. 1986). At that stage, fish swim side and belly-up with violent whirling movements interspersed with periods of inactivity followed by spasmodic convulsions (the moribund stage) leading to death.

Gross lesions in fish with GBT have been well described (Rucker 1972; Stroud and Nebeker 1976; Bouck 1980). Fish usually develop the classic signs of GBT such as subepithelia emphysema of the head, external and internal surface of the operculum, along the branchiostegal region, inside the mouth, and on the gills and in the fins.

In fish, detailed reports of the histopathology of GBT are limited (Pauley and Nakatani 1967; Nebeker et al. 1976; Bell et al. 1986; Smith 1987). Observations of lesions in these studies demonstrated primarily gas emboli in vessels of gill filaments, atrium, ventricle, bulbus arteriosus, ventral aorta and coronary arteries. In addition, associated with exophthalmia, gas emboli were observed (Bell et al. 1986) in the blood vessels of the retinal choroid gland.

Some of the difficulty in determining the nature of GBT in fishes may be explained by the rapid disappearance of the gas emboli after death (Harvey 1974; Bouck 1976; Weitkamp

and Katz 1980). A method for evaluation of gas embolism lesion was developed (Bell et al. 1986) and this fixation technique has shown to be useful for laboratory diagnosis of GBT since gas emboli were better preserved than with standard techniques.

A GBT pathogenesis hypothesis by Stroud and Nebeker (1976) is that undefined physiological conditions may trigger a "cascading bubble effect" of gas emboli formation which cause blockage of blood flow within the vessels of the gills leading to an acute death. Previous to this report, Bell and Farrel (1972) suggested a hypothesis based on the assumption that violent oxygen unloading, characteristic of some fish hemoglobin, forms microbubbles of oxygen. If the blood is normally saturated with atmospheric gases, these bubbles will dissolve by diffusion (Epstein and Plesset 1950). On the other hand, in GBT conditions, once the nuclei are formed, the high gas tensions of blood may cause these microbubbles to build and form larger emboli.

This study was performed in an attempt to elucidate the bubble nucleation condition, by further investigating the Bell and Farrell hypothesis. Our objectives, performed in three series of experiments were: (1) to determine if the early phase emboli distribution was associated with specific tissue/organ types under variable, graded water supersaturation conditions; (2) to test the hypothesis that

a CO-induced hemoglobin dysfunction could prevent the development of GBT and (3) to determine the effects of blood and red cells pH changes in fish affected by GBT.

CHAPTER 1. Histopathology

A. Introduction

The major objective of this study was to determine if the early-phase emboli distribution was associated with specific tissue/organ types under variable, graded water supersaturation conditions similar to those found in high-mortality hatcheries.

B. Materials and Methods

Two trials were conducted using the same experimental design and apparatus. Fish used in trial 1 were rainbow trout fry, nine weeks old, with an average total length of 4.5 cm. They were reared in the Aquaculture Research Laboratory at Michigan State University. For trial 2, the same size, age and type of fish were obtained from the Baldwin Fish Farm, Baldwin, Michigan. Fish were fed twice a day with Biodiet Grower (Bioproducts, Inc., Astoria, OR, USA). The fish were exposed to artificial light that simulated a natural photoperiod with a ratio of 14 light to 10 dark hours a day.

To create a higher than normal gas tension in the rearing trays, well water was supersaturated by pumping air

into a vertical column through an air-diffusing stone. Pressurized air was pumped into the base of the 4 m high by 15 cm in diameter water column.

The water column was contained in a (PVC) pipe, open on the upper end to allow excess gas to escape and fitted with an overflow system. Supersaturated water was released from the base of the column into the rearing trays placed 30cm above one another. Each tray measured 38cm x 50cm x 12cm and held 16.2L. In trial 1, the flow rate was adjusted to produce a water renewal in the trays of 200 mL/min. The waterflow was changed to 400 mL/min at the 46th day of the 60-day test. In trial 2, the waterflow in the trays was maintained at 400 mL/min during the course of the 20-day test, to determine if the mortality in trial 1 was a result of alterations in individual gas pressures resulting from change in waterflow.

Each trial was designed with four treatments. The first three treatments (A, B, C) had four replicates and the fourth reference treatment (D) had three replicates.

The desired level of supersaturation for each test tray was maintained by a waterflow cascading system. The water was allowed to cascade in free-fall through 30cm x 4cm PVC pipes in transit from tray to tray. The splashing action allows the water contact with air at atmospheric pressure therefore decreasing the level of supersaturation. In both

trials, treatment A had the highest supersaturation levels. The gas saturation levels progressively decreased as the water flowed down the trays to treatment B and C. Treatment D, which had the same well water source, but did not undergo supersaturation, served as a reference or the true control. Specimens taken from reference trays were used as control samples.

The well water supply at the Aquaculture Laboratory has a nitrogen supersaturation of 105% which is similar to that in hatcheries where problems with GBT have been occurring. In the control group, treatment D, in which the water used did not undergo artificial supersaturation, the levels of nitrogen ranged from 104 to 106% saturation throughout the test. The total dissolved gas saturation (TDGS) and oxygen saturation were below 100%.

Fish in the fifteen shallow test trays from the two thousand cohort holding tank were allocated to groups in a fashion similar to dealing a deck of cards. The first group of five fish netted from the holding tank was placed in the first test tray, the second group of fish in the second tank, etc. The sixteenth group of five fish was placed in the first tray, the seventeenth in the second tray, etc. This was continued until each test tray contained fifty fish.

Dissolved oxygen, total dissolved gas pressure,

barometric pressure, water temperature, and water vapor pressure were measured weekly in every test tray. Dissolved oxygen was determined by the modified Winkler method utilizing a Hach's Digital Titrator, Loveland Co. An electronic Tensionometer model 300C, Novatech Designs LTD, Victoria, British Columbia, Canada was used to determine supersaturation by measuring the total gas pressure in mmHg of all dissolved gases in the water, called differential pressure (dP). The tensionometer also measures the local barometric pressure (B). Bunsen's solubility coefficient for oxygen was obtained from tables in Weast (1980).

The test well water had the following known chemical and physical characteristics: hardness (as $CaCO_3$), 330 mg/L; alkalinity (as $CaCO_3$), 260 mg/L; pH, 7.5 to 7.7; and temperature, 12.0 $\pm$ 1.0°C.

Saturation levels of all gases were determined by computer using as a basic program the following formulae improved by Bouck (1982).

1. Total dissolved gas saturation = $\dfrac{100\ (dP + B\ )}{B}$

2. O_2 % sat. = $\dfrac{\text{(oxygen pressure in water)}\ \ 100}{\text{(oxygen pressure in air)}}$

$$= \frac{(PO_2)\ 100}{(B - PH_2O)\ O_2 \text{ mole fraction in dry air}}$$

$$= \frac{(PO_2)\ 100}{(B - PH_2O)\ 0.2095}$$

$$3.\ N_2\ \%\ \text{sat.} = \frac{(PN_2)\ 100}{(N_2\ \text{mole fraction in dry air})(B - PH_2O)}$$

$$= \frac{(PN_2)\ 100}{(0.78084)\ (B - PH_2O)}$$

When fish with typical signs of gas bubble disease became moribund they were collected from trial 1 for histological examination. Asymptomatic fish from the control trays were used for comparison. A recently developed technique utilizing heated Bouin's solution for rapid fixation of the tissues was used (Bell et al. 1986). During the test, 12 moribund fish were selected for histological study along with an equal number of control fish. At the end of 60 days of the experiment, the 422 survivors were examined and 104 were randomly selected for microscopical study. Representative gross and histologic lesions were photographed.

Each specimen was sampled so that whole fish, viewed with a dissecting microscope (macroscopic examination) as well as fixed multiple microscopic sections were surveyed. It was thus possible to make gross and microscopic comparisons. Lesions were categorized by making a blind comparison among treated and control fish. As gross lesions

were encountered, additional sections of gills, liver and kidney were made and examined.

Mortality curves (percent mortality versus time exposure) were plotted for each level of supersaturation. Mortality data were calculated to one half of one percent. Mortality rate among treatments was compared using chi-square tests. The Bonferroni chi-square test was used to compare each experimental group with the control group.

C. Results

1. Mortality

The exposure of fish to the highest supersaturation level, treatment A, resulted in a high morbidity and mortality, reaching 80% at the end of the 60-day test in trial 1, and 50% after the 20-day test in trial 2. In the latter trial, the exposure differed only in the constant water flow conditions and in the shorter duration of the exposure (Figure 1). Signs consistent with GBT were found in moribund or recently dead fish.

In both trials, fish held in treatments B, C and D experienced no mortality. The levels of nitrogen saturation in these treatments varied from 103 to 111%. It is emphasized that these test groups had oxygen levels below 100%, varying from 50 to 94% saturation (Tables 1 and 2).

Figure 1. The effect of oxygen on the rate of cumulative mortality (Treatment A, Tables 1 & 2). Exposure of 3-mo-old rainbow trout to nitrogen supersaturation ranging from 114 to 118% produces significant mortality only if oxygen is also supersaturated. Upward water flow adjustment during trial 1 (box) from 200 to 400 mL/min resulted in both oxygen supersaturation and mortality in treatment A of trial 1 but neither in treatments B, C and D (not shown in Figure 1). In trial 1 oxygen saturation (solid circles) was elevated from day 46 to 60 and the cumulative mortality was accelerated during that period. Trial 2, a 20-d experiment, was designed to repeat the final 14 d of trial 1 with constant water flow to test if mortality was due to elevation of oxygen (open circles) or due to changing the water flow. The oxygen saturation standard error is shown as bars extending from those values.

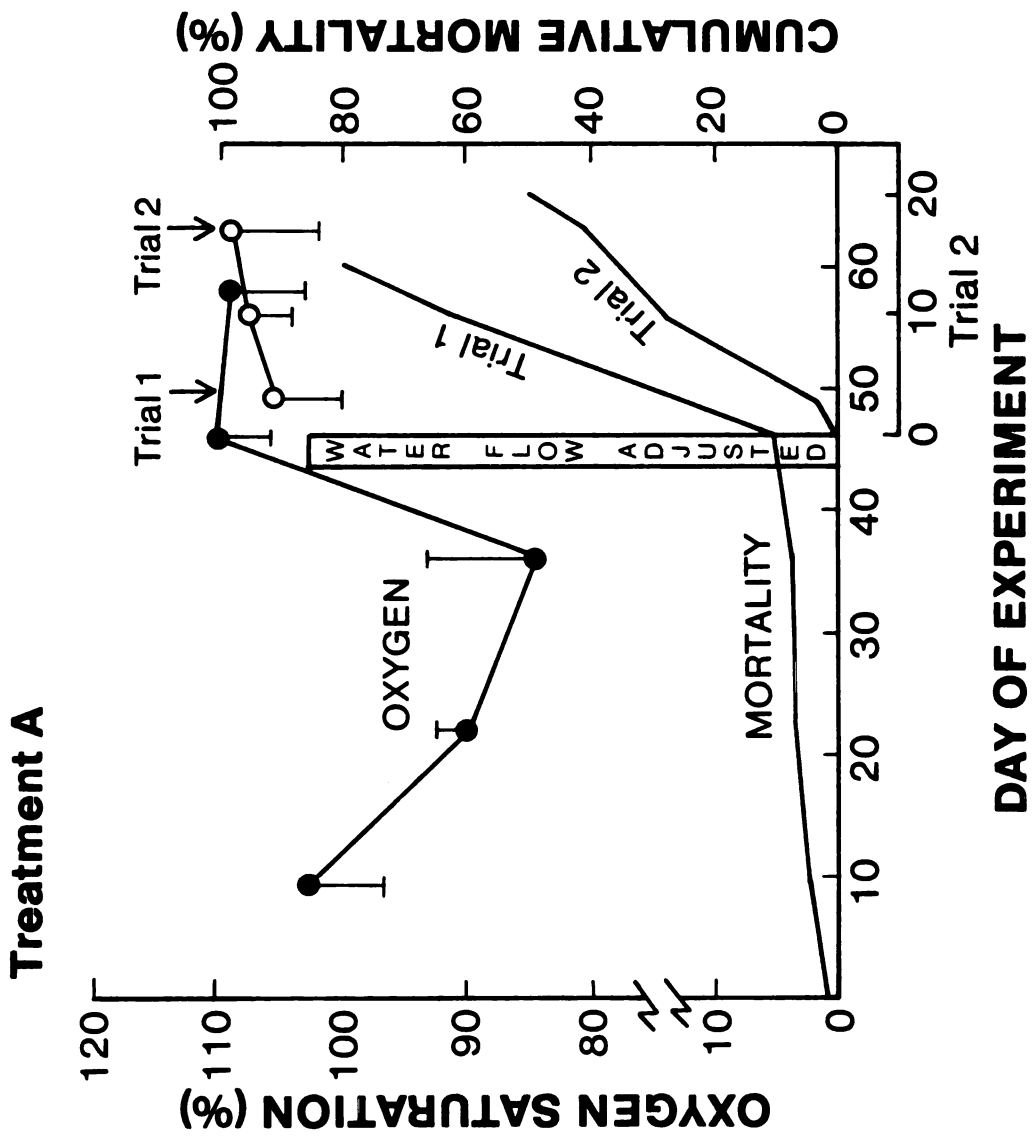
Treatment A
OXYGEN SATURATION (%)
CUMULATIVE MORTALITY (%)
DAY OF EXPERIMENT
Trial 1
Trial 2
OXYGEN
MORTALITY
WATER FLOW ADJUSTED
Trial 1
Trial 2
Trial 2

Figure 1

Table 1. Numbers of fish in exposure, mortality and dissolved gas content of the experimental water at 12°C. Rainbow trout were exposed for 60 days to varying levels of dissolved gases (Trial 1).

	% Cumulative[a] Mortality (test day)	Total dissolved gas in % satur. mean ± t 0.05 SE	Nitrogen in % saturation mean ± t 0.05 SE	Oxygen in % saturation mean ± t 0.05 SE
Treatment A	5.0 (9)	113.7 ± 0.9	116.9 ± 1.5	102.6 ± 6.2
(n = 200)	7.0 (22)	109.8 ± 0.5	115.2 ± 0.6	89.8 ± 2.2
	8.5 (36)	110.2 ± 1.2	117.2 ± 0.8	84.5 ± 8.4
	11.0 (46)	115.7 ± 1.2	117.5 ± 0.4	110.1 ± 4.6
	63.5 (56)	115.6 ± 1.2	117.8 ± 0.4	108.7 ± 5.4
Treatment B	0.0 (9)	103.2 ± 1.1	108.0 ± 0.1	85.3 ± 5.4
(n = 200)	0.0 (22)	98.7 ± 1.4	108.0 ± 1.0	63.0 ± 5.4
	0.0 (36)	98.3 ± 2.4	108.3 ± 2.4	60.5 ± 7.9
	0.5 (46)	105.1 ± 0.9	109.4 ± 0.6	89.9 ± 2.4
	0.5 (56)	105.1 ± 1.0	109.9 ± 0.3	87.7 ± 3.4
Treatment C	0.0 (9)	98.4 ± 1.0	104.5 ± 0.2	75.3 ± 2.9
(n = 200)	0.0 (22)	94.8 ± 0.9	104.7 ± 0.6	57.0 ± 5.7
	0.5 (36)	94.1 ± 0.7	105.6 ± 2.0	50.2 ± 9.9
	0.5 (46)	99.1 ± 0.5	106.4 ± 0.3	71.7 ± 3.4
	0.5 (56)	99.2 ± 0.5	106.9 ± 0.4	70.1 ± 4.1
Treatment D	0.0 (9)	97.6 ± 2.7	104.9 ± 3.1	70.2 ± 1.2
(n = 150)	0.5 (22)	96.9 ± 2.9	104.4 ± 3.1	68.4 ± 2.5
	0.5 (36)	94.7 ± 3.6	106.5 ± 3.9	49.9 ± 2.5
	0.5 (46)	96.3 ± 2.2	106.4 ± 1.2	57.9 ± 5.9
	0.5 (56)	96.2 ± 2.1	106.5 ± 0.9	57.3 ± 7.2

[a] Total mortality after 60-day test for treatments A through D: 80.0, 0.5, 0.5 and 0.5 percent, respectively.

Table 2. Numbers of fish in exposure, mortality and dissolved gas content of the experimental water at 12°C. Rainbow trout were exposed for 20 days to varying levels of dissolved gases (Trial 2).

	% Cumulative[a] Mortality (test day)	Total dissolved gas in % satur. mean ± t 0.05 SE	Nitrogen in % saturation mean ± t 0.05 SE	Oxygen in % saturation mean ± t 0.05 SE
Treatment A	2.5 (3)	111.6 ± 0.7	113.5 ± 1.5	105.1 ± 5.7
(n = 150)	28.0 (10)	113.0 ± 0.5	114.6 ± 0.2	108.1 ± 4.0
	41.5 (17)	113.4 ± 1.7	114.9 ± 1.7	109.0 ± 7.4
Treatment B	0.5 (3)	105.7 ± 1.0	109.2 ± 1.2	93.1 ± 0.7
(n = 150)	0.5 (10)	105.8 ± 0.7	109.2 ± 1.0	93.5 ± 7.4
	0.5 (17)	105.8 ± 2.2	111.4 ± 3.0	85.2 ± 3.7
Treatment C	1.0 (3)	101.9 ± 0.2	106.1 ± 1.0	86.2 ± 4.5
(n = 150)	1.0 (10)	101.8 ± 1.0	107.4 ± 2.7	81.1 ± 7.9
	1.0 (17)	100.7 ± 0.7	107.4 ± 3.0	75.1 ± 8.9
Treatment D	0.5 (3)	99.0 ± 2.7	104.3 ± 0.7	79.2 ± 6.4
(n = 150)	0.5 (10)	100.0 ± 1.7	104.3 ± 0.2	83.5 ± 8.9
	0.5 (17)	100.3 ± 1.5	103.2 ± 1.0	89.4 ± 6.4

[a] Total mortality after 20-day test for treatments A through D: 50.0, 0.5, 1.0 and 0.5 percent, respectively.

The onset of mortality, in trial 1, occurred in treatment A two days after exposure to 116.9 $\pm$ 0.7% nitrogen and 102.6 $\pm$ 6.2% oxygen saturation. Progressive mortality reached 6% in the first ten days. Thereafter, the nitrogen levels remained constant at about 116%, but the oxygen levels diminished gradually to 84.5 $\pm$ 8.4%. There was insignificant mortality during the period from day ten to day 46. On day 46 the waterflow was changed from 200 to 400 mL/min. The increase in waterflow increased the oxygen saturation level to 110.1 $\pm$ 4.6% in treatment A. Death began to occur rapidly at that point and continued, reaching 80% at the termination of the test. Mortality in trial 2 started after two days and constantly progressed until the end of the test.

Mortality rates were significantly different for the four treatments ($P = 0.001$; Chi-square test). Using the Bonferroni chi-square test to compare mortality rates between each treatment A, B and C with treatment D, only treatment A was significantly different from treatment D ($P < 0.01$).

Because of the rapid growth of the fry and an initial waterflow of 200 mL/min, there resulted a decrease of the level of oxygen in the water as the fish grew. This caused the oxygen tension to decrease to 5 mg/L in the lower trays. Since the system did not have an external oxygen

supply, the hatchery recommended minimum level of 6 mg/L oxygen was not maintained. Therefore, the waterflow in trial 1 was increased on the 46th day of the 60-day experiment. In trial 2, oxygen tension was maintained above 7 mg/L.

Coincident with the trial 1 waterflow change, even while the nitrogen levels remained constant, the oxygen levels increased substantially in all treatment trays. These fortuitous adjustments in waterflow resulted in the significant observation that oxygen and nitrogen levels each play a different role in determining the mortality at relatively low total dissolved gas saturation.

Even though the injected volume of air produced by a 1/2 HP motor at 1.4 atmosphere of pressure was constant throughout the test, the oxygen level at the 36th test day was significantly different ($P < 0.01$) from the level on the 46th test day, after the adjustment of water flow. The levels of nitrogen and total dissolved gas saturation were not significantly different ($P > 0.01$), however. It is important to note that the elevated level of nitrogen combined with the increased oxygen levels could have raised supersaturation above the lethal threshold, and this was associated with extensive mortality. Because the percent saturations of nitrogen, oxygen and total dissolved gases were not directly comparable as a result of variation within

groups and between determinations, the threshold saturation level could not be calculated.

2. Pathology

The signs usually associated with the disease were rarely observed prior to the convulsive stage which occurs a few minutes before death. Signs noted in rainbow trout fry included loss of equilibrium, abnormal buoyancy and aimless swimming as the convulsive stage commenced. Fish started swimming side and belly-up with violent whirling movements interspersed with periods of inactivity followed by spasmodic convulsions (the moribund stage) leading to death. Fish frequently died with the mouth agape and the gills and operculum flared.

Gross pathology findings confirmed reports of Shirahata (1966), Rucker and Kangas (1974), Stroud et al. (1975). The most common grossly detectable lesion was subcutaneous tissue emphysema elevating the skin. Another common lesion was emphysema of the caudal fin, dorsal fin, pectoral fins and anal fins.

Gross lesions of GBT also included periorbital hemorrhages in some affected fish. Petechial hemorrhages were commonly observed in the gills, and in the opercular and branchiostegal areas. Fish appeared to die most frequently during periods of stress such as feeding, gas

monitoring, or any manipulation that increased muscular activity.

3. Exophthalmia

In 5 of 50 fish, in treatment A, there was unilateral exophthalmia (Figure 2) and one fish had bilateral exophthalmia. In the severe form, the globe of the eye was prolapsed outward from a point adjacent to the optic disk (Figure 3) resulting in a space forming between the capillary layer of the choroid and the pigment epithelial layer of the retina. This space occupied up to one half the volume of the normal globe. No particular inflammatory lesion was associated with the prolapse.

The earliest histologic ocular lesion was an emphysematous space within a vessel of the choroid body of the eye (Figure 4). Another lesion, more advanced and presumably the precursor lesion to globe prolapse, was a separation of the retina from the choroid with an intact optic nerve. The dissecting separation resulted in formation of an oval tent of retinal tissue around the optic nerve. The separation occurred at the disk which served as the point of remaining attachment (Figure 5). In the normal eye there was an intimate association between the choroid gland, choroid layer and the pigmented epithelium of the innermost retinal layers. In the affected eye, degenerative

Figure 2. Three-month-old rainbow trout fry exposed to supersaturation levels of 117% N_2 and 110% O_2. Marked unilateral exophthalmia, H & E Stain, (Bar = 1 mm).

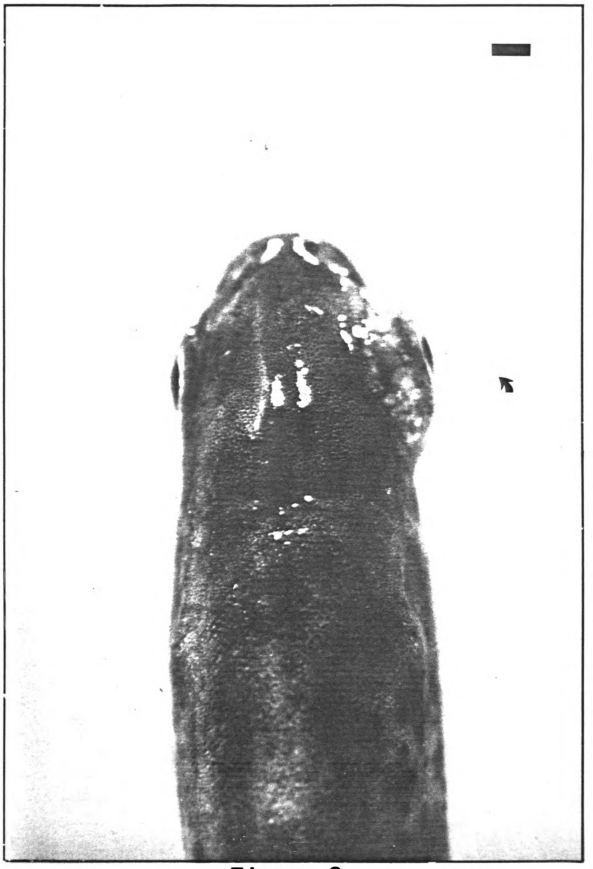

Figure 2

Figure 3. Three-month-old rainbow trout fry exposed to supersaturation levels of 117% N_2 and 110% O_2. Macroscopic lesion of severe unilateral exophthalmia, H & E Stain, (Bar = 0.5 mm).

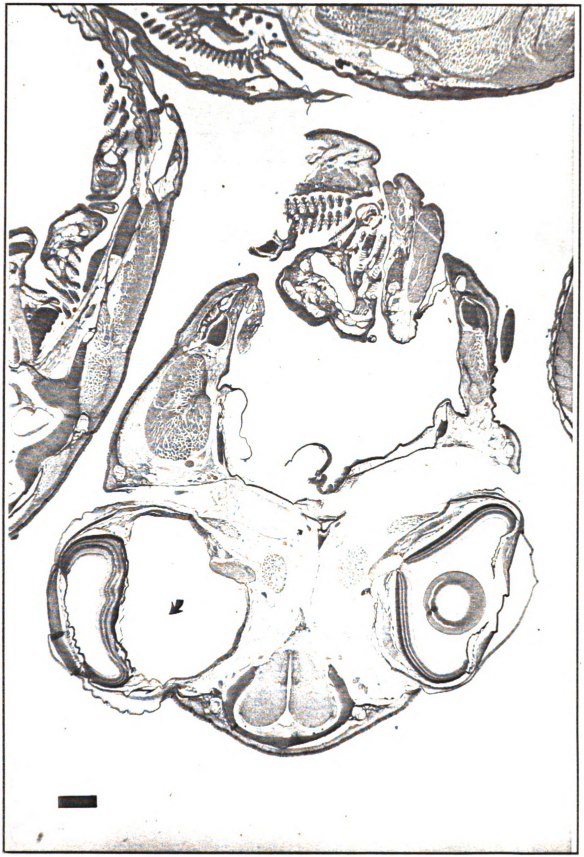

Figure 3

Figure 4. Three-month-old rainbow trout fry exposed to supersaturation levels of 117% N_2 and 110% O_2. Lesion of the early exophthalmia manifested by the presence of an air space in a vessel of the choroid gland, H & E Stain, (Bar = 50 microns).

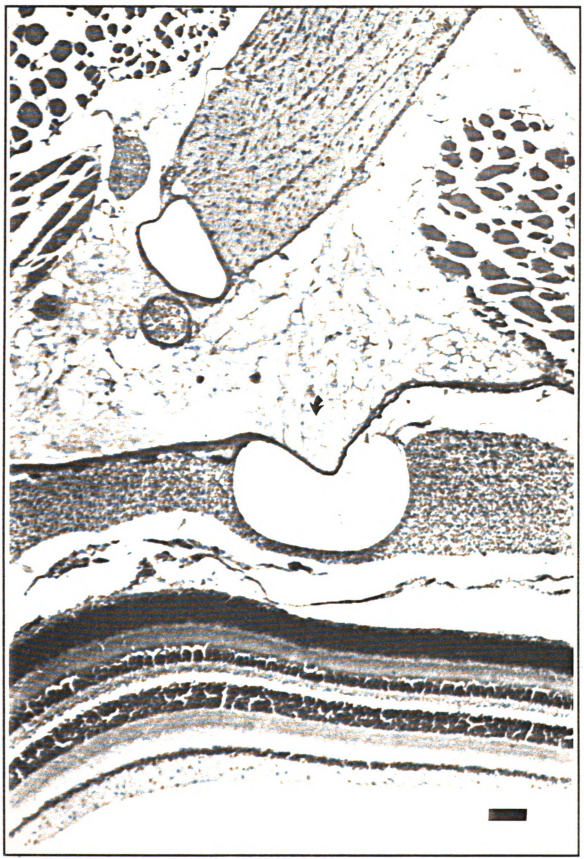

Figure 4

Figure 5. Three-month-old rainbow trout fry exposed to supersaturation levels of 117% N_2 and 110% O_2. Precursor lesion to occular prolapse in GBT induced exophthalmia, H & E stain, (Bar = 50 microns).

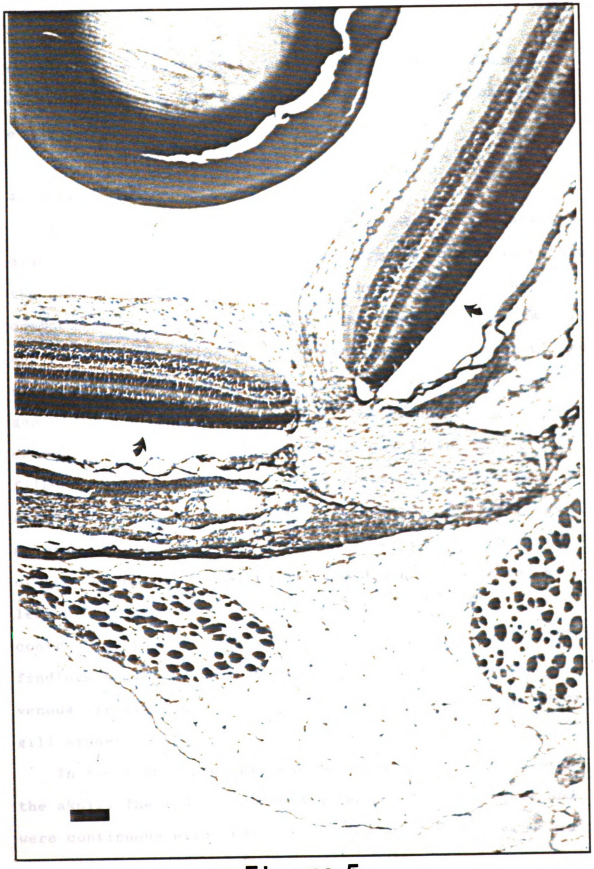

Figure 5

vacuolization of the optic nerve, musculature, adjacent connective tissue and glandular tissue was occasionally evident.

4. Gill Lesions

A lesion found in every moribund treated fish was gas displacement of the blood from the afferent arteriole within the gill filaments. The lesion was never observed in controls. The afferent arteriole was identified by its anatomical association with the cartilaginous support of the gill arch. The efferent arteriole, which was not marked by gas-blood displacement, was identified by anatomical characteristics of the fleshy, broad end of the gill filament Figures 6 through 9. In addition, the ventral aorta, which supplies blood in branches into the afferent arterioles immediately after leaving the bulbus arteriosus of the heart, was occasionally recorded as also having a lesion of gas-blood displacement (Figure 11) while the control sections did not have such lesions (Figure 10). The findings suggested that the origin of gas was from the venous circulation with build-up in the afferent side of the gill arches.

In two fish, an emphysematous space was observed within the skull. The spaces surrounded the medulla oblongata and were continuous with semi-circular canals. The lesion was

Figure 6. Oblique sections of the gill filaments of 3-mo-old rainbow trout fry. The afferent arteriole is identified by its anatomical association with the cartilaginous support of the gill arch. The efferent arteriole is identified by anatomical characteristics of the fleshy, broad end of the gill filament. Normal afferent and efferent arterioles of control group specimen, H & E Stain, (Bar = 50 microns).

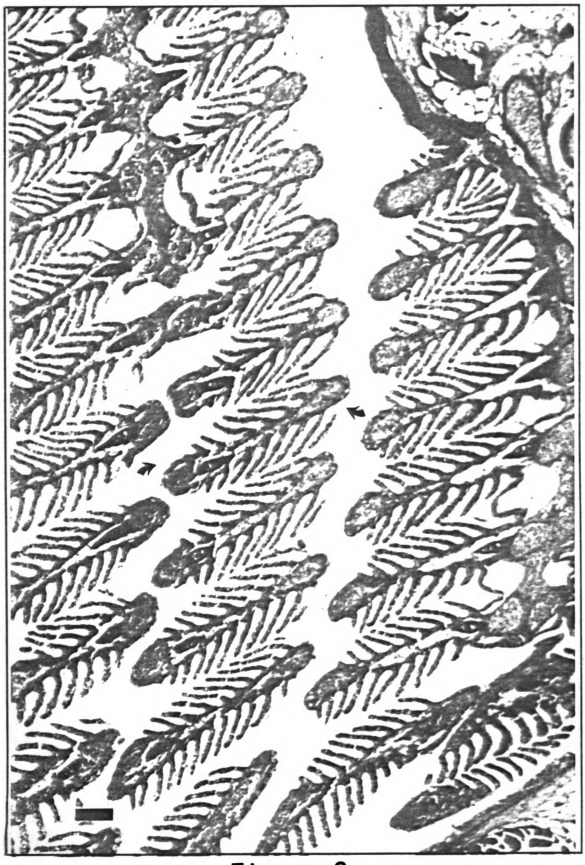

Figure 6

Figure 7. Oblique sections of the gill filaments of 3-mo-old rainbow trout fry. Treated fish indicating gas displacement of the blood from the afferent arterioles and normal blood content of the efferent arterioles, H & E Stain, (Bar = 50 microns).

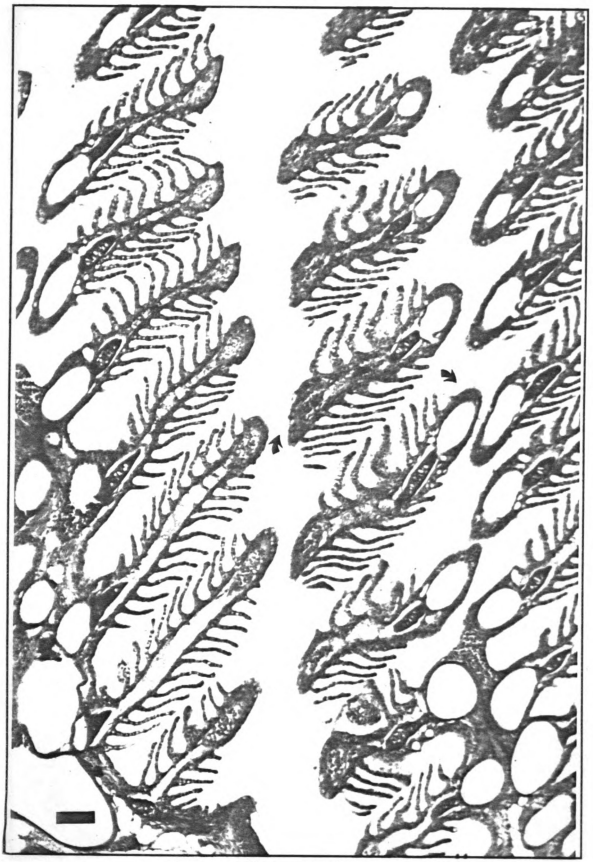

Figure 7

Figure 8. Oblique sections of the gill filaments of 3-mo-old rainbow trout fry. Detail of the gill filaments of a control specimen showing the appearance of normal arterioles, H & E Stain, (Bar = 200 microns).

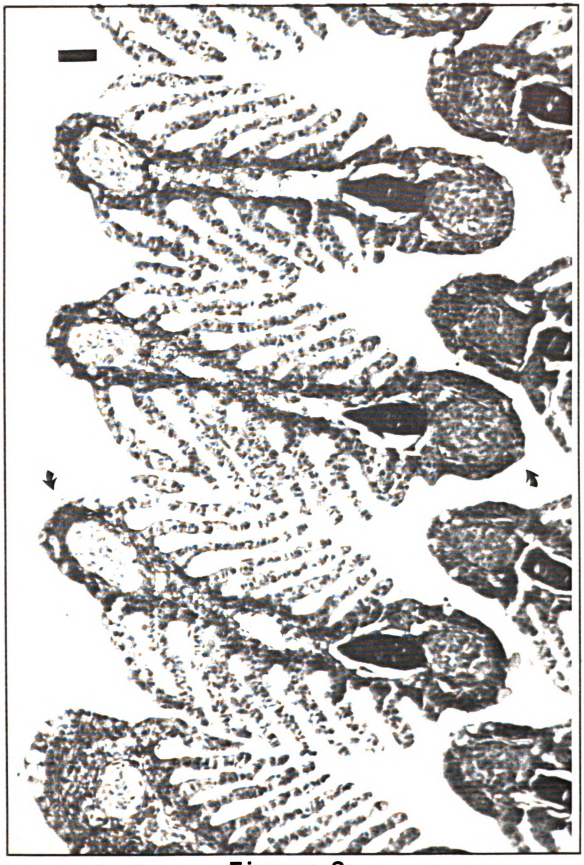

Figure 8

Figure 9. Oblique sections of the gill filaments of 3-mo-old rainbow trout fry. Detail of the gill filaments of a treated specimen demonstrating gas displacement of the blood from the afferent arterioles, H & E Stain, (Bar = 200 microns).

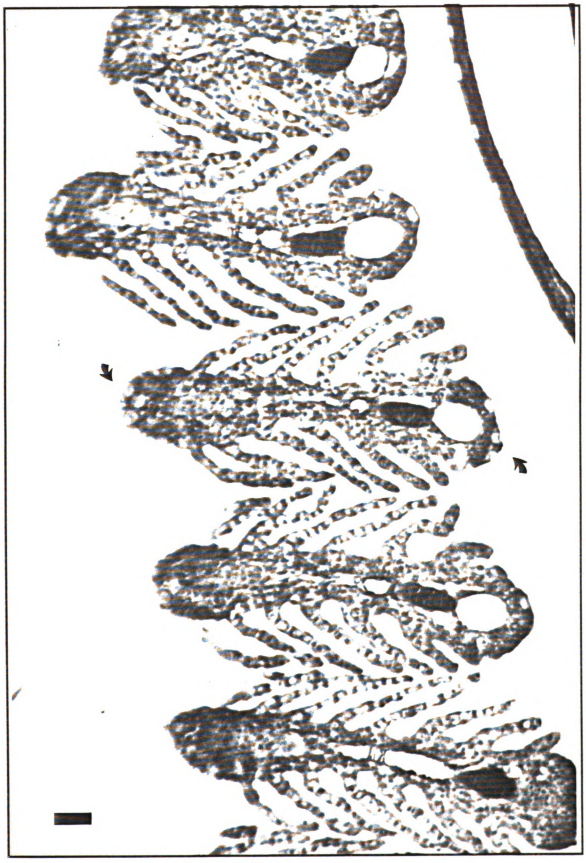

Figure 9

Figure 10. Longitudinal sections of the gill arch of 3-mo-old rainbow trout fry. Control specimen indicating the normal appearance of gill structure and ventral aortic vasculature without gas-blood embolization, H & E Stain, (Bar = 50 microns).

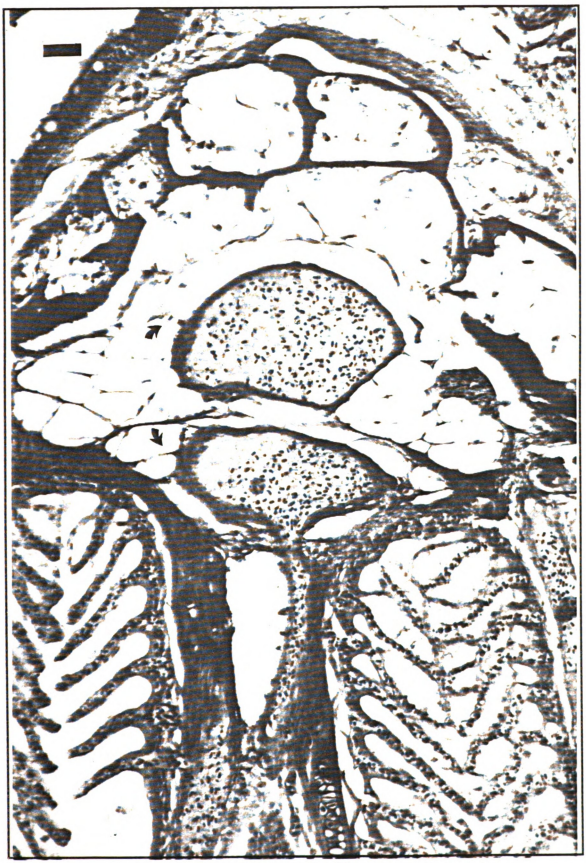

Figure 10

Figure 11. Longitudinal sections of the gill arch of 3-mo-old rainbow trout fry. Treated specimen showing the ventral aorta with evidence for gas displacement of blood close to the origin of the afferent branchial arteries, H & E Stain, (Bar = 50 microns).

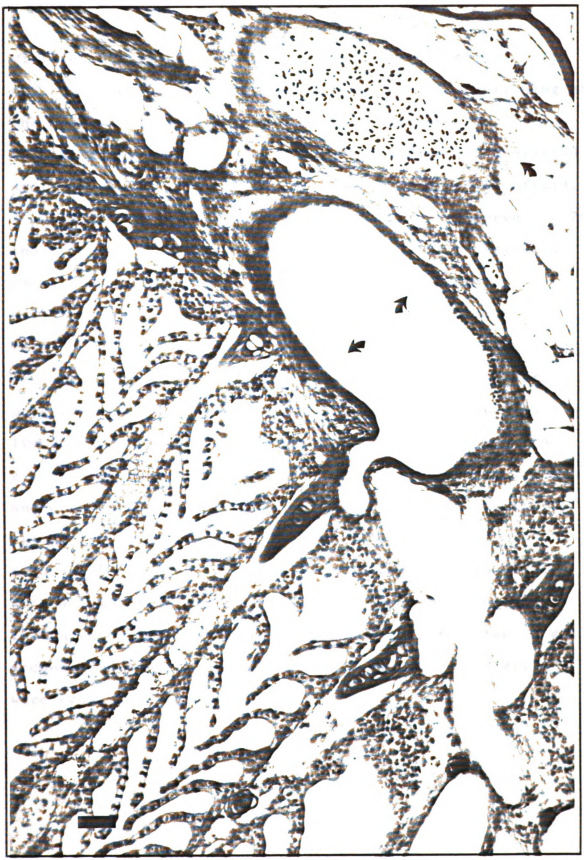

Figure 11

visible on sagittal and on cross section in one fish. It may have been an artifact caused by an oblique sectioning of the auditory passages which gave the appearance of emphysematous lesions. Control sections revealed undilated semi-circular canals containing an amorphous protein fluid.

Additional non-emphysematous lesions were observed in 70 individual fish. These lesions were randomly distributed among treated and control groups with no frequency association with a particular supersaturation exposure. They consisted of various gill lesions, extraordinary liver glycogen accumulation, fatty vacuolization of the liver, possible mineralization of kidney tubules and suspected lymphoid hyperplasia of the thymus gland associated with gill alterations. Gill lesions, seen in 21 fish on gross and/or histological examination, consisted predominantly of epithelial hyperplasia. This change was segmental or nodular in two fish, but most often diffuse and associated with accumulation of mucus. Gill filament blunting and fusion was observed sporadically when sections of whole gill arches were sagittally cut but not frequently when gills were cross sectioned.

CHAPTER 2. Carbon Monoxide Exposure

A. Introduction

The present study was designed to test the hypothesis that an accelerated oxygen transfer from oxyhemoglobin during exposure to supersaturation conditions initiates GBT. To accomplish this, fish pretreated with carbon monoxide (CO) were compared to control fish during a standarized supersaturation exposure. Mortality, lesion development and respiratory rate were recorded. It was anticipated that a CO-induced hemoglobin dysfunction could prevent the development of GBT.

B. Materials and Methods

1. Experimental Animals

Rainbow trout fry were utilized as the experimental animal in this study. Fish obtained from the Baldwin Fish Farm, Baldwin, Michigan were transported to the Aquaculture Research Laboratory at Michigan State University. Fish were fed twice a day with Purina Trout Chow, Purina Company. Fish were exposed to artificial light which simulated a natural photoperiod with a ratio of 14 light hours to 10

dark hours per day.

One thousand rainbow trout, 8 weeks old, with an average total length of 4.0 cm, were maintained in a holding tank and provided with well water. The fish were acclimated in the holding tank for two weeks before the beginning of the experiments.

2. Experimental Series

A pilot study (Test 1) and a series of experiments, I-V, were conducted. The pilot study examined superficially the effect of CO treatment on survival and enabled approximate mortality calculations in fish treated with CO and exposed to supersaturation conditions utilizing a similar experimental design and the same apparatus as described below. Two hundred fish, half of which were exposed to CO for 90 minutes when the hemoglobin conversion to carboxyhemoglobin was approximately 70%, were introduced into water which was supersaturated to a total dissolved gas saturation of approximately 133%. The results revealed an apparent protection from mortality by CO treatment. The remaining experiments were designed to establish the nature of the effect.

(i) The Series I experiments defined the supersaturation methods to be used in the later trials. In order to precisely determine the time necessary to supersaturate a

specific volume of water so that trials would involve an appropriate exposure, supersaturation was produced by pressurizing a 25 L plastic capped jar filled with 10 L of well water at 12°C with compressed air (30 psi) from a 223 Ft^3 cylinder. The jar was constantly agitated by an electric shaker at 110 rpm. The water was then depressurized to ambient creating a supersaturation. Pressurization times of 5, 10, 15 and 20 min were used.

(ii) Series II experiments, in order to maximize the probability of detecting an effect of CO exposure, determined the TDGS level which killed about 50% of control fish. Under conditions and supersaturation methods defined by series I experiments, four tests were conducted at various supersaturation levels achieved by pressurization as described above for 15 min at 36.7, 22.0, 18.4 and 7.3 psi. For each of these tests, 10 fish were placed in 10 L of supersaturated water at 12°C, and the mortality was recorded every 5 minutes during 180 minutes of observation.

(iii) Series III experiments determined the carboxyhemoglobin (COHb) conversion of fish exposed to 3% CO in air mixture, certified standard, Union Carbide Co., for varying periods of time. The efficiency of conversion was determined by measuring the percent saturation of carboxyhemoglobin in blood after exposure. Since it was technically impossible to take enough blood from 10 week old

fish for this purpose, for each of these tests, one adult rainbow trout was used so that 1 ml of blood could be drawn. Before placing the fish in the tank, 10 L of water at 12°C was saturated with the gas mixture through an aquarium air-diffusing stone for 15 min at a flow of 80 ml/min. Thereafter, fish were placed in the tank and blood samples for each of the five tests were taken after 15, 30, 60, 90 and 120 minutes of carbon monoxide exposure. Samples of blood were taken by cardiac puncture using 2.5 ml capacity blood gas syringes containing 125 USP units of lyophilized lithium heparin, Terumo Co., Tokyo, Japan, and 18G 1 x 1/2 inch hypodermic needles. Fish were immobilized in a wet towel and care was taken to assure that the time that elapsed until blood collection was less than one minute. Samples were immediately placed on ice and transported within three hours to the clinical chemistry laboratory at St. Lawrence Hospital, Lansing, Michigan, for blood gas analysis. The percent saturation of carboxyhemoglobin in deoxygenated whole blood samples was determined by the method of Siggaard-Andersen (1977) using the OSM-2 Hemoximeter, Radiometer America Inc., Westlake, Ohio.

(iv) To determine if the hemoglobin function influenced GBT, series IV experiments (Test 2) examined the effects of blocking oxyhemoglobin formation by carbon monoxide

exposure. In this phase of the investigation, both control and CO-treatment groups of fish were to be exposed to supersaturated water. First, the CO-exposure and the sham exposure for controls were conducted concurrently for 120 minutes under identical conditions. The CO-treatment protocol developed for Series III was the same for these experiments, including the water volume, temperature, and the flow for CO injection. Ten trials were conducted using a total of 200 fish. Twenty rainbow trout fry were used in each trial: 10 in the treatment group tank and 10 in the control tank.

For each trial, randomly selected fish were CO treated and then treatment fish were simultaneously tested with control fish to eliminate minor inconsistencies between the groups. To minimize external stress, the sides of the tanks were covered with sheets of black polyethylene, and particular care was taken to avoid any sudden movements, since stress may precipitate GBT.

All the procedures performed with treatment fish were performed with controls. In the CO-treatment groups, after saturating the water with CO-air mixture, 10 fish were placed into the tank and the same mixture was added at a fixed rate of 80 ml/min for 120 minutes. In the control groups, after saturating the water with air, 10 fish were placed into the tank and air was added at a fixed rate of 80

ml/min for 120 minutes. Then both treatment and control fish were transferred to other identical tanks containing 10 L of supersaturated water at 130% TDGS. The duration of each supersaturation test was 180 minutes.

Fish were observed every 5 minutes throughout each 180 minute trial for mortality and dead fish were removed from the tank. Five moribund fish from each of the four groups were fixed by the technique of Bell et al. (1986) and then stained by the H&E method for histopathologic examination.

The tests were performed under static flow conditions, and the total gas pressure (dP) could be measured only at the end of each trial since the use of the tensionometer requires a frequent shaking of the instrument for proper readings. If measurements were to have been performed continually during the tests, the manual agitation of the water would inconsistently reduce the total gas pressure. Prior to performance, it was determined that the initial supersaturation was 5% TDGS above of the final measured level at the end of the 180 minute observation.

(v) Because of observations of altered respiration in previous tests, series V experiments examined the respiratory performance of trout using the same carbon monoxide and supersaturation exposure and protocol described above for Series IV experiments (Test 2). The breathing movements (buccal and opercular) of four fish groups were

recorded at 30, 60, 90, 120, 150, 180 and 210 minutes. Significant disturbance to the fish was avoided by simply placing a black polyethylene sheet with a peep-hole along side the tanks. Lighting was supplied by overhead fluorescent lamps placed 0.25 m from the water surface. After observing the control and CO-treated test subjects in normally saturated water, supersaturation was imposed. At each time interval, the measurement was the average of three or more determinations. The gill ventilatory rate was determined with the aid of a stopwatch.

3. Statistical Analysis

Statistical analysis for Series IV experiments (Test 2) was performed utilizing a two-way factorial analysis of variance (General Linear Model, GLM, SAS Institute Inc.,Cary, N.C.) with post-analysis comparison of means utilizing the least significant difference (LSD) test, at $P < 0.05$ for significance.

Analysis of mortality data was performed by comparing control and treatment groups. The mortality data obtained during the 180 minute observation from the 10 trials were pooled and divided into 12 blocks of 15 minutes each. For each of these time intervals, the total mortality of treated vs. control was analyzed by means of the paired Student's two-tailed t-test ($P < 0.05$) and a computer program for

polynomial regression equations was used to plot the best fit mortality rate curves (Enertronics Research Inc.).

C. Results

Carbon monoxide treatment prior to supersaturation exposure significantly delayed the onset of mortality. The pilot study (Test 1) revealed that 60 minutes of exposure to supersaturation resulted in a mortality of 44% in controls while 33% of the CO-treatment group succumbed. These results suggested that CO had a protective effect. In the more refined series IV experiments (Test 2), a simultaneous induction of GBT in the CO-treatment group and controls resulted in a mortality of 26% in controls while 17% of the CO-treatment group were killed after 60 minutes. In CO-treated fish there was a significant delay in the onset of mortality. The time required for fatal exposure to supersaturation in the pilot study and Series IV experiment are charted on Figures 12 and 13. Mortality data comparisons using Student's two-tailed t-tests for each of the 15 minute time interval indicated significant ($P < 0.01$) difference between CO-treated and control groups for the 30 to 45 minute interval of exposure; however, the analysis of variance indicated that the accumulated total mortality of the treatment group was not statistically significantly different from control at $P < 0.05$.

Figure 12. Effect of carbon monoxide (CO) treatment on gas bubble trauma susceptibility (Pilot study, Test 1). Treatment rainbow trout fry were exposed to water containing 3% CO for 90 minutes prior to supersaturation. Treatment fish (n=100) in which carboxyhemoglobin conversion was estimated to be about 70% and controls (n=100) were placed in 12°C water supersaturated to approximately 133% TDGS. The pooled mortality data represent 10 trials with determinations every 10 minutes during 90 minute observations.

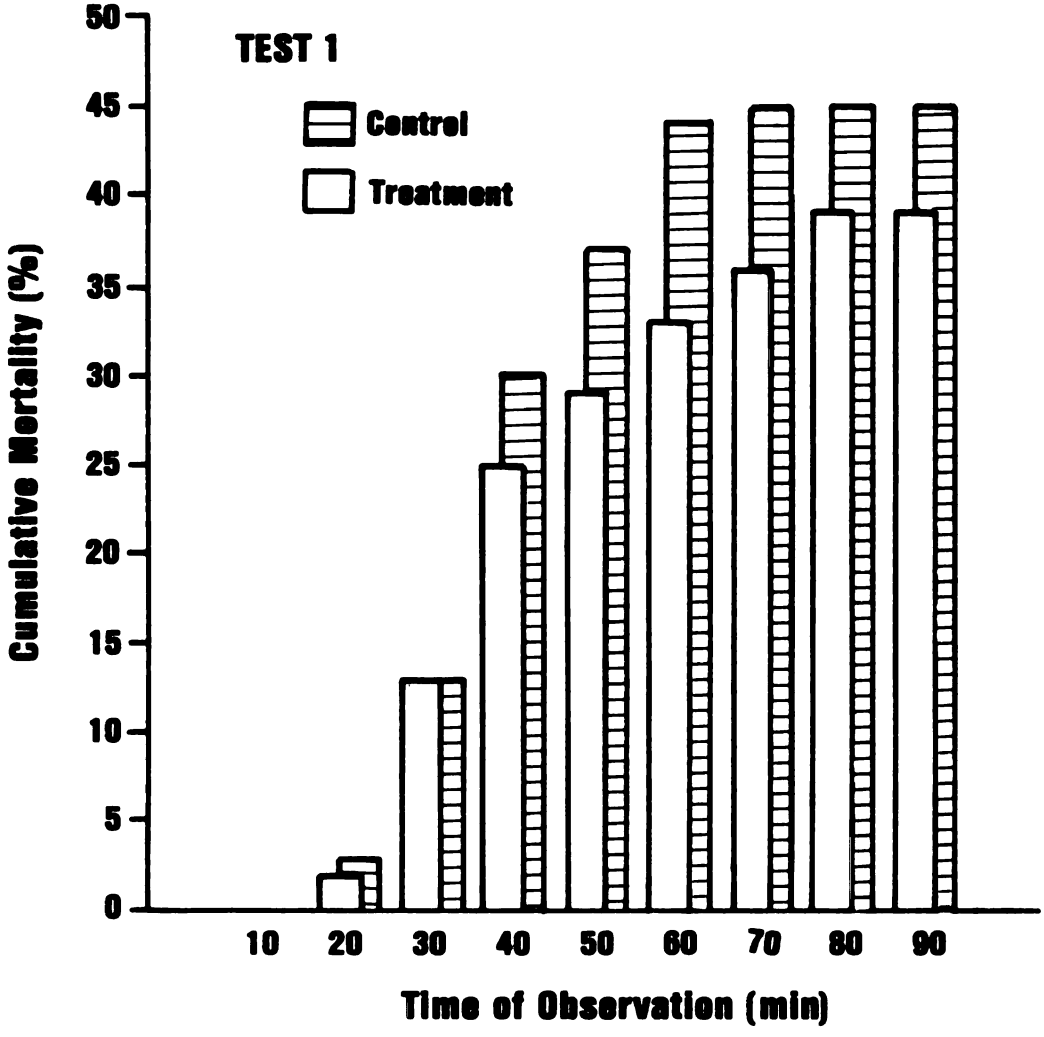
TEST 1
Control
Treatment
Cumulative Mortality (%)
0
5
10
15
20
25
30
35
40
45
50
10
20
30
40
50
60
70
80
90
Time of Observation (min)

Figure 12

Figure 13. Effect of carbon monoxide (CO) treatment on gas bubble trauma susceptibility (Test 2). Treatment rainbow trout fry were exposed to water containing 3% CO for 120 minutes prior to supersaturation. Treatment fish (n=100) in which carboxyhemoglobin conversion was estimated to be about 80% and controls (n=100) were placed in 12°C water supersaturated to 130% TDGS. The pooled mortality data represent 10 trials with determinations every 30 minutes during 180 minute observations.

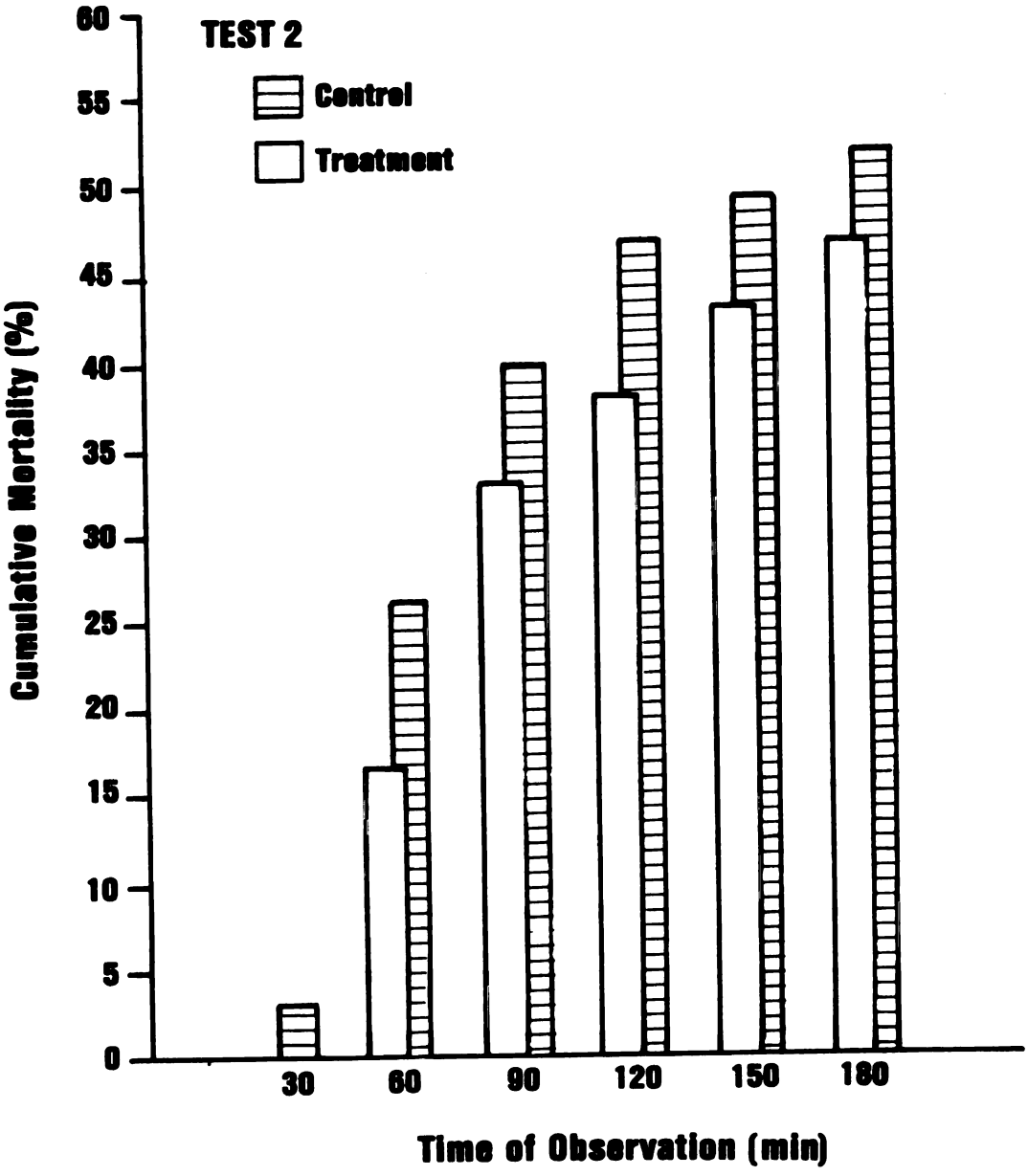
TEST 2
Control
Treatment
Cumulative Mortality (%)
60
55
50
45
40
35
30
25
20
15
10
5
0
30
60
90
120
150
180
Time of Observation (min)

Figure 13

In a graphic representation of the difference in the onset of mortality from series IV experiments, the data are plotted and curves are fitted by developing a polynomial regression equation for each set of data. The data demonstrate that there was a delay of about 15 minutes in the onset of mortality in CO-treated fish and it can be inferred that, in the CO treatment, the mortality was increased between 130-175 minutes such that cumulative mortality was not different (Figure 14).

The histopathologic examination of moribund supersaturation exposed fish revealed the cause of death to be GBT for both CO-treated and control fish. Microscopic examination of branchial lesions was characterized by a gas displacement of blood in the arterioles of the gill filaments. Gas emboli were evident in 80% and 60% of the retinal choroid gland sections of the control and CO-treated fish that succumbed to supersaturation treatment (see Figure 4). Similar extent and distribution of lesions were found in CO-treated and control moribund fish with GBT, but no lesions of gas embolization were detected in any CO-treated or control fish prior to supersaturation exposure.

Carbon monoxide treatment had the effect of temporarily delaying the characteristic reduction in the gill ventilatory drive normally observed in fish exposed to high levels of supersaturation. The rate and amplitude of

Figure 14. Graphic representation of mortality data (Test 2) of rainbow trout fry: carbon monoxide treatment group (n=100) and control group (n=100). Fish were exposed to supersaturated water of 130% TDGS at 12°C. The best fit curves were plotted using polynomial regression equations obtained by computing the number of dead fish at 15 minute time intervals during 180 minutes of observation. The curves demonstrate a delay of about 15 minutes on the onset of mortality in CO-treated fish when compared to control groups.

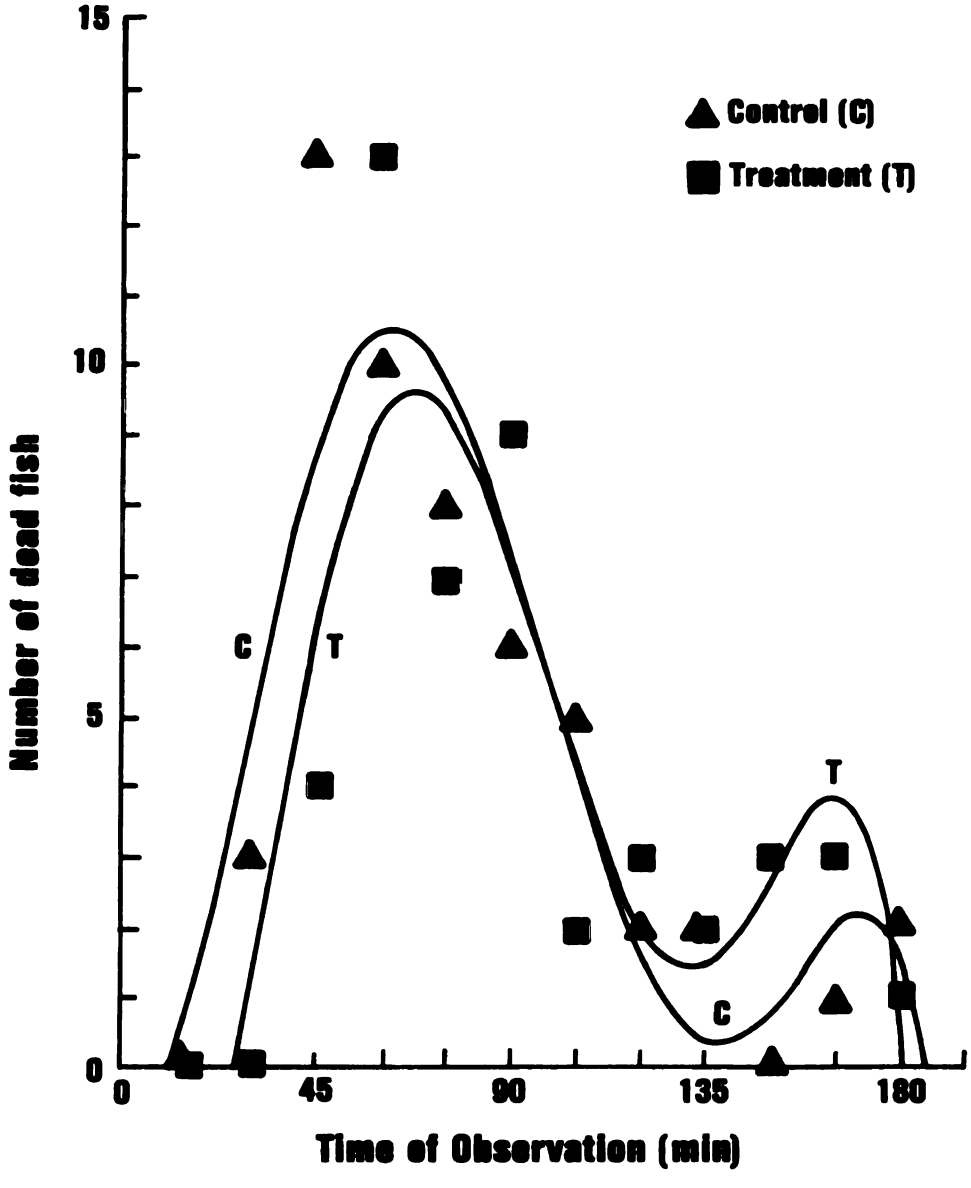
15
10
5
0
Number of dead fish
Control (C)
Treatment (T)
C
T
T
C
0
45
90
135
180
Time of Observation (min)

Figure 14

respiratory movements did decline during supersaturation exposure in both groups; however, the initial respiratory rate and the rate during exposure was greater in CO-treated fish than in controls. Although both treated and control groups had similar total mortality, CO-treated fish had prolonged survival for a period roughly equal to the delay period that occurred before the ventilation was markedly reduced.

The respiratory rate of control fish declined from 104 to 90 cycles/min during the initial 120 minute observation in the tank containing normally saturated water. Immediately after being transferred to supersaturated water, breathing movements were greatly reduced for the next 30 minutes. The respiratory rate during the period of reduced ventilation was estimated to be aproximately 20 cycles/min and remained slow until the onset of the convulsions. By comparison, CO-treated fish had a higher initial respiratory rate of 120 cycles/min with a gradual decrease to 82 cycles/min after the 120 minute rest period. When CO-treated fish were transferred to supersaturated water, a respiratory depression was observed, but the respiratory rate was 2 times the control fish rate for a period of 15-30 minutes (40 versus 20 cycles/min).

The general experimental design for the cumulative mortality experiments was based on series I experiments

which revealed that it took between 5 and 10 minutes to air supersaturate 10L of water at a pressure of 30psi. An additional period of pressurization did not result in any increase in supersaturation; thus, to ensure a consistent level of supersaturation, the pressurization time was 15 minutes. The volume of 10L of water was utilized because tests previously had shown that 10 trout fry can survive for at least 48 hours in 10L of static, normally saturated water.

Because of the pilot study results, it appeared that the most sensitive detection of a CO-treatment effect would be obtained by having a cumulative mortality of about 50%. Series II experiments are charted in Figure 15 and examine the mortality rate for fish under the conditions to be utilized for CO-treatment tests. By varying the level of supersaturation with different injection pressures, the mortality data for 10 fish groups exposed for 180 minute in 10 L of water demonstrates that the lowest TDGS level for 100% mortality was 136% (36.7 psi), for 50% mortality was 130% (22.0 psi), for 20% mortality was 124% (18.4 psi) and the highest level that kills no fish was 116% (7.3 psi).

Because there was no available information about the rate of carboxyhemoglobin conversion under the conditions of these tests, the series III experiments determined the percent saturation of carboxyhemoglobin of blood of adult

Figure 15. Effects of various levels of supersaturation expressed as total dissolved gas saturation by air on the mortality of rainbow trout fry exposed to 10L of water at 12°C during 180 minutes.

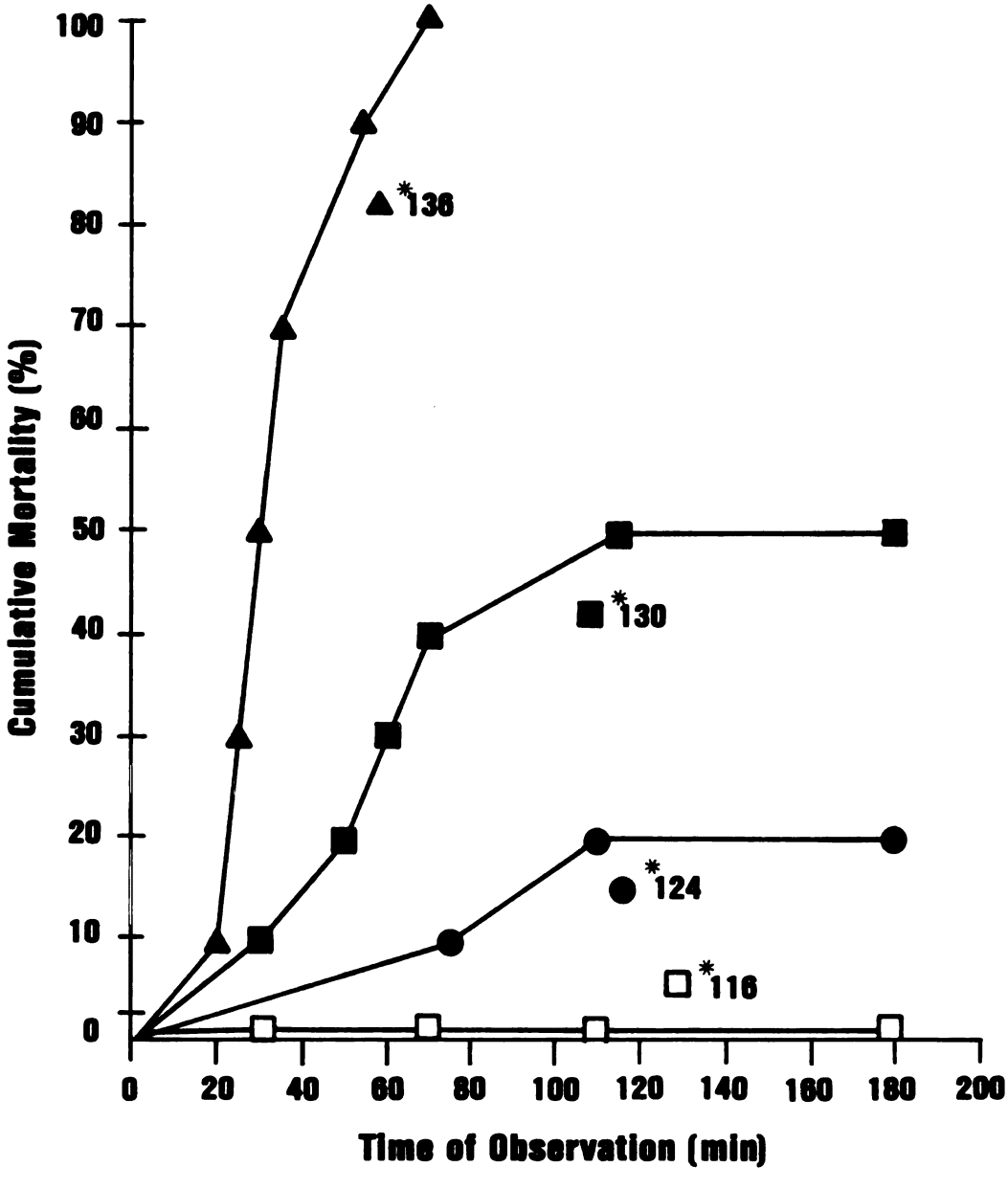
100
90
80
70
60
50
40
30
20
10
0
Cumulative Mortality (%)
*136
*130
*124
*116
0
20
40
60
80
100
120
140
160
180
200
Time of Observation (min)

Figure 15

trout exposed to 10 L of water equilibrated with an air mixture containing 3% CO (Figure 16). The data show that COHb conversion increased progressively with exposure time, reaching 82% after 120 minutes. Fish exposed for a longer period evidenced an apparent narcosis. Because of the blood sample size requirement of 1 ml, COHb conversion was determined in large trout; thus, an extrapolation was necessary to arrive at the conclusion that approximately 80% of the hemoglobin in small fry similarly exposed would be converted to COHb.

Figure 16. Concentrations of carboxyhemoglobin (COHb) of adult rainbow trout venous blood after exposure to varying periods of time to water at 12°C containing carbon monoxide.

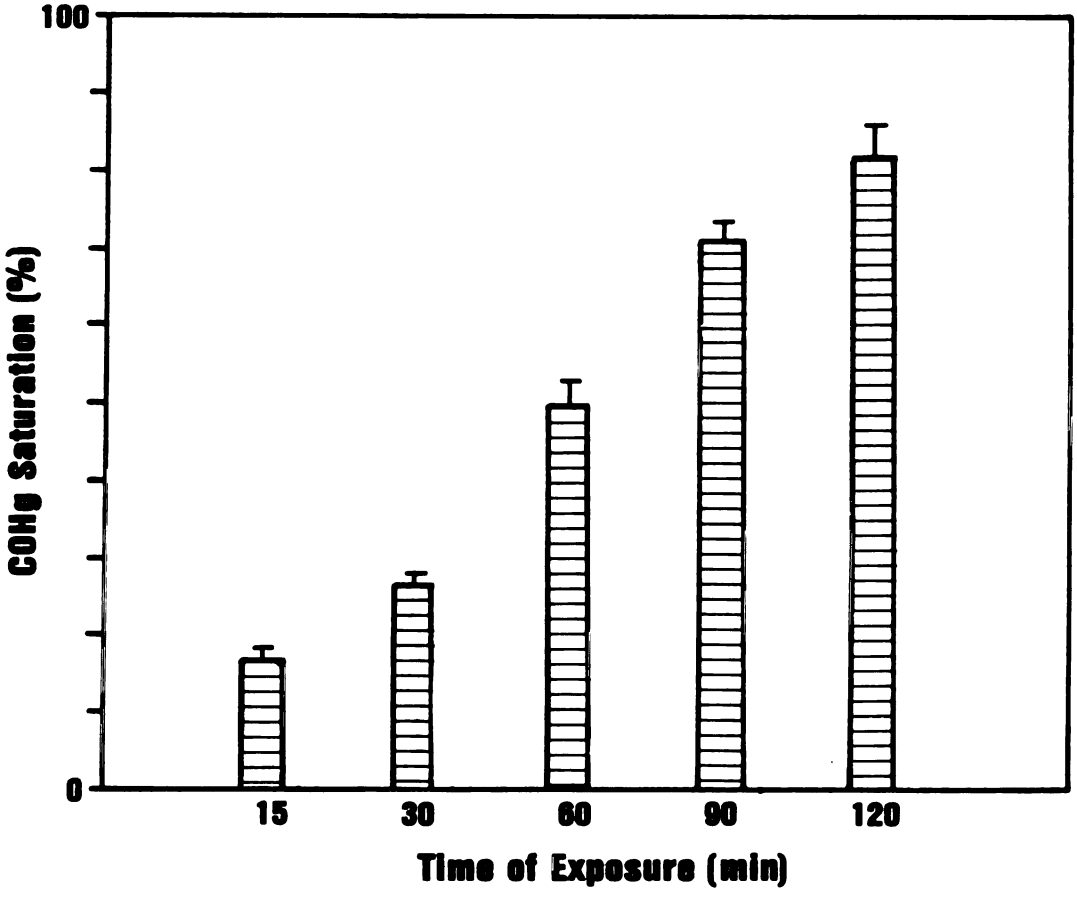

Figure 16

CHAPTER 3. Blood and Red Cell pH

A. Introduction

Many studies have been published which deal with the effects of various stressing factors upon the blood pH of fish (see Discussion). Lower blood pH decreases both oxygen affinity (Bohr effect) and oxygen carrying capacity of hemoglobins (Root effect). A significant drop in blood pH may accelerate the unloading of oxygen from Root effect hemoglobins which could be responsible for the bubble nucleation in fish affected by GBT. These experiments were conducted to determine if there is an association between blood pH and supersaturation-induced stress in fish.

B. Materials and Methods

Rainbow trout were obtained from a commercial fish farm (Baldwin, Michigan) and transported to the Aquaculture Research Laboratory at Michigan State University. One hundred fish with an average total length of 28 cm and average weight of 300 g, were maintained in a holding tank and provided with well water. Fish were fed once a day with Purina Trout Chow, Purina Company and were exposed to artificial light which simulated a natural photoperiod with

a ratio of 14 light hours to 10 dark hours per day. The fish were acclimated in the holding tank for two weeks before the beginning of the experiments.

Supersaturation was produced by pressurizing a 25L plastic capped jar filled with 10L of well water at 12°C with compressed air (40 psi) from a 223 Ft^3 cylinder. The jar was constantly agitated by an electric shaker at 110 rpm for 15 min. The water was then depressurized to ambient pressure creating a supersaturation of 140% total dissolved gas saturation. It had been previously observed that this amount of supersaturation would result in approximately 100% mortality in the test fish after a 90 min exposure.

Before conducting the experiments, a test was performed to establish the reference control values (sampling group) for selected hematological parameters. The values obtained do not represent the normal levels (resting conditions), since the handling and blood sampling methods used in these experiments cause considerable stress to fish. The reference fish (N = 6) were sampled immediately after netting and removal from the holding tank. The same blood sampling method was used as in the experiments (see below).

Two experiments were conducted using a total of 24 fish. For each test, two randomly selected trout were simultaneously used, one treatment and one control. The experiments determined selected hematological changes of

control and supersaturation treated fish. In the first experiment, the fish (N = 12) were sampled after 15 minutes. This was to determine the effect of the transfer from the holding tank to the test aquarium and the effect of the initial 15 minutes in the test tanks. In the second experiment, sampling (N = 12) was done when treated fish had convulsions (the moribund stage). The maximum duration of the second experiment was dictated by the fact that treated fish succumb to 60 min of exposure to 140% TDGS.

The treatment fish were placed in a 10G tank containing 10L of supersaturated water at 140% TDGS. The control fish were exposed to normally saturated water and air was supplied through a diffusing air-stone, but otherwise the conditions were identical. An earlier test had been performed to assure that a fish can live at least 24 hours under the control conditions. At the appropriate times, blood samples were quickly taken. At least 0.5 mL of whole blood was drawn by cardiac puncture into a gas blood sampler syringes containing lyophilized lithium heparin (Terumo Co) using 20G, 1" hypodermic needles. Fish were immobilized in a wet towel, and care was taken to assure that the time that elapsed until blood collection was less than one minute. Samples were immediately placed on ice, and were transported within two hours to the Veterinary Clinic Laboratory at Michigan State University, for blood analysis.

Samples were analysed for extraerythrocytic pH (pHe), packed red cell volume (PCV), red blood cell (RBC) count and intraerythrocytic pH (pHi). The RBC count was calculated with a hemocytometer and the PCV was determined by the microcapillary method, using micro hematocrit centrifuge (3 min), Damon/IEC Division. The RBC count and PCV values were used to calculate the mean corpuscular volume (MCV) in femtoliters. The extra- and intracellular pH were determined by use of a Corning semimicro combination pH glass electrode connected to a Corning model 10 pH meter.

The whole blood pH measured in the extraerythrocitic fluid, was determined at a temperature of 23°C after transferring 0.5 mL of the sample from the syringe in ice bucket to a plastic centrifuge tube. After measurement of the extracellular pH, blood samples were taken for PVC and RBC count. Afterwards, the remaining whole blood was then centrifuged (10 min; 2,000 rpm) in a Beckman centrifuge, model TG-6, connected to a Beckman refrigeration unit, model TG-R at 20°C. The plasma fraction and the uppermost layer of red cells were discarded and the red cells were washed three times by alternate centrifugation (5 min; 2,000 rpm) and resuspension in 154 uM NaCl unbuffered saline solution. After the last washing cycle, the saline was removed and the packed red cells were frozen for 5 minutes in a bath consisting of dry ice and ethanol to lyse the cells. These

hemolysed cells were finally suspended in one volume of deionized and distilled water and the pH of this suspension, was determined at 23°C. The pH of the suspension was taken as the intraerythrocytic pH.

Statistical analysis for these experiments was performed utilizing One-Way ANOVA test for analysis of variance (MSTAT computer program, Michigan State University), at $P < 0.05$ for significance. A MSTAT subprogram (Plot) was used for linear regression equations.

C. Results

The effect of acute air-supersaturation exposure on selected hematological variables is illustrated in Table 3. This table shows the recorded values of red cell mean corpuscular volume (MCV), blood pH (pHe) and red blood cell pH (pHi) of venous blood during a reference control period, then during a 15-min period of supersaturation and subsequent convulsive period (moribund stage). The convulsions generally occurs a few minutes before death in fish affected by GBT. Analysis of blood samples of supersaturation group, taken during the convulsive stage, revealed a significantly ($P < 0.05$) lower pHe and pHi values than control termination group (Figures 17 and 18). The results (Figure 19) revealed a high degree of correlation

TABLE 3. Effects of supersaturation exposure at 140% TDGP on selected hematological parameters of rainbow trout (12° C).

	CONTROL			SUPERSAT.		SIGNIFICANCE TEST							
Variable	Sampling (N = 6 ± S.E.)	15 min (N = 6 ± S.E.)	Termination (N = 6 ± S.E.)	15 min (N = 6 ± S.E.)	Convulsion (N = 6 ± S.E.)	1	2	3	4	5	6	7	8
M C V (fL)	274 (± 11)	275 (± 9)	303 (± 9)	291 (± 4)	290 (± 7)	NS	NS	NS	NS	0.05	NS	NS	NS
Blood pH (pHe)	7.37 (± 0.02)	7.14 (± 0.01)	7.19 (± 0.02)	7.15 (± 0.01)	7.10 (± 0.03)	0.05	0.05	0.05	0.05	NS	NS	NS	0.05
RBC pH (pHi)	7.05 (± 0.01)	6.97 (± 0.01)	6.95 (± 0.01)	6.99 (± 0.01)	6.90 (± 0.02)	0.05	0.05	0.05	0.05	NS	NS	0.05	0.05

One-Way ANOVA test was used for Analysis of Variance (p . 0.05) where: Sig. 1 - Control sampling compared to control 15 min; Sig. 2 - Control sampling compared to control termination; Sig. 3 - Control sampling compared to supersat. 15 min; Sig. 4 - Control sampling compared to supersat. convulsion; Sig. 5 - Control 15 min compared to control termination; Sig. 6 - Control 15 min compared to supersat. 15 min; Sig. 7 - Supersat. 15 min compared to supersat. convulsion; Sig. 8 - Control termination compared to supersat. convulsion.

Figure 17. Time-dependent changes (mean ± S.E.) of venous blood pH (pHe) in rainbow trout during paralleled tests of exposure to normally saturated water (controls, N = 6) and to supersaturated water of 140% TDGS (treated, N = 6) at 12°C. Arrow, control (reference) sampling; (15), sampling time at 15 min; (Conv.), sampling time at supersaturation convulsion; (Term.), sampling time for controls at termination. The duration of each of the 6 tests (25-60 min) depended upon the susceptibility of the treated fish to GBT. See Table 3 for significance tests.

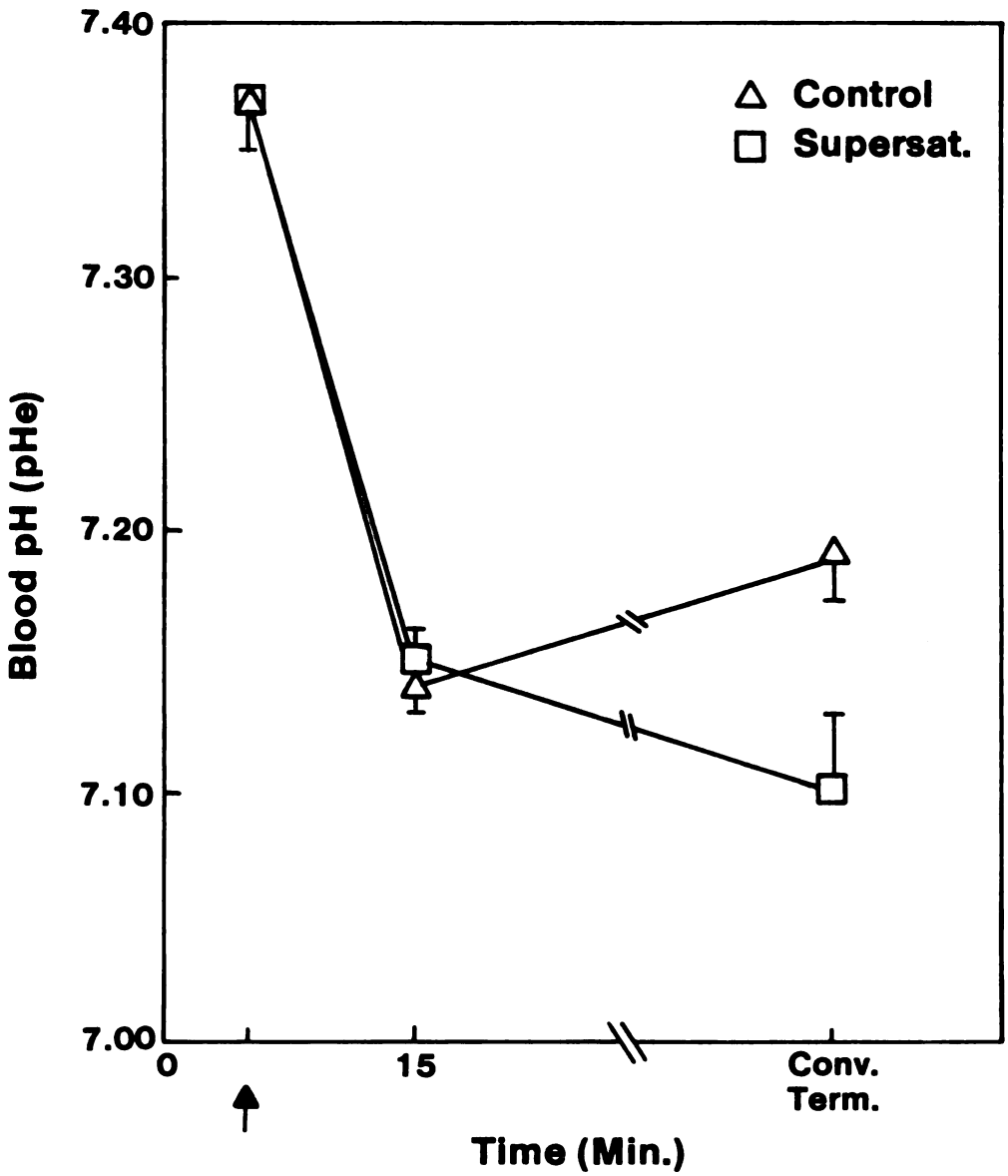
7.40
7.30
7.20
7.10
7.00
Blood pH (pHe)
Control
Supersat.
0
15
Conv.
Term.
Time (Min.)

Figure 17

Figure 18. Time-dependent changes (mean ± S.E.) of RBC pH (pHi) in rainbow trout during paralleled tests of exposure to normally saturated water (controls, N = 6) and to supersaturated water of 140% TDGS (treated, N = 6) at 12°C. See Fig. 17 for further explanation.

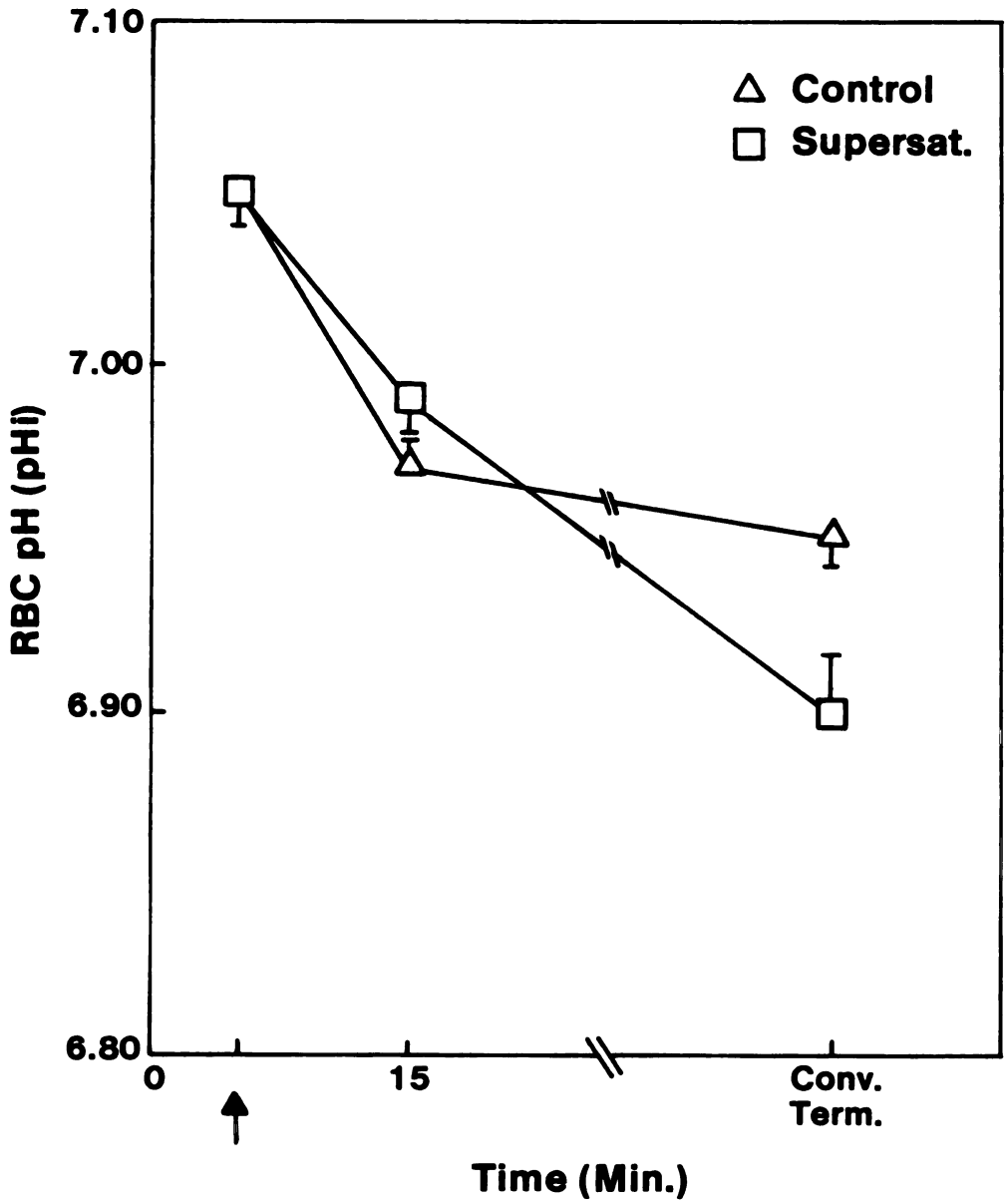
7.10
7.00
6.90
6.80
RBC pH (pHi)
Control
Supersat.
0
15
Conv.
Term.
Time (Min.)

Figure 18

Figure 19. The relationship between RBC pH (pHi) and blood pH (pHe) in rainbow trout exposed to supersaturated water of 140% TDGS at 12°C., for variable period of time (25-60 min). Blood samples were taken during the GBT convulsive stage. The regression equation for the line is: pHi = 2.319 + 0.65 pHe, r = 0.961, n = 6 ($P < 0.05$).

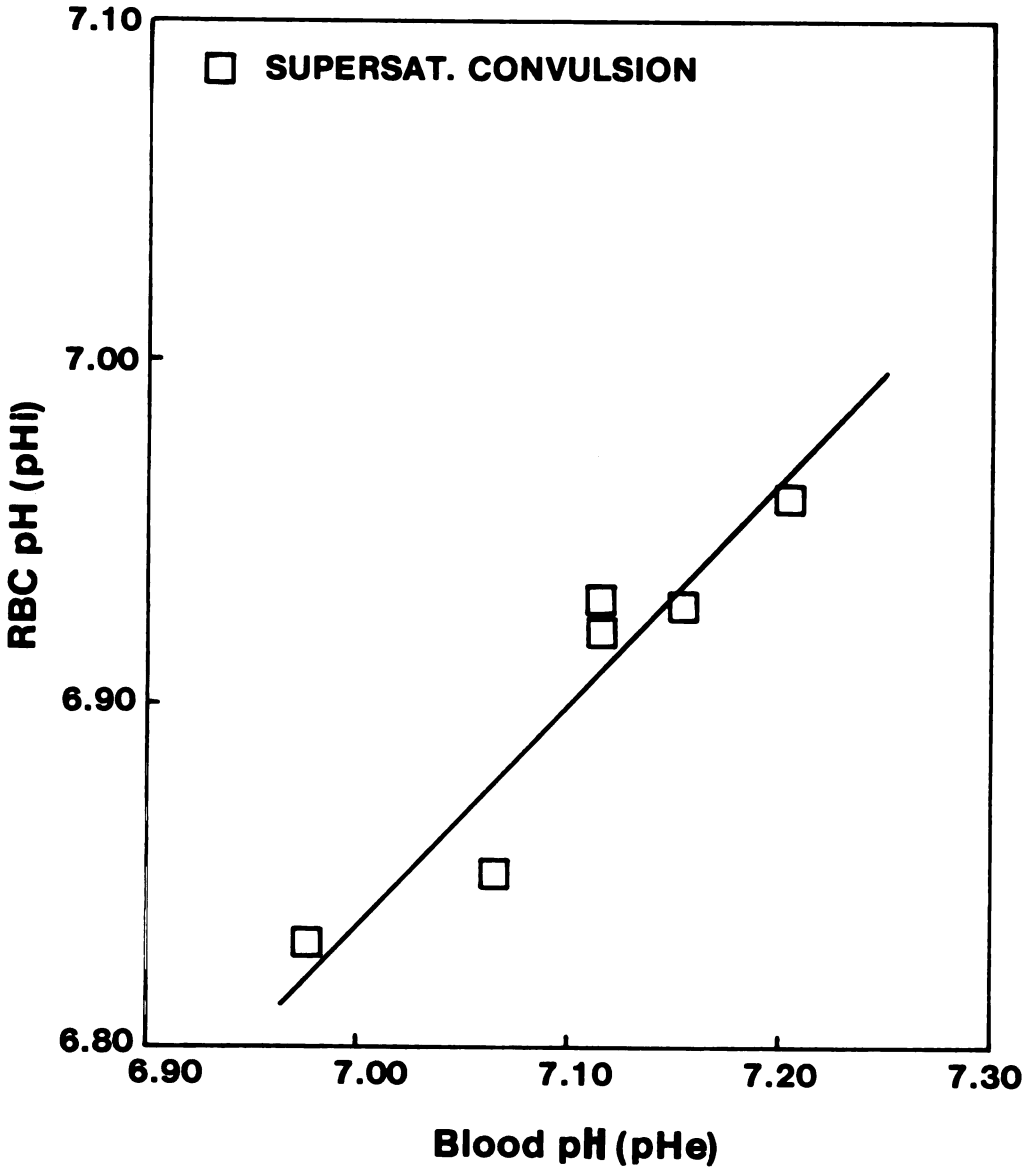
7.10
7.00
6.90
6.80
SUPERSAT. CONVULSION
RBC pH (pHi)
6.90
7.00
7.10
7.20
7.30
Blood pH (pHe)

Figure 19

between pHe and pHi in treated fish during convulsions. A decrease in pHi to 6.90 $\pm$ 0.02 during convulsions was associated with a decrease in pHe to 7.10 $\pm$ 0.03 (means $\pm$ S.E. for 6 fish). A positive correlation was not observed in termination controls (Figure 20).

The average of pHe and pHi of reference control fish from the holding tank affected by the sampling stress only was 7.37 and 7.05 units respectively. This is in agreement with the results of (Cossins and Richardson 1985) who reported that trout pHi was between 0.3 and 0.4 units lower than pHe. The fifteen minutes exposure (Table 3) showed that the stress caused by transferring fish from the holding tank to the test aquarium depressed pHe and pHi values similarly in both control and treatment groups when compared with the control sampling (reference) group. The substantial acidosis observed in the supersaturation group represented an average drop in pHe down to 7.15 and in pHi to 6.99. The control values were 7.14 and 6.97 respectively.

After the 15 min period, however, the supersaturation treatment caused a significant ($P < 0.05$) drop in pHi mean value from 6.99 to 6.90 at convulsions, while the pHe did not significantly decrease. Moreover, in the control termination group, the pHe and pHi decrease was not

Figure 20. The relationship between RBC pH (pHi) and blood pH (pHe) for termination controls. Rainbow trout were exposed to normally saturated water at 12°C, for a period (25-60 min) corresponding to the supersaturation convulsion exposure time. The regression equation for the line is: pHi = 6.366 + 0.08 pHe, r = 0.185, n = 6 (not significant at p. 0.05).

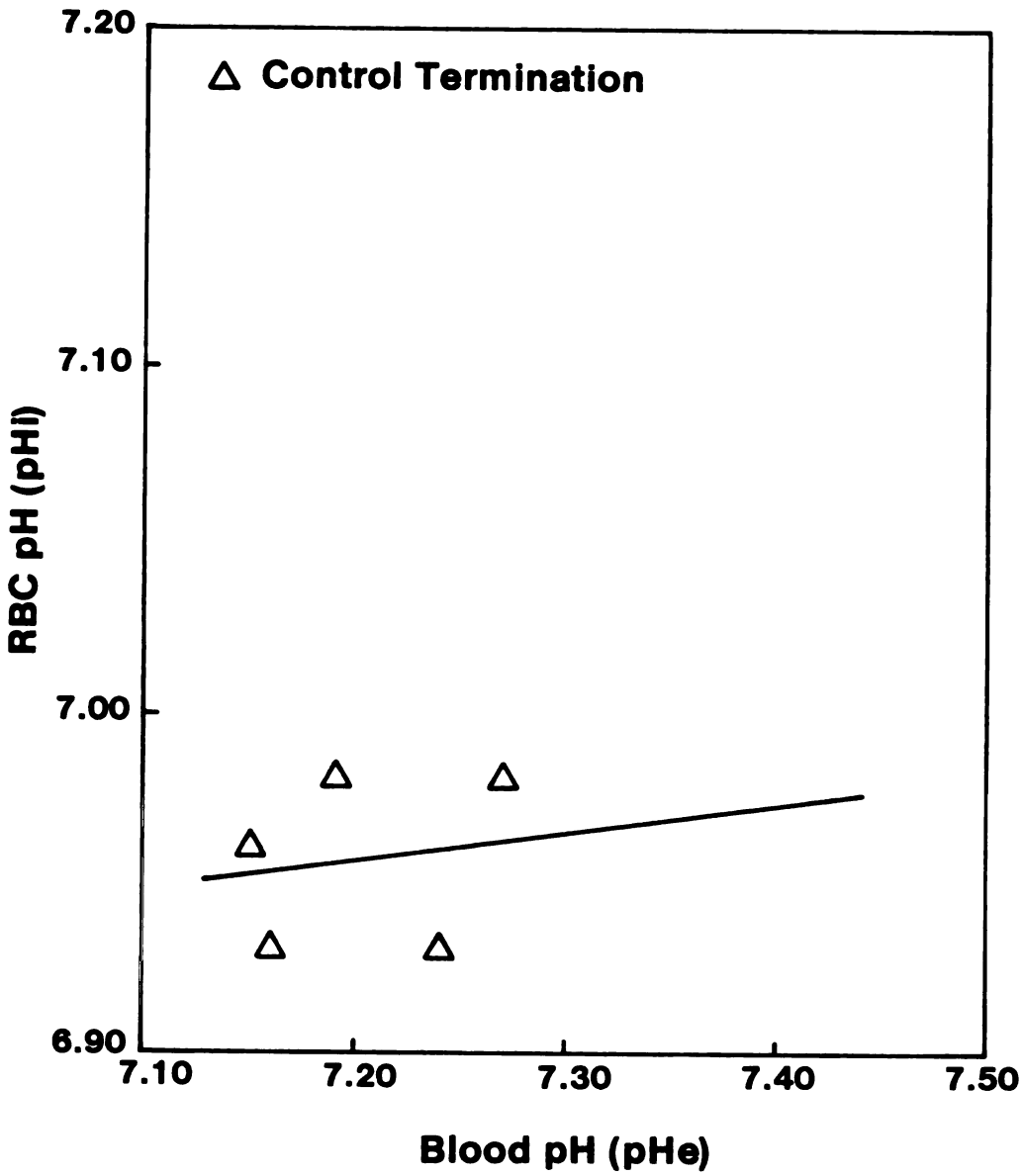
7.20
7.10
7.00
6.90
RBC pH (pHi)
Control Termination
7.10
7.20
7.30
7.40
7.50
Blood pH (pHe)

Figure 20

significant ($P < 0.05$).

The extracellular acidosis was at least partially respiratory in origin as indicated by the compensatory hyperventilation observed in controls. On the other hand, upon exposure of fish to supersaturated water, the ventilatory drive was markedly depressed throughout the supersaturation exposure period (see Chapter 2). This may explain the significantly ($P < 0.05$) lower pHe of 7.10 and pHi of 6.90 observed in treated fish during convulsions in comparison with the termination controls which had a pHe of 7.19 and pHi of 6.95, respectively. The lower pHe and pHi observed in the treatment group, may be the result of stress since blood lactate levels have reportedly been elevated after stress (Milligan and Wood 1986).

As seen in Table 3, the extra- and intracellular pH in controls after the 15 min period did not decrease further until the termination of the tests. This may be associated with the significant ($P < 0.05$) elevation of the mean corpuscular volume of erythrocytes during that period from an average of 275 to 303 fL (Figure 21). In contrast, no evidence of red cell volume elevation was found in supersaturation treated fish. The increased MCV is an indicator of RBC swelling which dilutes the hemoglobin and organic phophates within the RBCs. The erythrocytic

Figure 21. Effect of supersaturation (140% TDGS, 12°C) on RBC mean corpuscular volume (MCV) in rainbow trout. Control group, N = 6; treatment group, N = 6. All other details as in Fig. 17.

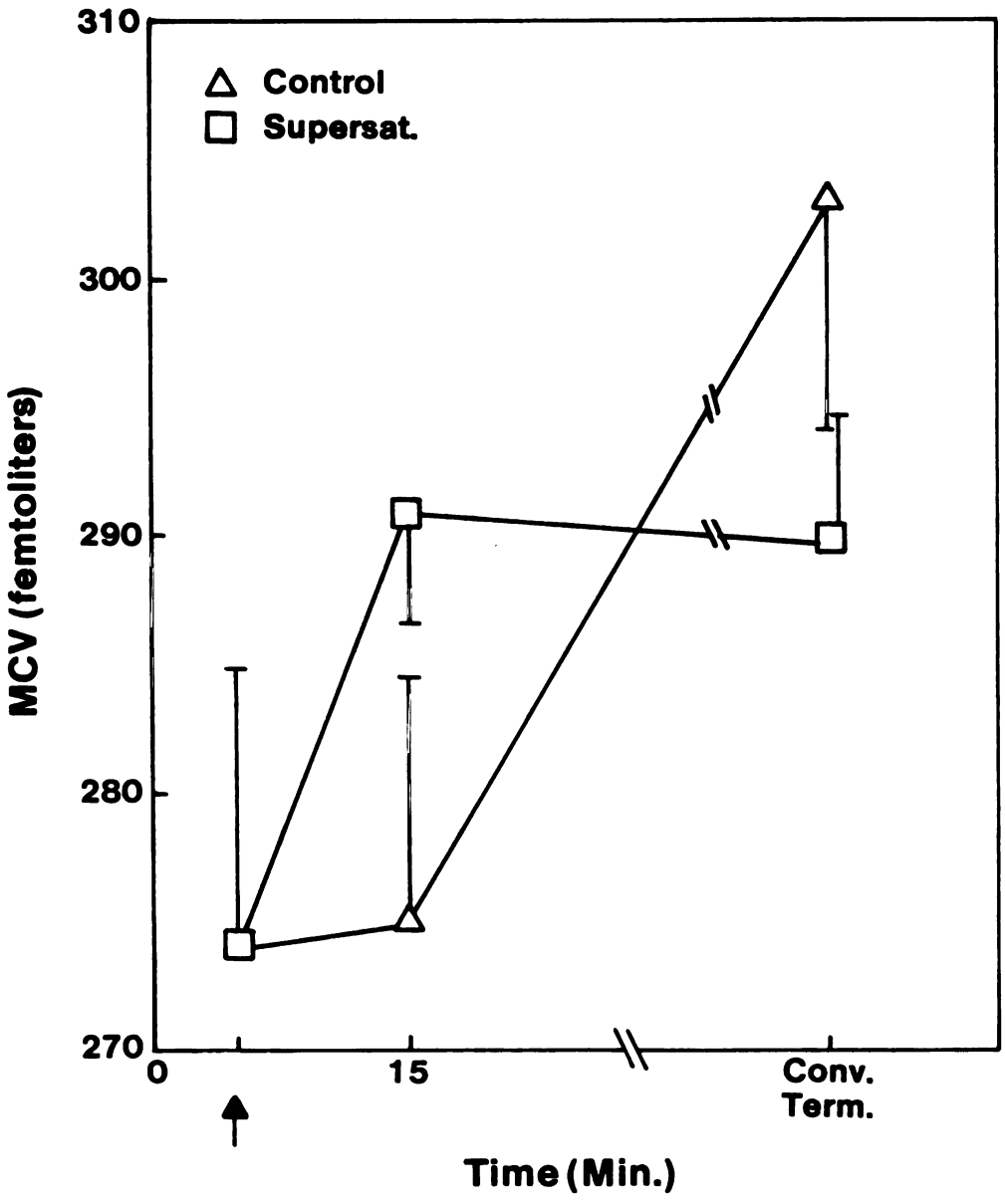
310
300
290
280
270
MCV (femtoliters)
Control
Supersat.
0
15
Conv.
Term.
Time (Min.)

Figure 21

swelling has been associated with the release of systemic catecholamine during stress (Nikinmma 1983). Catecholamine release in trout was suggested by Milligan and Wood (1987) as having a protective effect on blood oxygen transport during a period of acidosis. The stress-induced swelling of erythrocytes is associated with an increase in blood oxygen affinity (Soivio and Nikinmma 1981). Circulating catecholamines, known to induce an elevation in intracellular pH (Primmett et al. 1986), may explain why pHi stabilized after 15 min in controls.

In the supersaturation group, the red cell volume was marginally elevated at 15 min (not statistically significant) in comparison with the sampling control group and remained virtually constant until the moribund stage. The catecholamine response with cell swelling and rise in RBC pH has been reported to be associated with hypoxia and hypercapnia, but not during normoxic adrenergic stimulation (Jensen 1986; 1987). He suggested that this defense mechanism may be activated only during hypoxia when pO_2 is decreased. Since, in exercised normoxic conditions, the blood remains oxygenated, the increase of pHi is absent. Based on the absence of increased red cell volume in this study, supersaturation exposure appears not to induce the adrenergic response that regulates the RBC pH.

It should be emphasized that in neither control nor treatment groups, was there the expected transient increase of pHi. We conclude that, if an acute pHi elevation occurred in either one or both groups, it was not detected due to the sampling protocol used.

DISCUSSION

A. Mortality/Supersaturation

The significant mortality observed in the long term exposure trials (Chapter 1) occurred only when both oxygen and nitrogen levels were above 100 percent saturation. The influence on mortality of altering the PO_2 in the gas mixture is suggested by the findings in trial 1, since nitrogen as high as 117% was not lethal in the absence of oxygen supersaturation. The lethal level under these experimental conditions is confirmed in trial 2 (Figure 1).

Since well water normally utilized in fish operation systems is frequently supersaturated, a practice currently used in fish hatcheries to remedy nitrogen supersaturation is to increase aeration. However, in those situations, oxygen commonly increases above saturation level and the nitrogen excess is not completely bubbled out. Thus the problem may become aggravated. High oxygen levels in the field have not been assessed with regard to GBT. It is suggested that, when a case of GBT is reported, oxygen saturation be documented in addition to nitrogen and/or total dissolved gas saturation.

We endorse the stratagem of reducing oxygen saturation

when nitrogen supersaturation is present to reduce mortality from GBT.

B. Lesion Distribution

Histological examinations (Chapter 1) revealed that the distribution of lesions is specific and is located in tissues associated with acid secreting glands or with high metabolic requirements. Lesions were found earliest in the gill arches, choroid gland of the eye, the skull and in the peripheral tissues. Since each of these anatomical sites are associated with acid secretion resulting in accelerated oxygen exchange, the lesion distribution supports the hypothesis that oxyhemoglobin may play a role in the initial generation of GBT emboli.

C. Carbon Monoxide Study

The reduction of oxyhemoglobin formation by CO-treatment (Chapter 2) did significantly delay the onset of mortality, but did not prevent the eventual, fatal formation of intravascular bubbles in fish exposed to a supersaturation of 130% TDGS. During exposure, the respiratory rate of CO-treated fish was temporarily higher than that of the controls for a period of time similar in length to the delay of onset of mortality. This study documented that shortly after exposure to supersaturation, markedly decreased

ventilation occurred. These findings first suggested that a respiratory acidosis developed before the onset of GBT mortality. The fish reduces its ventilatory drive in response to supersaturation apparently because of the high O_2 tensions. The altered respiratory effort may augment the effect of the hemoglobin mediated gas nucleation.

It can be concluded that the initiation of embolus formation is significantly influenced by oxyhemoglobin function since an 80% CO-hemoglobin formation prolonged the survival time. The increased respiratory rate observed in fish exposed to CO confirms Holeton's (1971b) findings. He subsequently reported that CO treatment did greatly increase breathing movements and cardiac output but did not alter the blood pH (Holeton 1977). Dysfunctional CO-hemoglobin formation is associated with a high respiratory rate because of the need to maintain tissue O_2 delivery in the face of the decreased O_2-carrying efficiency of blood. The rapid respiration is also associated with hypertension (Holeton and Randall 1967a; Stevens and Randall 1967a). Even though trout are more sensitive to the loss of functional hemoglobin than are some other aquatic species, they are able to survive by utilizing O_2 transported through the plasma (Holeton and Randall 1967b; Stevens and Randall 1967b).

In both the CO-treated and control group exposed to

normally saturated water, the buccal and opercular respiratory movements decreased during the 120 minute observation period, presumably due to the initial stress of transference into the experimental tanks.

Carbon monoxide combines with trout ferrohemoglobin and interferes with oxygen transport by two major mechanisms. One mechanism is by displacement of O_2 from hemoglobin in the arterial blood, thereby decreasing the O_2 carrying capacity of the hemoglobin. The other is by interference with O_2 release in the tissues through alteration of the shape of the O_2 hemoglobin dissociation curve. Holeton (1971a) calculated that in trout the affinity of CO for hemoglobin is about 66 times greater than of O_2, but the specific affinity for Root effect hemoglobins has not been completely determined (Brunori 1975).

By utilizing carbon monoxide to inhibit the oxyhemoglobin formation, it was anticipated that fish would be more resistant to GBT. This proposition was based on the knowledge that CO exposure decreases the oxygen carrying capacity of hemoglobin. The CO treatment with an 80% carboxyhemoglobin conversion did achieve a delay in lethal gas emboli formation (Chapter 2, Test 1 and 2). It did not, however, significantly change the accumulated mortality. Perhaps this is because a conversion of 80% is just not enough to prevent the eventual formation of lethal emboli by

the remaining 20% unconverted oxyhemoglobins. Altering the oxygen affinity and release did not change the basic mechanism that is responsible for the formation of large, fatal gas emboli at the high TDGS of 130%. The usual hatchery mortality occurs with supersaturation levels of roughly 115% TDGS (Chapter 1), about half of the TDGS in these carbon monoxide experiments.

D. Proposed Mechanism

The physical-chemical explanation of how gas bubbles are formed within solutions has long challenged researchers and remains incompletely resolved. The assumption that bubbles propagate from pre-existing gas nuclei is well accepted, but how the initial nuclei are generated in various systems is still unknown. In microbial models, intrabacterial gas bubble formation apparently requires the presence of gas nuclei a priori or decompression from pressures above 150 atms (McLeod 1978) for internal bubble nucleation to occur (Hemmingsen 1982). Gas bubble trauma in fish occurs at relatively low TDGS, much less than a 2 atm differential pressure. In fish, therefore, it should come as no surprise that a search is on for an intrinsic mechanism which might explain the generation of blood gas nuclei in GBT.

Attempting to explain the physiological processes that occurs in fish dying of GBT, it is important to distinguish

between the respiratory gases, oxygen and carbon dioxide, and nitrogen. Translation of the oxygen tension from water into the blood depends on blood flow, water flow over the gills and hemoglobin affinity. The equilibrium between hemoglobin and oxygen is influenced predominantly by the PCO_2, the pH, and the body temperature. Fish hemoglobin is relatively sensitive to fluctuations of each of these factors when compared to the mammalian hemoglobins (Powers 1975).

Trout hemoglobin is especially sensitive to such changes and, therefore, the Root effect and the Bohr effect are prominent (Eddy 1971). The binding affinity of hemoglobin for oxygen varies with fluctuations of temperature and acidity (Powers 1975). In fish, the difference between the PCO_2 of inspired and expired water is small, but that of PO_2 is great since oxygen affinity for hemoglobin is high.

Laboratory investigations have shown that oxygen sets the main ventilatory drive in water-breathers; therefore, fish probably only respond to the levels of oxygen in the water and blood rather than carbon dioxide levels as do air-breathers (Eddy 1971). Therefore, changes in environmental nitrogen, blood carbon dioxide, or total gas saturation in water which do not involve changes in oxygen are not directly conpensated by altered respiratory effort. Nitrogen passively diffuses across the gill membrane and the

tension in the blood should reflect that in the water. Nitrogen supersaturation of blood has not been demonstrated (DeJours et al. 1968).

Fish respond to excess of oxygen by markedly depressing ventilation even though their tissues may be accumulating lactic and carbonic acids resulting in decreased blood pH (Wood and Jackson 1980). These hematological changes were also observed when violent exercise and physical disturbance produced low pH values of fish blood (Jensen et al. 1983). In addition, when fish are exposed to supersaturated water, gas comes out of solution and may form bubbles which adhere to the skin and gill membrane. Adhering bubbles significantly block the translation of partial pressure of gases in solution and may affect gas exchange from blood to water and vice versa. Consequently, excretion of carbon dioxide may be greatly reduced at the gills.

Changes in blood pH affect the hemoglobin oxygen binding properties such as P_{50} value, Root effect and Bohr effect. The P_{50} value is the oxygen partial pressure at which the hemoglobin is 50% saturated with oxygen. The Root effect is a phenomenon by which the oxygen carrying capacity of blood is drastically lowered at acidic pH, and the Bohr effect is the decrease in the oxygen affinity of hemoglobin as the pH is lowered. Decreased pH from metabolic activity shifts the blood oxygen dissociation curve to the right,

decreases hemoglobin oxygen affinity and lowers the oxygen carrying capacity. These three factors combine to suppress oxygen binding at the gills and facilitate oxygen unloading at the tissue level (Powers 1972).

During supersaturation exposure, metabolic processes markedly decrease the blood pH and RBC pH (Chapter 3), suggesting that a precipitation of violent oxygen unloading from red cells could form initial gas nuclei. This process may occur even faster during physical stress, as in feeding or handling, because muscular activity raises the partial pressure of carbon dioxide in venous blood from 2.5 to up to 8 mmHg (Holeton 1971a,b; Randall 1975).

E. Root Effect Hemoglobin

In trout, there are four main hemoglobin components (Hb trout I, II, III and IV) characterized by different functional characteristics (Brunori 1975). The oxygen binding properties (Root effect) of the Hb trout IV are greatly affected by pH change, while in the other three hemoglobin components are characteristically independent of pH over the physiological pH range. Because of this, Hb trout IV is also called Root effect hemoglobin (Ingermann 1982).

The Root effect hemoglobins (REH) represent 65% of the total of the hemoglobins and behave similarly to the other

Hbs at a pH above 7.5. REH show marked changes in oxygen affinity as the pH decreases below 7.5. At about pH 6.5 the oxygen affinity is reduced so that the REH is only 20% saturated when pO_2 is at about 150 mmHg (Brunori et al. 1973).

In trout, REH have the important role of oxygen secretion in highly metabolizing tissues. REH can unload oxygen against high oxygen tensions when exposed to low pH (Ingermann 1982).

The eye of many teleosts, including trout, require oxygen tensions approximately 20 times higher than those of blood or ambient water and, therefore, a specialized secretory mechanism has evolved (Wittenberg and Wittenberg 1974). The mechanism (Figure 22), aerobic glycolytic secretion and release of lactic acid by the cells of the retina into the choroid rete (Fairbanks et al. 1974), lowers the pH of entering blood, forcing oxyhemoglobin to rapidly unload oxygen. This increases oxygen diffusion from the choroid rete into the retina. Due to the anatomic configuration of the choroid rete, a countercurrent effect causes rediffusion of lactic acid and oxygen. A high concentration of O_2 in the choroid rete capillaries is maintained by the Root effect and salting out (Fange 1983).

Under supersaturation conditions, as evidenced by our histological studies (Chapter 1) and by lesions in 70% of

Figure 22. Diagrammatic representation of the vascular countercurrent multiplier for oxygen between the choroid rete mirabile and the retina, demonstrating the Root effect hemoglobins (REH) physiological role in oxygen secretion to the trout retina (modified from Ingermann 1982).

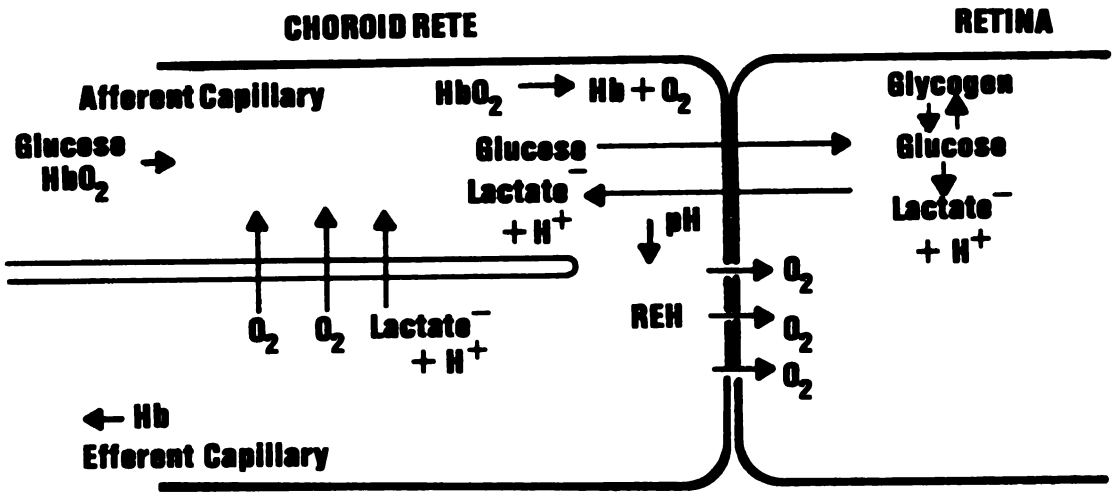
CHOROID RETE
RETINA
Afferent Capillary
HbO_2 → Hb + O_2
Glycogen
Glucose
HbO_2
Glucose
Lactate⁻
+ H^+
Glucose
Lactate⁻
+ H^+
pH
O_2
O_2
Lactate⁻
+ H^+
REH
O_2
O_2
← Hb
Efferent Capillary

Figure 22

GBT fish in Chapter 2 study, the choroid gland is the site of deposition of intravascular gas.

F. Blood pH

The supersaturation-induced stress in fish is associated with acidemia (Chapter 3). Under these conditions, metabolic processes can decrease the RBC pH to 6.90 which corroborates the tested hypothesis that a precipitation of violent oxygen unloading from Root effect hemoglobin could form the initial gas nuclei.

Our observations reveal that a fish exposed to supersaturated water at 140% TDGS react by markedly decreasing its ventilatory drive. This respiratory response, which is more pronounced in smaller fish (Chapter 2), persists until the fish reaches the convulsive stage. This period is quite variable, between 25 and 60 min in the tested experimental conditions (Chapter 3), depending upon the fish's individual resistance to GBT presumibly due to an ability to regulate blood pH.

Under conditions which lower blood pH, stressed fish are known to activate a physiological defense mechanism which stabilizes RBC pH. Catecholamine is released, the red blood cells swell and an increase, rather than decrease, in the RBC pH occurs. The result is that the oxygen uptake at the gills can be maintained due to the increased oxygen

affinity.

The catecholamine response under conditions of supersaturation has not been studied. The findings of the present study (Chapter 3) imply that, under supersaturation conditions, catecholamines have no major influence on RBC pH regulation, because of the lacking MCV change. The extracellular acidosis was followed by an acidosis inside the red cells. The evidence, however, is indirect since it is based only on changes in MCV, and not on catecholamine levels.

To explain an inhibition of the catecholamine response, one might postulate that, in fish, high nitrogen tensions cause an anesthetic effect similar to nitrogen narcosis in mammals (humans). This "calming effect" could be associated with the absence of catecholamine regulation of RBC pH. This, in turn, might explain why gas bubble trauma occurs only when both nitrogen and oxygen supersaturation are present (see Chapter 1).

According to our findings in Chapter 3 experiments, the gas embolization which leads to the convulsive stage appears to be initiated when the intraerythrocytic pH drops below 7.0, a pH at which the Root effect becomes operative. Brunori's (1975) findings strongly suggested that the functional property of the Root effect, to a great extent, is in the "off shift" process, which lends support to the

proposed mechanism that a violent unloading "off shift" of oxygen from REH causes the bubble nucleation.

In summary, when fish are exposed to supersaturation, metabolic processes cause a decrease in the blood pH. Some factors that contribute to the resulted acidemia are: the decreased gill ventilation, physical stress, and the blockage of gas exchange at the gills by gas that comes out of solution in the form of bubbles from the supersaturated water. A marked decrease in the RBC pH follows the acidemia. At that point, the Root off-shift becomes a predominate function of hemoglobin. It is proposed that this accelerated release of oxygen from Root effect hemoglobins at low pH, may initiate the formation of oxygen microbubbles. Because of the supersaturation of blood, bubbles are postulated to enlarge and gas emboli to form, leading to death with a few minutes.

SUMMARY & CONCLUSIONS

Gas Bubble trauma is a pathologic appearance of gas bubbles in the circulation and tissues of fish when exposed to air supersaturated water.

This problem first became extremely economically important in the United States as a result of hydroelectric projects in major salmon migratory systems. These construction projects caused the water of rivers to become supersaturated with atmospheric gases and very high fish mortality occurred due to gas bubble trauma. The river fisheries in the western United States were nearly eliminated because of this condition. For various reasons which remain obscure, the mortality rate has subsided. It was after the construction of new hatcheries in 1980 that GBT was first recognized as a serious problem in the state of Michigan.

Gas bubble formation in organisms is believed to occur as a result of pre-existing gas nuclei. This explanation is sufficient when the organism is subjected to decompression. But, currently there is no accepted explanation for gas nuclei formation in fish where there is relatively low pressure differences.

This study was conducted with two major goals; first, to determine how it is possible for bubble nucleation to occur in fish affected by GBT when pressure differences are relatively low and secondly, to investigate why salmonids are more susceptible to supersaturation.

The GBT pathogenesis hypothesis (Bell and Farrell 1972) was investigated in an attempt to elucidate the bubble nucleation phenomenum. This hypothesis postulated that an accelerated unloading of oxygen from some hemoglobins causes the formation of oxygen nuclei. These microbubbles would tend to grow when released in gas supersaturated blood.

A series of experiments were conducted with the objective of testing the above hypothesis. Firstly, for histopathologic examination, rainbow trout were exposed to supersaturated water at approximately 115% TDGS and samples of whole fish were preserved when fish affected by GBT reached convulsions (the moribund stage). The specimens were fixed using a rapid fixation technique which consists of placing the fish into Bouin's solution at 48°C for 10 min. This technique preserves the GBT lesions better than the standard methods.

The earliest lesions were found mainly in the gills and in the choroid gland of trout eye. The gill lesions were characterized by gas displacement of blood from the afferent arterioles of the gill filaments. This lesion was found in

every supersaturation treated fish, when sampled during the convulsive stage, but was never observed in controls. The most striking ocular lesion was an emphysematous space within a vessel of the choroid body of the eye. These histological findinds suggested that this eye lesion may be associated with the release of oxygen from hemoglobin within the capillaries of the choroid gland. It was concluded that the initial GBT lesions are associated with acid secreting glands and with high metabolic requirements.

Based on the mortality (Chapter 1) data, nitrogen supersaturation may induce GBT in fish if the levels of oxygen are above 100 percent. It may be recommended that, in situations where maitenance of oxygen below saturation levels is possible when nitrogen supersaturation is present, reduced oxygen saturation may be a method to reduce mortality.

Since the initial study supported the hypothesis that the oxygen unloading process is responsible for gas nuclei formation, a second study (Chapter 2) was designed in which the hemoglobin function could be inhibited, so that, the bubble formation would be decreased. Carbon monoxide (CO) was used to block the formation of oxyhemoglobins. A pre-exposure to CO which converted 80% of the hemoglobins to carboxyhemoglobins did significantly delay the onset of mortality, but did not prevent the eventual, fatal formation

of intravascular bubbles in fish exposed to a supersaturation of 130% TDGS.

The gill ventilation of both controls and CO treated fish markedly decreased when supersaturation was imposed. Carbon monoxide treated fish maintained relatively higher ventilatory rate for a time period which was similar to their prolonged survival time. It was concluded that the initial formation of gas emboli was delayed as a result of a carbon monoxide inhibition of hemoglobin function.

One can only infer from these findings that, if CO-exposed fish were tested at a minimal level of TDGS for GBT, then the onset of mortality would be delayed indefinitely. The significance of the demonstrated lethality of slightly elevated environmental oxygen when TDGS was 111-116% (Chapter 1) was that those conditions induced a critical point at which any nucleation may suddenly induce GBT. It is possible that those same conditions would demonstrate a dramatic CO prevention of GBT; however, the long term use of CO within the laboratory has been deemed inadvisable for safety of personnel.

Rainbow trout are characterized by having a pronounced Root effect. Since the Root and Bohr effects are operative when blood pH is lower, a third experiment (Chapter 3) was conducted to determine if there were any change in blood and red cell pH in fish affected by GBT. It was observed that,

the blood and RBC pH markedly and progressively decreased during supersaturation exposure. The most remarkable finding in this study was that, in trout, the onset of GBT occurs when the RBC pH drops to below 7.00 (approx. 6.90).

In summary, the pronounced Root effect of rainbow trout is believe to be disadvantageous during supersaturation exposure. It is suggested that the GBT condition occurs as a result of the combining effects of blood supersaturation, nitrogen narcosis, decreased ventilatory drive, blocked action of gas bubbles at the gill membranes, acidemia, a drop in RBC pH, Root off-shift, and consequent generation and enlargement of bubbles.

The mechanism proposed may explain the "cascading bubble effect" reported by Stroud and Nebeker (1976). Additionally, the mechanism may explain the strain specific GBT resistance reported for certain fish (Cramer and McIntyre 1975), since genetic selection for responses in hemoglobin function may confer resistance. If this were so, then a resistant hemoglobin type could be identified in the field at the time of selection of broodstock.

It must be emphasized that these studies have not yet revealed the exact meaning of the unique phenomenum of GBT in water-breathers. The hypothesis suggested as a basic mechanism of bubble nucleation must be further examined to reveal its exact role in the pathogenesis of GBT.

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LITERATURE CITED

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