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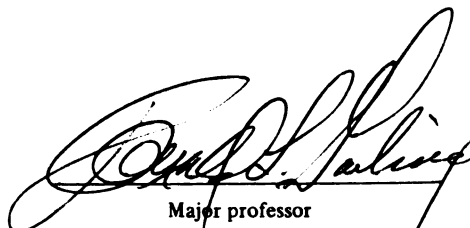
HISTOPATHOLOGIC STUDY OF RAINBOW TROUT FRY
WITH GAS BUBBLE DISEASE

presented by

Joao Paciano Machado

has been accepted towards fulfillment
of the requirements for

Master of Science degree in Fisheries & Wildlife



Major professor

Dr. Donald L. Garling, Jr.

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HISTOPATHOLOGIC STUDY OF RAINBOW TROUT FRY
WITH GAS BUBBLE DISEASE

By

Joao Paciano Machado

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Fisheries and Wildlife

1984

3179357

ABSTRACT

HISTOPATHOLOGIC STUDY OF RAINBOW TROUT FRY WITH GAS BUBBLE DISEASE

By

Joao Paciano Machado

A method for gas bubble disease (GBD) lesion preservation was developed using rainbow trout acutely exposed to total dissolved gas saturation (TDGS) of over 130%. Fixation for 10 minutes at 48°C in Bouin's solution preserved GBD lesions. Chronic exposure to graded levels of TDGS up to 115% for 60 days caused 80% mortality at the highest level coincident with adjustment of waterflow and elevation of oxygen.

Acute and chronic pathology were compared. Gross and histopathologic lesions of moribund fish were similar except for a 10% incidence of exophthalmia which occurred in chronically exposed fish. Lesions were located in tissues associated with acid secreting glands or with high metabolic requirements. Violent hemoglobin unloading apparently produced small emboli which enlarged to block circulation and caused death.

Based on these experimental data, it is suggested that hatchery oxygen levels be maintained below 100% if nitrogen supersaturation is observed.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my major professor Dr. Donald L. Garling, Jr., for his advice and assistance throughout my graduate program.

I deeply appreciate the guidance of Dr. Thomas G. Bell and Dr. Allan Trapp during the course of this project. They shared their expertise in laboratory technique, in histopathological examinations, and in interpreting the results. I am especially indebted to Dr. Thomas G. Bell for generously spending extra time to accomplish this project.

Recognition is due to all my colleagues at the Aquaculture Laboratory for helpful discussions. I am grateful to Tony Ostrowski and Mike Masterson in particular.

I would like to thank Susan Hazard for her work with Dr. Donald L. Garling on the computer program and for typing the thesis.

The study could not have been carried out without the continued financial support, including monthly allowance, health insurance and tuition fees, of the "Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq-BRASIL).

The technical assistance of John Hnath and Harry Westers, Michigan Department of Natural Resources, is gratefully acknowledged.

I would like to thank Dr. Donald L. Garling, Jr., Dr. Thomas G. Bell, Dr. Allan Trapp, and Dr. Patrick M. Muzzall for serving as committee members, for giving me invaluable direction and for reviewing this work.

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 Disease (GBD) was first recognized as a serious problem in Michigan State Fish Hatcheries in 1980. It has affected various fish species, particularly salmonids, and, in many situations, has resulted in unbearably high mortality rates.

GBD is a pathological condition produced in fish maintained in water supersaturated with atmospheric gases, which may result in gas bubble formation in tissues and blood vessels of fish, and may lead to death (Harvey 1974). The severity of GBD and its consequences depend mainly on the dissolved gas pressure in the water, depth of the water, type and species of the fish, duration of exposure, age of the fish, water quality and temperature, barometric pressure and stress on the fish during the exposure.

The recommended saturation level of atmospheric gases in water is approximately 100 percent (Speece 1969). An excess of atmospheric gases in water ranging from 103 to 107 percent of saturation has been determined to be detrimental by the Michigan State Hatcheries' investigators. Westers (1983) reported chronic low levels of gas supersaturation experienced in Michigan hatcheries which were harmful, creating a variable stress condition, making the fish more susceptible to other disorders. These sublethal levels of supersaturation may also cause abnormalities in surviving young fish that could be

responsible for later high mortality rates observed in Michigan hatcheries. Harrietta State Fish Hatchery personnel reported a 74.3 percent mortality in rainbow trout juveniles in 1980 and a 92 percent mortality in 1981. In 1982, over two thirds of the steelhead sac fry and fingerlings died at Wolf Lake Hatchery, and in 1983, losses of steelhead and brown trout were as high as 70 percent. Pendill's Creek National Fish Hatchery lost all 1.3 million lake trout in 1983. Losses of production of lake trout were 35-55,000 per day in two raceways at Platte River Hatchery in July, 1983. The Marquette Fish Hatchery lost 100 percent of their two million lake trout fingerlings in 1984. These events illustrate the devastating potential in relatively low level gas supersaturation conditions when present during the hatchery rearing cycle. These factors were reviewed in recent meetings on gas supersaturation in August, 1983, Milwaukee, Wisconsin and in December, 1983, Traverse City, Michigan.

The problem of GBD has been recognized in many fish culture systems, but in spite of the efforts to evaluate this disease, the cause and effect relationship of the gas supersaturation condition is still poorly defined. More precise information is needed to correlate the incidence of the disease lesions with the levels of gas saturation in the water and length of exposure to the dissolved gas. Pathological changes associated with the disease have been discussed (Marsh and Gorham 1905; Shirahata 1966; Bouck et al. 1975; and Stroud and Neveker 1976), but with a few exceptions (Pauley and Nakatani 1967; Nebeker et al. 1976), there is a paucity of histopathological information defining the location and effect of gas bubbles in the fish under supersaturation conditions.

Some of the difficulty in determining the nature of GBD in fishes may be explained by the rapid disappearance of the gas emboli after death. Harvey (1974) and Weitkamp and Katz (1980) pointed out that in GBD fish external signs were rapidly lost. Therefore, a study of the pathogenesis of gas saturation would require fixation methods which preserve otherwise transient gas bubbles for evaluation in the laboratory through histopathological examination. This study was designed to determine the pathogenesis of hatchery gas bubble disease in salmonid fry reared with well water containing varying levels of gas supersaturation. The specific objectives were: (1) to develop a rapid fixation technique to aid in assessment of the histopathological examination of GBD lesions and (2) to correlate the anatomical location and appearance of GBD lesions with the onset of signs of the disease in the acute and chronic conditions.

REVIEW OF THE LITERATURE

Gas Bubble Disease (GBD) has been observed and described in fishes by many investigators throughout the United States since the beginning of this century. GBD in fishes was first thoroughly studied and documented by Marsh and Gorham (1905) in an attempt to solve a water saturation problem at the United States Bureau of Fisheries Station at Woods Hole, Massachusetts. Interest in GBD reemerged in the late 1960's, when the fish mortalities associated with hydroelectric projects in the Pacific northwest nearly resulted in annihilation of the west coast fisheries. Technological advances have been introduced to alter supersaturation and those modifications have reduced gas supersaturation to tolerable levels at several hydroelectric dams on the Columbia and Snake Rivers (Ebel 1979). It is not known if natural selection among fish populations has played a role in reduced fish mortality.

The first recorded incidence of serious GBD in the midwestern United States occurred in 1978 due to gas supersaturation in the Osage Arm of Lake of the Ozarks below Harry S. Truman Dam, a United States Army Corps of Engineers hydroelectric project (Crunkilton et al. 1980).

The occurrence of gas supersaturation in Michigan State Fish Hatcheries is relatively recent, Spring 1980, and has prompted more questions than answers (Westers 1983). High mortalities of young salmonids due to GBD throughout the rearing cycle have been reported

by many Michigan Fish Hatchery biologists including: Vernon Bennett, Harrietta Fish Hatchery; Pete Drake, Pendills Creek National Fish Hatchery; John G. Hnath, Wolf Lake Fish Hatchery; John Driver, Marquette State Fish Hatchery.

The gas tension in water depends on the total dissolved gas. GBD results from high gas tensions in the water to which fish are exposed. Fish maintained in water supersaturated with air reach equilibrium with the gases dissolved in water. In the fish, gases tend to equilibrate with the atmosphere just as the gases in the water do (Rucker 1972).

Atmospheric air is composed principally of nitrogen (N_2), oxygen (O_2), and argon (A) in the following proportions: 78 percent, 21 percent and 1 percent, respectively. Since N_2 and A are biologically inert gases and A is present only in very small quantities, they will be considered together. The solubility of each gas is determined by the mass of the individual gas and its partial pressure in the atmosphere. Oxygen in air has only one quarter the partial pressure of nitrogen, but is two times more soluble than nitrogen. Therefore, in water, oxygen (35 percent) is half as plentiful as nitrogen (65 percent). Carbon dioxide, while very soluble in water, is present in minute amounts, usually less than three ppm.

The solubilities of atmospheric gases in water are affected principally by temperature and pressure (Henry's Law). Pressure is increased in water by hydrostatic head. Hydrostatic pressure increases rapidly with depth, greatly increasing the capacity of deeper water to hold dissolved gas as compared to shallow water. The solubility of nitrogen and oxygen in water decreases rapidly with increasing temperature.

An increase in water temperature will produce supersaturation in water that is initially unsaturated (Harvey 1974, Weitkamp and Katz 1980).

The respiratory gases, O_2 and CO_2 , are carried by hemoglobin in the blood while N_2 is not. Translation of the O_2 tension from the water into and out of the blood depends on blood flow and water flow over the gills. The equilibrium between hemoglobin and oxygen is influenced predominantly by pCO_2 , pH, and the temperature. Fish hemoglobin is relatively sensitive to slight fluctuations in each of these influencing factors when compared to mammalian species and trout hemoglobin is sensitive compared to other fish. The reduction of oxygen carrying capacity with increased pCO_2 is the Root effect and with reduced pH is the Bohr effect (Eddy 1971).

In fish, the difference between the pCO_2 of inspired and expired water is small but that of pO_2 is great since O_2 affinity for hemoglobin is high. Nitrogen passively diffuses across the gill membrane and the tension in the blood reflects that of the water, although demonstration of a nitrogen supersaturation of blood has not been reported (DeJours et al. 1968).

GBD is defined as a noninfectious, physically induced condition produced in fish exposed to water supersaturated with atmospheric gas. This process occurs when the sum total of all dissolved gas partial pressures (ΔP), is greater than the atmospheric pressure directly above the water surface. In this uncompensated condition, gas bubbles can be formed in the fish's body and produce lesions in blood vessels and in the tissues resulting in physiological disfunction which may lead to death (Bouck 1980).

Supersaturation of water is the cause of GBD. A number of natural and human-related processes can produce gas supersaturation leading to GBD. In general, these processes either cause an increase in the amount of air dissolved or they reduce the amount of air that water will hold (Bouck 1980).

The most common human sources of supersaturation are air injection, hydroelectric projects, and thermal pollution. Air injection was first described by Marsh and Gorham (1905) as a result of an air leak on the suction side of the water supply system for the Woods Hole aquarium system permitting air to be drawn in with the water, causing supersaturation. Hydroelectric facilities can cause supersaturation by gas entrainment below dam spillways. This phenomenon causes air and water to be mixed and carried to substantial depths where hydrostatic pressure is sufficient to greatly increase the solubilities of atmospheric gases producing supersaturation with respect to surface or atmospheric pressure. Thermal stations can produce gas supersaturation through the inverse relationship between water temperature and gas solubility. Heating water to promote fish growth has resulted in supersaturation (Weitkamp and Katz 1980).

Fish hatchery or aquarium water supplies are often derived from wells or springs. These waters are frequently supersaturated with dissolved gases. The aspirating effect of water flowing downward into aquifers carries air with it. The air is thus mixed with water under considerable pressure in the aquifers. Oxygen concentrations may be reduced prior to use of these waters; however, the inert dissolved nitrogen gas remains in solution, causing nitrogen supersaturation.

problems (Weitkamp and Katz 1980). Marsh and Gorham (1905) discussed such situations in the well water supplies at a Tennessee and a New Hampshire fish hatchery. Rucker and Tuttle (1948) described a Washington State hatchery water supply with 110 percent total gas pressure (120 percent nitrogen and 80 percent oxygen). Well water at the La Crosse National Fishery Research Laboratory was reported to contain supersaturation of nitrogen; levels ranged from 130 to 140 percent of saturation (Anon. 1982).

Photosynthesis can lead to high levels of dissolved oxygen in natural waters. Fish kills in lakes and in bays have been attributed to the supersaturation of water through photosynthetic activity of algae and warming of the surface water (Stroud et al. 1975 and Woodbury 1941).

Several studies have discussed the role of nitrogen and/or total dissolved gas saturation in causing GBD in aquatic organisms. Marsh and Gorham (1905) pointed out that only two gases, oxygen and nitrogen, are dissolved in water in significant quantities and that a large excess of oxygen is required to produce GBD using this gas alone. They concluded, based on the Woods Hole studies, that while both oxygen and nitrogen were in excess, the damage was probably done by nitrogen alone. Some reports reviewed by Weitkamp and Katz (1980) indicated that high levels of oxygen (above 300 percent) can produce GBD, but in those cases dissolved nitrogen was not measured. Rucker and Kangas (1974) exposed salmonid fry to constant nitrogen supersaturation at different total dissolved gas pressures. The test fish exposed to total gas saturation of 120 but not at 116 calculated as excess of the solubility

of oxygen and nitrogen at appropriate conditions were killed with GBD. The higher total gas pressure resulted in a significant upsurge of mortality at 25 days while the same increase in mortality did not occur at the lower gas pressure until 35 days of exposure. They concluded that those results indicated total gas is more important than the nitrogen saturation alone in producing the disease.

Meekin and Turner (1974) reported high mortalities of three species of juvenile salmonids tested (chinook salmon, coho salmon and steelhead trout) at nitrogen levels over 120 percent saturation and supersaturated oxygen although the actual level of the latter was not reported. These test fish died in less exposure time than those fish held at similar nitrogen levels with oxygen at less than saturation. They also found that salmonid juveniles survived nitrogen levels of 112 percent as long as oxygen was not supersaturated. From these results they concluded that both O_2 and N_2 are involved in GBD.

Marsh and Gorham (1905) reported that the fish vascular system becomes supersaturated as a result of the increase of osmotic pressure due to exchange of gases at the gills, between water and blood when fish are exposed to supersaturated water. In that new situation, the gills are subject to a very high osmotic pressure. The osmotic pressure, by diffusion, on the two sides tends to equalize; considering that the blood and water have approximately the same saturation point, the blood stream presumably would acquire the same excess of gas as that in the water.

The gas bubble formation within the vessels was explained by Marsh and Gorham (1905) as due to a rise in blood temperature ($1-7^{\circ}C$)

as it passes from the gills to the systemic system, decreasing the solubility of the gases which come out of solution. Secondly, the precipitation of gases was explained by the principle that supports the existence of a gas nuclei around which there is supersaturated blood. They concluded that the temperature change within the blood is the most important and a fundamental cause of the release of gases. The nuclei, generated by the red blood cells, provide loci for the change of state.

Weitkam and Katz (1980) added that bubbles will form most easily at an interface of water and another substance where gas nuclei are normally present in a supersaturated condition. They also point out that gas nuclei decrease either the solubility or the surface tension of the supersaturated water, allowing the excess of gas to come out of solution. Thus, they states that gas nuclei allow the formation of gas emboli in fish that reside on the vessel linings.

Harvey and Cooper (1962) pointed out that gases enter and leave solution in relation to their partial pressures and solubilities and hence bubble growth involves both oxygen and nitrogen. The gas content of bubbles in fish suffering from GBD was analyzed by Shirahata (1966). He reported that the composition of the gases was essentially the same as the dissolved gas content of water that had been supersaturated with air.

The physiological and environmental factors that contribute to release of visible gas emboli in the circulatory system of fish exposed to supersaturated water are unknown. However, Stroud and Nebeker (1976) observed that fish frequently died of gas emboli shortly after stress was induced. Disturbances such as the netting of live fish,

removing dead fish, and taking gas measurements, all appeared to contribute. Their data indicated that initiation of bubble growth from pre-existing nuclei and/or bubble dislodgement from the periphery of the body may occur under physiological conditions such as muscular activity or excitement that can trigger a "cascading bubble effect" of emboli into the gill capillaries with resultant stasis.

Gas emboli were first described as the cause of the death in fish exposed to supersaturated water by Marsh and Gorham (1905). They found lesions within blood vessels primarily in the heart and main vessels of the gill filaments which usually were filled with gas. They concluded that the stasis of the blood caused by emboli in the major vessels resulted in anoxia and death of the fish. External signs observed by Marsh and Gorham such as exophthalmia and gas blisters in the fins, the lining of the mouth, the head or along the lateral line were not considered to be sufficient to cause death. They suspected that pressurization at the brain could account for some mortalities; therefore, they examined a butter-fish Peprilus triacanthus, specimen taken at Woods Hole aquarium with moderate exophthalmia on each side. The brain and optic nerves appeared normal; therefore, they concluded that if there had been a traumatic injury, evidence of it had disappeared.

The conclusion that the cause of death of fish by acute GBD as stasis was supported by Stroud and Nebeker (1976). They observed that fish prior to death, exposed to 120 percent total gas pressure, determined by the Weiss saturometer, did not have visible accumulations of gas within major vessels, heart and gills. However, at death, fish exposed to 120 percent had large accumulations of gas emboli in the

heart, ventral aorta and gills. They concluded that the formation and/or transport of macroscopic emboli within the blood vascular system which can obstruct normal blood flow appears to be an acute terminal or near terminal phenomenon. Dawley et al. (1976) found bubbles in the gills of dead fish but seldom in live fish. They concluded that those signs are directly associated with death. Several species of fresh-water fish exposed to nitrogen supersaturation usually died by asphyxiation from gas embolism in the circulatory system (Egusa 1959). Pathological changes associated with GBD were described by Pauley and Nakatani (1967) and the death in fish was reported to be caused by necrosis of vital organs and tissues presumably due to anoxia resulting from capillary occlusion by gas emboli. Stroud et al. (1975) supported the roles of emboli in death caused by GBD, but also mentioned other factors. They suggested that sublethal effects such as blindness, stress, and decreased lateral line sensitivity could lead to death from other causes such as predation. They pointed out that sublethal lesions can also increase susceptibility to other diseases. Weitkamp (1976) found that fish which were not able to recover from GBD apparently died due to secondary fungal infection.

GBD syndrome is characterized by a wide variety of physiological and behavioral signs exhibited by the fish exposed to supersaturated water.

Marsh and Gorham (1905) indicated that cod fry exposed to supersaturated water at Woods Hole for a few days did not show any GBD symptoms. They noted; however, that very young fry are not necessarily resistant under all conditions of supersaturation. Nebeker et al. (1978)

observed that steelhead trout fry (16 day posthatch) exposed to supersaturated water showed respiratory difficulties with gas bubbles in the mouth, gill cavity and yolk sac, resulting in flotation and/or rupture of yolk sac membranes which led to death. Sockeye salmon alevins were reported by Harvey and Cooper (1962) to be especially susceptible to GBD, with symptoms developing within five days of exposure to water varying in nitrogen saturation from 108 to 120 percent. Gas bubbles accumulated rapidly in the yolk sacs, buoying the fish helplessly to the surface.

Jones and Lewis (1976) observed that channel catfish fry (6-8 weeks old) after 1 week of exposure to supersaturated water showed large air emboli lodged in the peritoneal cavity. They reported that some of the bubbles were large enough to interfere with equilibrium and cause the fry to swim on their back. Erratic swimming behavior of coho salmon fry having large gas bubbles in the yolk sac was described by Adams and Twole (1974).

Bioassays were carried out by Shirahata (1966) who exposed rainbow trout fry to nitrogen supersaturated water and observed gas bubbles adhered on the body surface of fish around the head, in the oral cavity and gill covers. These bubbles caused them to rise to the surface and/or suffocate by blocking the normal flow of water through the oral cavities. Blisters filled with gas appeared in the following subcutaneous tissues: the basal part of fins, especially the adipose and caudal fins; the bottom of oral cavity; the interbranchiostegal membrane; the lateral lines and the orbits of eyes.

In work with newly hatched coho and chinook salmon fry, Rucker and Kangas (1974) found them tolerant to nitrogen supersaturation up to

128 percent. They developed normally without adhesion of bubbles or any noticeable external abnormality. The fry of the two species developed symptoms of GBD at 10 and 20 days respectively after hatching. Mortality ensued chiefly from rupturing of the vitelline membrane. They noted that fry that were able to survive bubbles in the yolk sac later developed signs of GBD. Stroud et al. (1975) exposed cutthroat trout fry (Salmon clarki) to supersaturated water and observed similar symptoms described by Shirahata (1966), and Rucker and Kangas (1974). They pointed out that lethal levels of supersaturation to salmonid larvae and fry vary widely according to exposure circumstances and that the overall impact of supersaturation at these life stages is not yet clear. Their observations also suggested that the effects of GBD on embryos or fry may result in long term low vigor and poor survival.

Symptoms of GBD in juvenile and adult salmonids are similar to those described for young but generally occur with a different frequency in the various organs and tissues (Dawley et al. 1976). The cutaneous bubbles were the most common symptoms reported by Meekin and Turner (1974) in juvenile chinook salmon exposed to nitrogen supersaturated water. They also found exophthalmia in less than 5 percent of dead fish. Westgard (1964) reported in his bioassay studies that adults frequently developed exophthalmia and bubbles in the roof of the mouth near the branchiostegal region. Bubbles along the lateral line were observed by Dawley et al. (1976) in 50 to 100 percent of juvenile chinook salmon and steelhead trout exposed to supersaturated water. Bouck (1976) found adult chinook salmon swam aimlessly, were unresponsive, and exhibited a "coughing syndrome" as they

approached death. They noticed that those fish also tended to remain at the maximum available depth during the bioassays, thus utilizing the available hydrostatic pressure to compensate for supersaturation.

Distention of the swim bladders of juvenile salmonids were reported by Stroud et al. (1975) and Chamberlain et al. (1980). Gas supersaturation caused the swim bladders of the fish to inflate, resulting first in upward drift and then in downward swimming to restore neutral buoyancy.

The effects of sublethal levels of supersaturation such as blindness, stress, decreased lateral line sensitivity, increased disease susceptibility or damage to vital tissues such as the gills and nares have not yet been thoroughly studied (Stroud et al. 1975).

Lesions associated with GBD are especially dependent on the levels of supersaturation, duration of exposure, and species and life stage of fish. Lesions in GBD affected fish were found by Marsh and Gorham (1905) within the main blood vessels of the heart and gills. The walls of the auricle and ventricle were often emphysematous. Gas bubbles in the gill filaments were described as the most constant and significant lesion. Gas bubbles found in the pyloric caeca, in the walls of the intestine and within the intestine itself, were not considered by Marsh and Gorham (1905) to be related to GBD.

Rucker and Kangas (1974) reported that coho and chinook salmon fry (3 weeks after hatching) exposed to supersaturation, developed GBD. Macroscopic examination of moribund fish revealed bubbles of gas in the bulbus arteriosus and ventral aorta. Frequently gas bubbles were observed in the gills. Histological examination was done; however, no significant changes were observed.

Necropsy of adult sockeye salmon exposed to 115 to 120 percent supersaturation was performed by Nebeker et al. (1976). Necropsied fish after death contained subdermal emphysema (gas blisters) in the mouth, in the fins, on the opercle, at the base of the gill filaments and the gill rakers. The gills appeared hemorrhagic and swollen, and bacterial and fungal infections became apparent in tissue devitalized by these lesions. Gas emboli filled the bulbus arteriosus and ventral aorta, and emboli were observed in the coronary arteries. Gas emboli and hemorrhage were observed in the eye. Blood vessels in the brain were congested and free gas bubbles were observed in the kidney. No emboli were observed in the heart or associated blood vessels in fish held at 115 percent saturation that died after 21 days of exposure. No external lesions were observed in the fish held at 110 percent saturation.

Chinook salmon fingerlings were diagnosed by Pauley and Nakatani (1967) as having GBD, and their tissues were fixed and stained for histopathological examination. They reported that gas bubbles filled the branchial arterioles, causing degeneration of gill filaments, which became edematous, developed aneurysms, and sometimes contained hemolyzed red blood cells. The most striking pathological changes were observed in the roof of the mouth where the squamous epithelium had undergone hydropic degeneration and was edematous and hypertrophied. They stated that these pathological changes appear to be unique to GBD. The epithelial layer of the skin showed pathological changes similar to those they observed in the epithelium of the roof of the mouth. The muscle fibers were atrophied, edematous, and separated from the sarcolemma. The muscle cells exhibited a light staining

cytoplasm which had lost its characteristic striated appearance. The muscle nuclei were observed to be pyknotic and, in many instances, completely separated from the muscle fibers. The liver cells had undergone degenerative changes, with vesicular, hypertrophied cytoplasm and pyknotic nuclei. Necrosis was observed in the intestines and kidneys of the diseased fish. The kidney tubules possessed epithelial cells exhibiting vesicular, lightly stained cytoplasm and pyknotic nuclei. The kidney also had an increased number of hemolyzed red blood cells. The spleens of all infected fish were reported by Pauley and Nakatani (1967) to be enlarged with hemolyzed red blood cells and a reduction of white pulp. They reported that those histological changes were observed in GBD affected fish in its early stages.

Stroud et al. (1975) reported that a necropsy of juvenile salmonids dying from acute GBD at 125 and 130 percent TDGS revealed hyperemia at the base of the fins and hemorrhagic gills. Gas emboli and hemorrhage were observed in the kidney, liver and spleen. Gas emboli were also observed in the coronary vessels, intercostal vessels, and sinusoidal tissue throughout the body. However, some fish died at 125 percent and 130 percent levels without visible external lesions.

After long term exposure of adult chinook salmon to 115 percent saturation, Stroud et al. (1975) observed extensive emphysema of the fins, skin and opercula. In those fish surviving extended exposure (more than 200 hours), the gills and skin were invaded by fungus. Gas emboli in the vascular system were infrequently observed in fish dying after chronic exposure. Petechial hemorrhages were observed in the fins, skin, muscles, gonads, nares, brain and other tissues in many fish. Edema of the mesenteries often accompanied by ascities

were observed in 100 percent of the fish examined. Hemorrhage and edema were observed in the opercular and branchiostegal areas. They reported emphysema beneath the peritoneum covering the kidney and ribs, and occasionally under the epicardium in the region of the bulbus arteriosus, in several juvenile salmonids. Exophthalmia was due to gas accumulation in the fatty tissue of the orbit. Bubbles of air directly under the cornea were also observed in a few fish.

A unique lesion, muscular emphysema, occurred following prolonged exposure (more than 400 hours) to 115 percent TDGS. The darker (red) muscle along the lateral line had a greater number of cavities than the remaining somatic musculature. They attributed that difference in incidence to be related to differences in metabolic activity, fat content or vascularity. Histological investigation of tissues by Stroud et al. (1975) failed to confirm the characteristic hydropic degeneration of some tissues observed by Pauley and Nakatani (1967).

The diagnostic methods for GBD have not yet been well defined. Positive identification of the disease should include confirmation of the excess of dissolved gases, the presence of gas bubbles in the body surface (external signs), and gas emboli in the blood and tissues (internal signs). The observation of structural lesions should also be included.

Some investigators have suggested some procedures to distinguish gas bubble disease from other others based mainly on clinical diagnosis. Shirahata (1966) reported that it is possible to make early diagnosis of GBD by examining whether gas bubbles adhere on the surface of the fish's body, especially on the fins, a few minutes after exposing fish

to new water. This phenomenon was observed on the body of rainbow trout fry younger than five weeks after their initial feeding, in the water containing more than about 120 percent of nitrogen gas. In addition, Shirahata (1966) suggested that if fish kept in super-saturated water exhibit rushing, whirling, and spasmodic movements and die, it is desirable to examine carefully whether gas emboli are seen in the bulbus arteriosus and ventricle of heart.

Signs of GBD can disappear after death and leave the carcass without evidence of the lethal agent (Bouck 1976). Bouck (1980) suggested that both emboli and signs of emphysema disappear because the heart has stopped supplying hyperbaric gases for continued inflation.

Newcomb (1976) attempted to determine the physiological changes of the disease based on laboratory findings during the life of the fish. He investigated the sublethal effects of exposure to nitrogen supersaturation and found no differences in blood chemistry of juvenile steelhead trout cultured in water of 110 percent saturation or less. Exposure to 116 percent resulted in decreased serum calcium, albumin, cholesterol, protein and alkaline phosphatase, and increased serum potassium and phosphate.

Pathologic diagnosis may be determined by observing the structural lesions present. Pauley and Nakatani (1967) preserved chinook salmon fingerling specimens for histopathological examination that exhibited a light case of the disease. They concluded that degenerative changes in the epithelium of the roof of the mouth appeared to be unique to gas bubble disease. This lesion was considered by them as the diagnostic characteristic for detecting GBD in its early stages.

Differentiation between spurious mortality and GBD may be a problem. Lethal levels of supersaturation to salmonid species vary widely according to lifestage and exposure circumstances. In general, GBD may be chronic at low supersaturation levels or acute at high levels. Salmonid eggs have been shown not to be affected by supersaturation. Marsh and Gorham (1905) reported that cod eggs incubated for two weeks at Woods Hole in supersaturated water, at levels fatal to juveniles and adults, were not affected. Embury (1934) found that increasing total dissolved gas levels above 111 percent saturation by raising the water temperature (5°C), did not affect trout eggs but the resulting fry developed GBD. No signs of GBD were found by Meekin and Turner (1974) in chinook salmon eggs at 122 percent nitrogen saturation but reported heavy mortality (50 percent) of steelhead trout eggs reared in supersaturated water at the same level, although they did not describe GBD signs in them. The development of embryos of steelhead trout was not affected when held at 126 percent TDGS (Nebeker et al. 1978). These results were also observed by Rucker and Kangas (1974) who found that coho and chinook salmon eggs were not affected until hatching, when exposed to 128 percent saturation. The alevins also tolerated those levels for one week post-hatching.

Nebeker et al. (1978) found that the onset of GBD does not occur until swim-up stage of steelhead trout (16 day posthatch) exposed to 126 percent saturation. Death occurred rapidly through the course of the 40 day test with mortality rate up to 99 percent at 126 percent and 45 percent at 115 percent saturation. Similarly, Shirahata (1966) indicated that the tolerance of trout fry to gas supersaturation was

considerably higher for the period from hatching to swim-up, and that the tolerance decreased with the development of fish. He reported that nitrogen saturation levels which did not cause GBD in young fry were 130 percent, 120 percent in older fry, 110 percent in fry three weeks after the beginning of feeding and less than 110 percent in the more advanced fry.

Rucker and Hodgeboom (1953) noted that dissolved nitrogen gas about 115 percent created excessive mortality in trout fry. These fish were more susceptible to parasite diseases than those held at lower levels of nitrogen supersaturation. Surviving juvenile salmonids exposed to nitrogen levels over 120 percent were listless and did not actively feed (Meekin and Turner 1974). They observed that smaller juveniles survived high nitrogen levels for longer periods than larger fish.

The hypothesis that larger fish tend to be more susceptible to gas supersaturation than smaller ones, was also supported by Egusa (1959). He stated that since larger fish have stronger muscular activity and since the bubbles occur in blood vessels as a result of the contraction of muscles between which blood vessels pass, the stronger muscular activity must be accompanied by a more active bubble formation. He also suggested that at nitrogen levels below 115 percent almost every fish could survive indefinitely without showing any signs of GBD.

Nebeker et al. (1976) exposed adult sockeye salmon to air-supersaturated water. The first mortality occurred after 3 days of exposure to 120 percent saturation and 40 percent of the fish were

dead after 5 days. At 115 percent saturation, the first mortality occurred after 21 days and 40 percent were dead after 35 days. No further mortality occurred at 120 or 115 percent. However, at 120 and 115 percent saturation, survivors exhibited many sublethal lesions such as hemorrhaging and emphysema (bubbles) in the mouth, on the gill arches, body surface, and fins, etc. No deaths or signs of GBD occurred in fish held at 110 percent saturation. The lethal threshold was reported to be 114 percent saturation.

MATERIALS AND METHODS

Experiment 1 Fixation Techniques: Temperatures

I. Objectives

The specific objective of this experiment was to develop a rapid fixation technique to aid in assessment of the histopathological examination of gas bubble disease lesions in fish after exposure to acutely lethal gas supersaturated water. For this purpose, control fish and fish with acute, fatal GBD were utilized. Various fixation procedures were tested to determine if a method could be devised to preserve indefinitely the lesions of GBD.

II. Hypothesis

The hypothesis to be tested was that by increasing the temperature of the fixative, a temperature greater than ambient at which preservation of the size and distribution of tissue emphysema induced by acute GBD could be determined.

III. Test Animals and Design

The rainbow trout used in this experiment were reared at the Aquaculture Research Laboratory facilities operated on the campus of Michigan State University. The same facilities were used to conduct the remaining experiments.

Supersaturated water was produced by injecting compressed air (30.0 ± 0.5 PSI) for 5 minutes into a one liter plastic capped jar half filled with water at 12°C . The jar was constantly and violently agitated by hand during this process. The water was returned to normal atmospheric pressure creating a level of supersaturation previously determined to exceed 130 percent total dissolved gas saturation (TDGS).

Rainbow trout fry (3.5 cm long and 5 weeks old) were randomly selected in four lots of six fish on each from a homogeneous two thousand member tank. Each lot was separated in two groups of three experimental and three control fish.

IV. Experimental Procedure

Experimental fish were placed into the supersaturated water for approximately 20 minutes. After reaching the moribund stage, they were rapidly transferred to a Bouin's solution which was previously heated to achieve the desired temperature in a temperature control water bath. These procedures were repeated with fixative at 12, 24, 48, and 80°C . The fish groups placed in 24, 48, and 80°C heated Bouin's solution were fixed for 20, 10, and 5 minutes respectively. After the procedure they were transferred to 30 ml vials filled with fixative heated to each temperature. The specimens were left in the fixing solution for 36 hours and then trimmed, processed, cut at $6\text{ }\mu\text{m}$ and finally stained by the Hematoxylin and Eosin Method at the Animal Health Diagnostic Laboratory (AHDL) of Michigan State University for histopathological examination. All the fixation procedures detailed above were simultaneously performed on the control fish. The samples from

each temperature treatment were assessed by microscopic comparison of liver, kidney, brain and gill morphology and compared for the clarity of detail, uniformity of staining and presence of tissue emphysema. Any pathological alterations were recorded. All observations were made without knowledge of the treatment or control category to maximize objectivity.

Experiment 2 Fixation Techniques, Time

I. Objectives

The objectives of this experiment were: (1) to investigate the effects of exposure time variation with heated Bouin's solution at the optimal temperature derived from experiment #1 on tissue and lesion preservation in acute GBD; (2) to define and describe the location and effects of gas bubbles formed in the fish's body in gas supersaturation; and (3) to evaluate the hypothesis that the fixative solution temperature and gas pressure influence the gas bubble size formed in the tissues.

II. Hypothesis

The hypothesis to be tested was that by varying the time of fixation, an optimal time at which preservation of the size and distribution of tissue emphysema induced by acute GBD could be determined.

III. Test Animals and Design

Rainbow trout from the same population used in experiment #1 were used for these experiments. Four lots of six fish, each from the same population, were separated in two groups of three experimental and three control fish.

IV. Experimental Procedure

Following the previous procedure (Experiment #1), three fish were placed into the supersaturated water chamber. After reaching the moribund stage, they were rapidly transferred to a Bouin's solution which was previously heated to achieve the desired temperature (48°C) in a temperature controlled water bath. These procedures were repeated four times varying the exposure time for 5, 10, 20 and 40 minutes. Afterwards, they were placed in 20 ml vials filled with Bouin's solution at ambient temperature (12°C). The fish samples were transferred to 70% ethyl alcohol after fixation in Bouin's for 24 hours. The samples were taken to the ADHL for staining procedures and histopathological examination.

Experiment 3 Chronic Gas Bubbie Disease

I. Objectives

The objectives of this experiment were: (1) to expose well water reared rainbow trout fry to chronic low levels of supersaturation and fix the lesions by utilization of the methods developed in experiments #1 and #2, (2) to investigate the location and effect of emboli within the tissue of fish by histopathologic examination, (3) to determine the pre-lethal histopathologic lesions of chronic GBD by comparing histopathologic lesions from fish in experiments #1 and #2 with fish from experiment #3.

II. Hypothesis

The hypothesis to be tested is that: (1) there is a difference between lesions of acutely lethal and chronic gas bubble disease determinable by fixation of tissue emphysema and other lesions through histopathologic

examination and (2) there are lesions of chronic supersaturation exposure which precede induction of gas bubble disease detectible through histopathologic examination.

III. Test Animals

The fish used in this experiment were rainbow trout reared at the Aquaculture Research Laboratory facilities of Michigan State University. Eggs taken from spawning fish by laboratory personnel on November 23, 1983 hatched by December 23, 1983. Sac fry started actively feeding by January 10, 1984.

The experiment commenced on February 25, 1984, using nine week old rainbow trout fry with an average total length of 4.5 cm. Fish were fed twice a day with Biodiet Grower (Bioproducts, Inc., Washington). The fish were exposed to artificial light which simulated a natural photoperiod with a ratio of 14 light to 10 dark hours a day.

IV. Test Design

In order to create a higher than normal gas tension in the rearing trays, the water was supersaturated by pumping air into a vertical column. This was accomplished by bubbling pressurized air from a one-half horse power motor into the base of the water column which was 4 m high and 15 cm in diameter creating a maximum hydrostatic pressure of 1.4 atmospheres. The air was forced through an air-diffusing stone into the water, facilitating dissolution. The water column was contained in polyvinyl chloride (PVC) pipe, open on the upper end to allow excess gas to escape and fitted with an overflow system to drain off excess volume. Supersaturated water was released from the base of the column to the rearing trays through a

tygon tube which directed water to four vertical stacks of three plastic trays placed 30 cm above one another, each 38 cm by 50 cm by 12 cm. The flow rate was adjusted to produce a water velocity in the test units of approximately 200 ml/min.

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

A reference vertical stack of three trays serving as the control had the same waterflow system. The water source was also from the same well but did not undergo supersaturation.

In this experiment, the shallow rectangular test trays contained 10 cm of water and held a volume of 16.2 liters. With a waterflow of 200 ml/min. for each of the trays, the amount of time required to replace the body of test water was estimated to be 6 hours.

V. Experimental Procedure

A. Randomization

Allotment of fish in the fifteen shallow test trays from the two thousand member holding tank was performed in a fashion similar to dealing a deck of cards. The first groups of five fish netted from the holding tank was placed in the first test tray, the second group of five fish into the second tank, etc.; the sixteenth group of five fish into the first tank, the seventeenth into the second tank, etc.; this was continued until all test trays each contained fifty fish.

B. Water Analysis

Dissolved oxygen, total dissolved gas pressure (ΔP), barometric pressure, water temperature, Bunsen's coefficient, and water vapor pressure were taken biweekly in every test tank (Figure 1). 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 (ΔP). The tensionometer is an electronic instrument, which contains a pressure transducer connected to a sensing membrane made of silastic tubing. The tube is permeable to gases and water vapor; therefore, in water, gases diffuse through the tubing wall, until the pressure inside the tube is equal to the pressure outside the tube. An electronic digital gauge displays the total gas pressure of all gases in the water (ΔP), when the equilibrium is reached. The tensionometer also measures the local barometric pressure. Bunsen's solubility coefficient for oxygen and water vapor pressure were obtained from tables in the Handbook of Chemistry and Physics (Weast 1980). The water temperature was measured by a manual mercurial thermometer.

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, $12.0 \pm 1.0^\circ\text{C}$.

All gas saturation levels were determined by computer using a basic program (Table 1) the formulae improved by Bouck (1982). The following data were recorded: barometric pressure (B , mmHg); water temperature

(C); differential gas pressure (ΔP , mmHg); total dissolved gas pressure (P, mmHg); total dissolved gas saturation (% of barometric pressure); water vapor pressure (P_{H_2O} , mmHg); oxygen pressure (PO_2 , mmHg) and saturation (% N_2 saturation); pressure of "inert" gases plus CO_2 (PI); Bunsen solubility coefficient for oxygen (BC). BC is a number indicating the volume of oxygen absorbed by a unit volume of water at $0^\circ C$ and a pressure of 760 mmHg.

These data were used in the following formulae:

I. Total dissolved gas pressure (P) = $\Delta P + B$

II. Total dissolved gas saturation = $\frac{100 (P + B)}{B}$

III. Oxygen pressure (PO_2) = $\frac{(O_2 \text{ mg/liter}) \frac{0.7 \text{ ml/liter}}{\text{mg/liter}}}{(BC) \frac{1,000 \text{ ml/liter}}{\text{mg/liter}}} \quad (760 \text{ mmHg})$

IV. Oxygen % saturation ($O_2\%$) = $\frac{(\text{oxygen pressure in water})}{(\text{oxygen pressure in air})} \frac{100}{(PO_2) 100}$
 $= \frac{(PO_2) 100}{(B - P_{H_2O}) O_2 \text{ mole fraction in dry air}}$
 $= \frac{(PO_2) 100}{(B - P_{H_2O}) 0.2095}$

V. Pressure of "inert" gases plus CO_2 (PI) = $P - PO_2 - P_{H_2O}$

VI. Nitrogen pressure (PN_2) = 0.9877 PI (N_2 is 98.77% of 'non-oxygen' pressure in dry air)

VII. Nitrogen saturation ($N_2\%$) = $\frac{(PN_2) 100}{(N_2 \text{ mole fraction in dry air}) (B - P_{H_2O})}$
 $= \frac{(PN_2) 100}{(0.78084) (B - P_{H_2O})}$

Table 1. The computer model used to determine gas saturation levels, based on the simplified formulae improved by Bouck (1982).

```

100 PRINT "Input in order: date, time, unit"
110 INPUT A$
120 INPUT B$
125 INPUT C$
160 PRINT
165 PRINT
200 PRINT "Input the following values in specified order."
201 PRINT
209 PRINT
210 PRINT
211 PRINT "Change in pressure"
220 INPUT A
230 PRINT "Barometric Pressure"
235 INPUT B
250 PRINT "Water Temperature"
255 INPUT C
260 PRINT "Dissolved Oxygen"
262 INPUT D
270 PRINT "Bunsen's Solubility Coefficient"
273 INPUT E
280 PRINT "Water Vapor Pressure"
283 INPUT F
300 'CALCULATIONS
310 LET G = A + B
315 LET H = (B/B) + 100
320 LET OP! = (D/E) + .532
325 LET OS! = OP!/(B-F) + 477.33
330 LET K! = G-OP!-F
335 LET NP! = K! + .9877
340 LET NS! = NP!/(B-F) + 128.067
349 LPRINT "Date: "A$,"Time: "B$,"Unit: " C$
350 LPRINT
351 LPRINT
352 LPRINT
353 LPRINT "Change in Pressure = " A, "Barometric Pressure = " B
355 LPRINT "Water Temperature = " C, "Dissolved Oxygen = " D
360 LPRINT "Bunsen's Solubility Coefficient = " E
362 LPRINT "Water Vapor Pressure = " F
370 LPRINT
372 LPRINT
373 LPRINT
375 LPRINT "***** RESULTS *****"
379 LPRINT
400 'results
410 LPRINT "Total Dissolved Gas Pressure = " G "mm"
415 LPRINT "Total Dissolved Gas Saturation = " H "%"
420 LPRINT "Oxygen Pressure = " OP! "mm"
425 LPRINT "Oxygen Saturation = " OS! "%"
429 LPRINT "Inert Gases = " K! "mm"
430 LPRINT "Nitrogen Pressure = " NP! "mm"
435 LPRINT "Nitrogen Saturation = " NS! "%"
990 PRINT "Would you like to run another test? type 1 for yes, 2 for no"
993 INPUT I
994 IF I=1 GOTO 100
995 IF I=2 GOTO 997
996 GOTO 990
997 PRINT "Be sure to save program when you are done - push F4 key and type
gassat"
1000 END

```

C. Fish Sampling

Specimens showing the typical signs of gas bubble disease were selected for histopathological examination during the course of the chronic GBD experiment. The criteria for sampling was the appearance of moribund fish. Also included were samples of asymptomatic fish taken from the holding tank for histological comparison between treatment and control fish. The samples of asymptomatic fish were not taken from the control trays, since such sampling would have affected the mortality calculations.

At the end of 60 days of the experiment, samples of up to 12 fish were randomly taken from every test tray and preserved for analysis of possible chronic sublethal effects of the treatment. The gross evaluation was recorded photographically.

D. Fixation and Staining Procedure

The fixative solution was standard Bouin's fluid, which consists of 95% of a stock solution and 5% of glacial acetic acid. The stock solution is a mixture of 75% saturated aqueous picric acid and 25% formaldehyde.

The technique developed in the preliminary studies was utilized for fixation. Treatment and control fish sampled were placed into Bouin's solution which was preheated to the determined optimal temperature (Experiment #1) in a water bath at the optimal fixation times (Experiment #2). After the procedure, they were transferred to 20 ml vials filled with Bouin's solution at ambient temperature (12°C). The specimens were left in the fixing solution for 36 hours and then transferred to 70% ethyl alcohol for dehydration. The specimens were stained by the hematoxylin and eosin method at the Animal Health Diagnostic Laboratory (AHDL) of Michigan State University for histopathological examination.

E. Anatomic Pathologic Evaluation

Examination of gross and histopathologic lesions in control and treatment animals were conducted utilizing methods established at the Animal Health Diagnostic Laboratory. Photomicroscopes for photography and transmission electron microscopy facilities were utilized for analysis.

F. Statistical Analysis

Means and Standard Errors were calculated for all gas saturation data.

Mortality curves (percent mortality versus exposure time) were plotted for each level of supersaturation. Data collected and stored from these experiments were analyzed at the Pathology Department (MSU).

RESULTS AND DISCUSSION

I. Fixation Techniques

A. Experiment #1 - Fixation Techniques, Temperature

That data show that gross and histopathologic lesions can be preserved by the rapid fixation techniques developed. The examination of the histological sections of the specimens fixed at four different temperatures of Bouin's solution revealed that fixation was complete at 48°C, while the preservation of tissue morphology was lost at a higher temperature (80°C) and less rapidly complete at lower temperatures (12 and 24°C) (Table 2).

Based on these observations, it was decided to conduct further investigations using Bouin's solution at a constant temperature (48°C) by varying the time of exposure to the heated fixative.

B. Experiment #2 - Fixation Techniques, Time

Histological examination of the specimens fixed in Bouin's exposure times showed that fixing fish specimens for ten minutes resulted in optimal preservation as judged by histological morphology. Therefore, it was decided to use these fixation techniques in conjunction with exposure of rainbow trout fry to chronic gas supersaturation, to investigate location and effect of emboli within the tissue (Table 3).

Table 2. Experiment -Fixation and Temperature: Physical data obtained by exposing rainbow trout fry to acutely lethal gas supersaturated water at 12°C (TDGS > 130%), for fixation techniques, varying the temperature of fixative solution.

Group	Number of fish		Supersaturation exposure time (min.)	Supersaturation Bouin's solution (°C)	Fixation time (min.)	Number of moribund over unaffected experimental fish at fixation
	Experimental	Control				
1	3	3	18	12	na	2/1
2	3	3	21	24	20	2/1
3	3	3	18	48	10	2/1
4	3	3	25	80	5	1/2

Table 3. Experiment 2 - Fixation Time: Physical data obtained by exposing rainbow trout fry to acutely lethal gas supersaturated water at 12°C (TDGS > 130% followed by fixation in Bouin's solution 48°C).

Group	Number of fish		Supersaturation exposure time (min.)	Fixation time (min.)	Number of moribund over unaffected experimental fish at fixation
	Experimental	Control			
1	3	3	30	5	3/0
2	3	3	20	10	2/1
3	3	3	31	20	1/2
4	3	3	30	40	2/1

The lesions produced in this experiment are considered below, section III, mortality data.

C. The Effects of Fixation Techniques on Gas Bubble Size

On the basis of these preliminary laboratory investigations, the rapid fixation technique was found useful in laboratory diagnosis of GBD, but raised questions concerning induction of artifactual lesions in treated and control fish. Since it is possible that histological appearance of emphysema may be influenced by fixative solution and temperature and gas pressure, a theoretical examination was also developed to evaluate this phenomenon. Considering that the atmospheric pressure is constant when the fish is transferred from supersaturated water at ambient temperature (12°C) to Bouin's solution at varying temperatures, Charles' Law is applicable: "The volume of a gas at constant pressure increases proportionately to the absolute temperature." The law may be expressed by the equation $V_1/V_2 = T_1/T_2$, where V_1 and T_1 represent volume of gas bubble and absolute temperature in one case (water) and V_2 and T_2 the quantities for the same mass of gas in another (Bouin's solution). For example, if a fish with a 1 cm² gas bubble were transferred from supersaturated water at 12°C to Bouin's solution at 48°C, the size of the bubble would increase to 1.126 cm, or approximately 12.6% (Table 4). It was determined that the small alteration induced could be better tolerated than complete loss of lesions resulting from standard fixation techniques.

II. Chronic Gas Bubble Disease: Experiment #3

A. Introduction

The high supersaturation level experimental group (Treatment 1)

Table 4. Theoretical influence of fixation temperature on GBD lesions: the effect of varying ambient temperature, 12°C, at constant pressure, on the volume of a gas bubble during fixation in Bouin's solution.

Temperature		Bubble size
°C	°K	cm ³
12	285	1
-200	73	.26
-150	123	.46
-100	173	.61
- 50	223	.78
- 20	253	.89
- 10	263	.92
- 5	268	.94
0	273	.96
5	278	.98
10	283	.99
20	293	1.03
50	323	1.13
100	373	1.31

of fish experienced a high morbidity and mortality. At the end of the 60-day test, 80% of the two hundred rainbow trout fry were dead. Signs consistent with GBD were found in nearly all moribund or recently dead fish. Groups of fish held in Treatment 2, 3 and Control experienced no mortality due to GBD or any other disease. Sporadic incidence tissue pathology occurred as frequently in controls.

The well water supply at the Aquaculture Laboratory was found to contain supersaturation of nitrogen. Therefore, the control group levels of nitrogen ranged around 105% throughout the test. The total dissolved gas saturation and oxygen saturation in the control trays were below 100%.

B. Water Flow Adjustment

As a result of utilization of an initial constant waterflow (200 ml/min), which did not take into account the rapid growth of the fry, the absolute level of oxygen in the water inadvertently decreased in proportion to the increased demands of growing fish. This resulted in slightly decreased oxygen tension amounting to five mg/l in the lower (Treatment 3) and control trays. In the remaining trays, there was less of a decrease. Since the system was not provided with a controlled external oxygen supply to keep a desirable minimum level (6 mg/l), it was decided to simply increase the waterflow from 200 to 400 ml/min, on the 46th day of the 60-day experiment. With the water-flow change, the nitrogen levels stayed approximately the same as before, but oxygen levels increased substantially in both the treatment and control trays.

III. Mortality Data

A. Acute Gas Bubble Disease

External signs developed within a few minutes after the fish had been exposed to the supersaturated water. Gas bubbles adhered to the surface of the fish body and fish tended to stay at the maximum available depth of the chamber. Shortly thereafter, in approximately 10 minutes, the fish exhibited abnormal behavior including erratic swimming and jumping free of the water. This was followed by spasmodic spiraling and drilling movements, and swimming side and belly-up. These symptoms culminated in convulsions, the moribund stage, frequently leading to death in approximately 20 minutes.

The most common external signs recorded from the gross examination were gas bubbles in the caudal fins. Gross examination of internal organs was not conducted before fixation.

Individual resistance to development of GBD occurred within groups (Tables 2 and 3). In each group of three experimental fish, usually one fish did not show behavioral changes, and did not die after three hours exposure even though visible gas bubbles were adhered to the surface of the body.

B. Chronic Gas Bubble Disease

1. Gas Saturation and Lethality

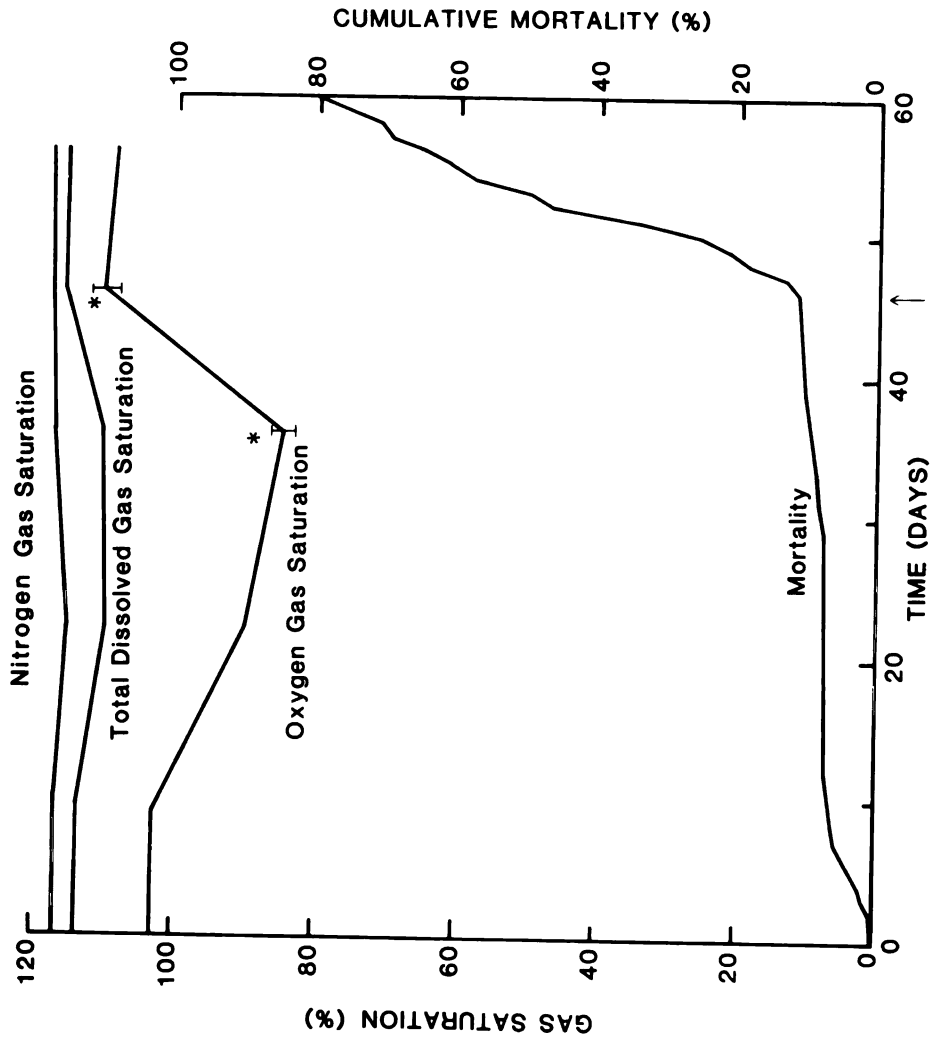
Rainbow trout fry were exposed to supersaturated water for 60 days at different experimental gas saturation levels. Plots of proportional mortality against gas saturation for each test treatment and control are shown in Figures 1 through 4.

Figure 1. Cumulative mortality curve^a for rainbow trout fry (n = 183) exposed to_b approximately 115 percent total dissolved gas saturation^b during 60 test-days (Treatment 1).

^a Mortality occurred after two days of exposure to supersaturated water, reaching 6% in the first 10 days. There was insignificant mortality during the period from day 10 to day 46. On day 46 the waterflow was changed from 200 to 400 ml/min. Death occurred rapidly at that point and continued, reaching 80% at the termination of the experiment.

^b Nitrogen saturation levels were constant at 117% throughout the experiment. Oxygen saturation levels which were approximately 103% at the beginning, diminished gradually from day 10 to day 46 to approximately 85% and increased vertically to 110% after the waterflow change at the 46th test-day.

TREATMENT 1



* SIGNIFICANTLY DIFFERENT ($P < .01$)

Figure 1

Figure 2. Cumulative mortality curve^a for rainbow trout fry (n = 191) exposed to_b approximately 103 percent total dissolved gas saturation^b during 60 test-days (Treatment 2).

^aNo significant mortality occurred (0.7%), throughout the experiment.

^bNitrogen saturation levels were constant at 109% throughout the experiment. Oxygen saturation levels which were approximately 85% at the beginning, diminished gradually from day 10 to day 46 to approximately 60% and increased vertically to 88% after the waterflow change at the 46th test-day.

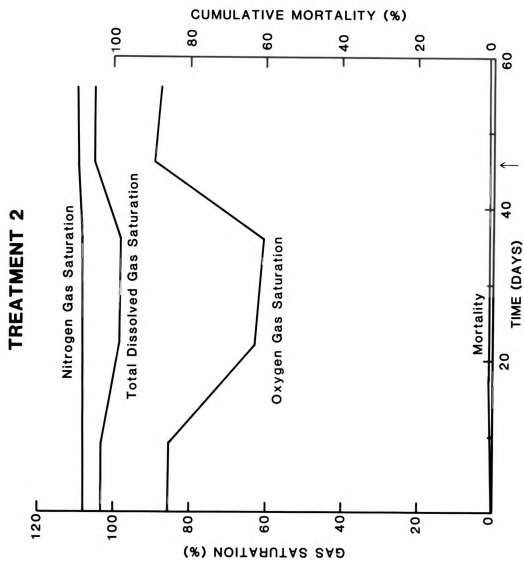


Figure 2

Figure 3. Cumulative mortality curve^a for rainbow trout fry (n = 196) exposed to approximately 97 percent total dissolved gas saturation^b during 60 test-days (Treatment 3).

^aNo significant mortality occurred (0.5%), throughout the experiment.

^bNitrogen saturation levels were constant at 106% throughout the experiment. Oxygen saturation levels which were approximately 75% at the beginning, diminished gradually from day 10 to day 46 to approximately 50% and increased vertically to 70% after the waterflow change at the 46th test-day.

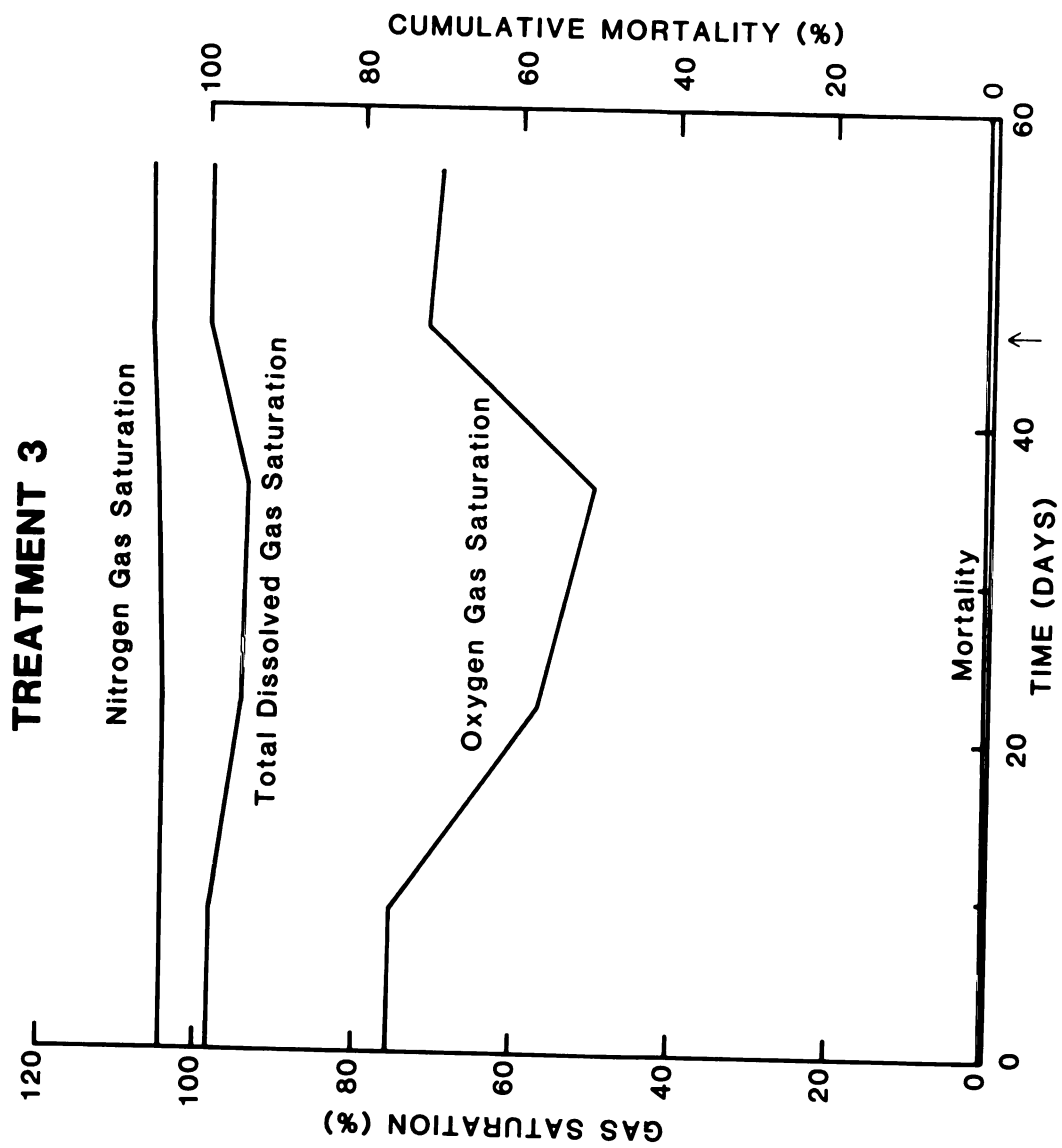


Figure 3

Figure 4. Cumulative mortality curve^a for rainbow trout fry (n = 145) exposed to approximately 96 percent total dissolved gas saturation^b (control group) during 60 test-days.

^aNo significant mortality occurred (0.5%) throughout the experiment.

^bNitrogen saturation levels were constant at 105% throughout the experiment. Oxygen saturation levels which were approximately 70% at the beginning, diminished gradually from day 10 to day 46 to approximately 50% and increased to 58% after the waterflow change at the 46th test-day.

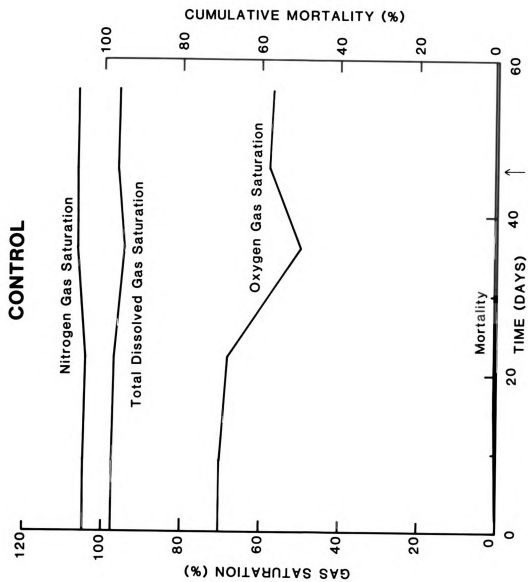


Figure 4

Figure 1 demonstrates that the initial onset of the disease occurred in Treatment 1 after two days of exposure to approximately 117% nitrogen saturation (oxygen 103%) causing a progressive mortality, reaching six percent in the first ten days. Thereafter, the nitrogen levels remained constant, but the oxygen levels diminished gradually to approximately 85%. There was insignificant mortality during a 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% in Treatment 1. Death began to occur rapidly at that point and continued, reaching 80% at the termination of the experiment.

The oxygen level at the 36th test day was significantly different ($P < 0.01$) from the level on the 46th test day, (Figure 1). The levels of nitrogen and total dissolved gas saturation were not significantly different ($P > 0.01$). The injected volume of air produced by $\frac{1}{2}$ HP motor at 1.4 atmosphere of pressure was constant throughout the test, however.

It is important to note in Figure 1 that the elevated level of nitrogen combined with the increased oxygen levels could have raised supersaturation above the lethal threshold, causing the extensive mortality. Unfortunately, the percent saturations of nitrogen, oxygen and total dissolved gas were not directly comparable due to variations occurring within groups between determinations. Therefore, the threshold saturation level was not calculated.

Figures 2, 3, and 4 show that mortality from GBD did not occur in Treatment 2, 3 and in Controls. Using Chi-square test, there was

a significant difference between survival rates for the four groups (Treatment 1, 2, 3 and Control) at 1% level of significance (Table 5). Using Bonferroni Chi-square to compare each experimental group with the control, only Treatment 1 was significantly different from control ($P < 0.01$). The levels of nitrogen saturation in Treatment 2, 3 and in Controls were approximately at 109, 106, 105%, respectively (Table 6). It is emphasized that these test groups had oxygen levels well below 100%, varying from 85 to 50% saturation. No 100% nitrogen saturation group was incorporated in the experiment because the normally saturated (100%) well water was not available at the laboratory facilities.

Observations conducted during the course of the long term experiment show that no changes in behavior or survival occurred in the test fish in Treatment 2 and 3 in comparison with the control fish (Table 5). On the other hand, in Treatment 1, initial mortality occurred within two days. However, the signs associated with the disease were rarely observed prior to the convulsive stage which occurs a few minutes before death.

2. Signs and Symptoms

The GBD syndrome exhibited during the initial mortality during the first ten days of exposure (Treatment 1) and the extensive mortality after the 46th test day (Experiment 3) was similar to that observed in the acute exposure (Experiment 1 and 2). That is, the acute lethality was indistinguishable from the chronic form.

Symptoms noted in rainbow trout fry in the Treatment 1 period included loss of equilibrium, abnormal buoyancy and aimless swimming

Table 5. Survival of rainbow trout fry in supersaturated water at 12°C for 60 days. Each test tray contained fifty fish at the beginning of the test.

	Test trays	Number of dead fish	Number of surviving fish	Total*	Survival (%)
Treatment 1	A1	45	4	49	8.2
	B1	36	9	45	20.0
	C1	34	10	44	22.7
	D1	30	15	45	33.3
Treatment 2	A2	0	48	48	100.0
	B2	1	46	47	97.9
	C2	1	46	47	97.9
	D2	0	49	49	100.0
Treatment 3	A3	1	47	48	97.9
	B3	0	48	48	100.0
	C3	0	50	50	100.0
	D3	0	50	50	100.0
Control	E1	0	49	49	100.0
	E2	0	48	48	100.0
	E3	1	47	48	97.9

*These fish escaped from the tray during the first day of experiment before the test tray had been covered.

Table 6. Dissolved gas content of the experimental water.

	Determ. test day	Total dissolved gas in % satur. mean + t.05 SE	Nitrogen in % saturation mean + t.05 SE	Oxygen in % saturation mean + t.05 SE
Treatment 1	9	113.7 + 0.9	116.9 + 1.5	102.6 + 6.2
	22	109.8 + 0.5	115.2 + 0.6	89.8 + 2.2
	36	110.2 + 1.2	117.2 + 0.8	84.5 + 8.4
	46	115.7 + 1.2	117.5 + 0.4	110.1 + 4.6
	56	115.6 + 1.2	117.8 + 0.4	108.7 + 5.4
Treatment 2	9	103.2 + 1.1	108.0 + 0.1	85.3 + 5.4
	22	98.7 + 1.4	108.0 + 1.0	63.0 + 5.4
	36	98.3 + 2.4	108.3 + 2.4	60.5 + 7.9
	46	105.1 + 0.9	109.4 + 0.6	89.9 + 2.4
	56	105.1 + 1.0	109.9 + 0.3	87.7 + 3.4
Treatment 3	9	98.4 + 1.0	104.5 + 0.2	7.53 + 2.9
	22	94.8 + 0.9	104.7 + 0.6	57.0 + 5.7
	36	94.1 + 0.7	105.6 + 2.0	50.2 + 10.8
	46	99.1 + 0.5	106.4 + 0.3	71.7 + 3.4
	56	99.2 + 0.5	106.9 + 0.4	70.1 + 4.1
Control	9	97.6 + 2.7	104.9 + 3.1	70.2 + 1.2
	22	96.9 + 2.9	104.4 + 3.1	68.4 + 2.5
	36	94.7 + 3.6	106.5 + 3.9	49.9 + 2.5
	46	96.3 + 2.2	106.4 + 1.2	57.9 + 5.9
	56	96.2 + 2.1	106.5 + 0.9	57.3 + 7.2

as the convulsion 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 were frequently observed to die with the mouth agape and the gills and operculum flared.

Gross examination of recently dead and moribund fish showed that in nearly all cases observed, gas bubbles on the surface of the fish's body were present. Fish developed the classic signs of GBD such as subepithelia emphysema of the head (Plates 1 and 2), external and internal surface of the operculum, along the branchiostegal region, inside the mouth, and on the gills and in the fins. The above external signs observed in this experiment confirm the results of Shirahata (1966), Rucker and Kangas (1974) and Stroud et al. (1975). The gills, in some cases, appeared swollen and were covered with excess mucus. Gas blisters on the margin of one or both opercula were observed in most of the affected fish. Gas bubbles were observed in all fins, with the exception of the adipose, and were most commonly observed in the dorsal, caudal anal fins (Plates 3 and 4). Additionally, gas bubbles appeared in the interbranchistegal membrane and the orbits of eyes directly under the cornea. These signs are similar to those described by Shirahata (1966) who exposed rainbow trout fry to super-saturated water.

It appears that the occurrence of these signs is associated with the convulsive stage. Evidence of subepithelia gas bubbles on the fish's body prior to that stage was apparent in only a few cases. Macroscopically visible emphysema without rapid Bouin's fixation

Plate 1. A photograph of a 3-month-old rainbow trout exposed for 48 days in supersaturated water at 115 percent TDGS, showing focal elevation of the epithelium over the caudal margin of the operculum and over the skull (arrow). (millimeter scale; 5X)



Plate 1

Plate 2. A photograph of a 3-month-old rainbow trout exposed for 53 days in supersaturated water at 115 percent TDGS, showing the separation of the epithelium from subcutaneous tissue filled with gas over the base of the skull (arrow) and at the caudal margin of the operculum. 5X

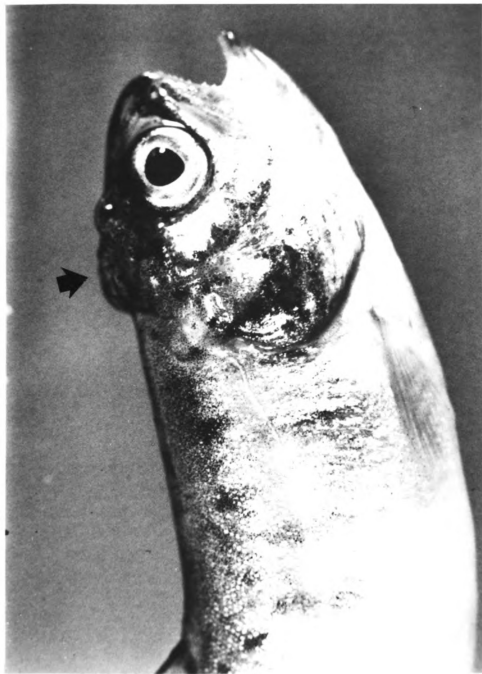


Plate 2

Plate 3. A photograph of a 3-month-old rainbow trout exposed for 50 days in supersaturated water at 115 percent TDGS, showing emphysematous lesions of the caudal fin (arrow). 5X

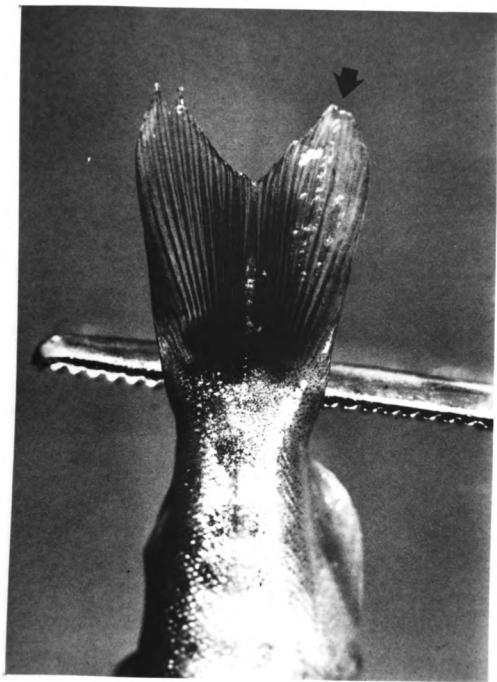


Plate 3

Plate 4. A photograph of a 3-month-old rainbow trout exposed for 50 days in supersaturated water at 115 percent TDGS, showing emphysematous lesions of the anal fin (arrow). 5X

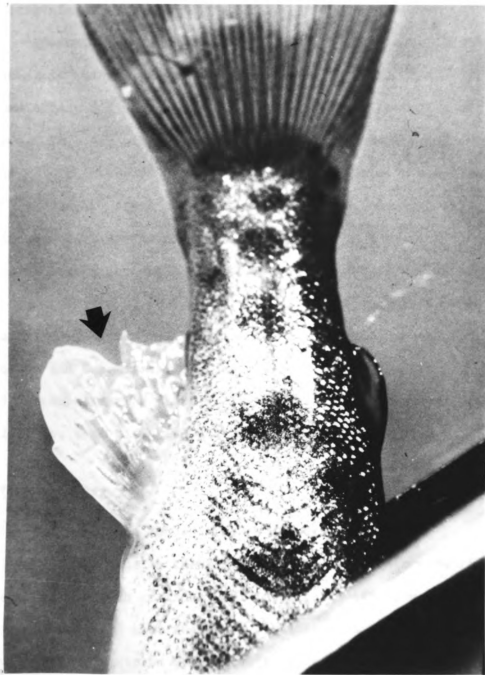


Plate 4

disappears within a few hours after death, when fish are removed from the supersaturated water. Signs of GBD were reported to have disappeared after death (Bouck 1976) and leave the carcass without diagnostic signs.

Approximately ten percent of the fish (Treatment 1) developed exophthalmia which did not necessarily lead to death (Plate 5). Over time, some affected fish that showed popeye suffered loss of one or both eyes. In a few other fish, the condition of exophthalmia regressed, leaving the eye intact. Tests for visual function were not conducted.

Gross GBD signs also included periorbital hemorrhage in some affected fish. Commonly in the gills, in the opercular and branchiostegal areas, petechial hemorrhages were observed. Fish appeared to die most frequently during periods of stress such as feeding, gas monitoring, or any manipulation which increased muscular activity. Stroud and Nebeker (1976) also observed that fish more frequently died of gas embolization shortly after physical stress was induced. In addition, some fish died without visible external signs.

IV. Pathology

A. Acute Gas Bubble Disease (Experiment 1 and 2)

All fish used in these preliminary experiments were fixed, examined and compared with controls. Histological sections were evaluated and it was concluded that the rapid fixation preserved lesions better than the standard fixation technique, since for laboratory diagnosis of GBD, macroscopic and microscopic emphysema was well preserved.

Acute exposure of experimental fish to supersaturated water ($\geq 130\%$ TDGS) caused death within approximately 25 minutes. Therefore, the

Plate 5. A photograph of a 3-month-old rainbow trout exposed for 55 days in supersaturated water at 115 percent TDGS. There is a marked unilateral exophthalmia (arrow). 5X

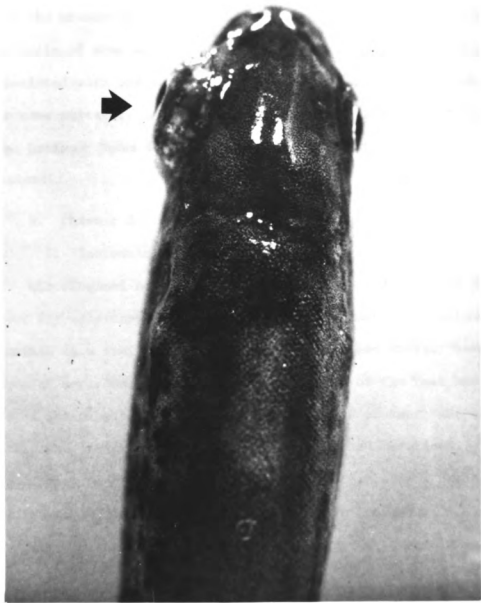


Plate 5

lesions were often less obvious than those observed in chronic exposure experiments. The lesions consisted of external gas blisters in the fins which arose immediately before the onset of convulsions. The most striking pathological changes were observed in the afferent branchial arterioles of the gill filaments which revealed spaces, suggesting fixed gas embolization. In some specimens, the air space was continuous with the branching of the ventral aorta. Microscopic examination of the brain of some specimens showed a large space in the skull, usually associated with and surrounding the brain stem. This lesion may account for some mortality in acute GBD through pressurization at the base of the brain. These lesions are considered below, (5. Additional lesions).

B. Chronic Gas Bubble Disease (Experiment 3)

1. Introduction

Histological sections of the major organs and tissues of rainbow trout fry specimens exposed to long term gas supersaturation were examined in a similar fashion to those described above. Samples were taken of moribund fish throughout the course of the test and at the end of the experiment, to determine the cause of death and to characterize the signs of gas bubble disease that had developed.

Gross, macroscopic and microscopic lesions of the organs were studied to locate involved organs and tissue components. The extent, severity and nature of the pathologic changes were determined in this manner.

In chronic exposure the histopathologic findings in moribund fish were very similar in extent and type to most lesions of the acutely

lethal (Experiment 1 and 2) supersaturation trials; however, in those fish surviving chronic exposure and in controls there were additional lesions which were distributed randomly among exposure and control groups.

Because the hypothesis predicted a chronic effect, changes were anticipated in various organ systems. Therefore, each treatment group was sampled so that whole fish, viewed with dissecting microscope (macroscopic examination) as well as fixed multiple microscopic sections were surveyed. These examinations revealed lesions which were then selected for additional microscopic sectioning. It was possible to make gross and microscopic comparisons.

The histopathologic results, split between the specimens from 149 moribund fish, and the specimens from 104 fish at termination were all examined grossly. There were 12 moribund fish selected for microscopical study along with an equal number of fish from the holding tank. There were 422 termination survivors examined and 104 were sectioned for microscopical study. Lesions were categorized by making a blind comparison among treatment and control fish. Because of lesions encountered, additional sections of gills, liver and kidney were made and examined.

2. Subcutaneous Emphysema

The most common grossly detectable lesion was subcutaneous tissue emphysema elevating the skin. This lesion took several forms. A ballooned-out epithelium elevating the basal layer of epithelium at the caudal margin of the operculum and over the skull (Plate 1 and 2) were the most common skin lesions in the chronic study. These lesions

occurred in all fish which died. The above confirm the results of Meekin and Turner (1976) who reported that cutaneous gas bubbles were the most common signs in juvenile chinook salmon exposed to gas supersaturated water. Another very common lesion was emphysema of the caudal fin (Plate 3), dorsal fin, pectoral fin and anal fins (Plate 4). These lesions occurred in all of the acute supersaturation and the majority of the moribund, chronically exposed fish.

3. Exophthalmia

In 5 of 50 fish in Treatment 1 of the chronic study, there was unilateral exophthalmia (Plates 5 and 6) and one case of bilateral exophthalmia. Meekin and Turner (1979) reported exophthalmia in less than 5% of dead juvenile chinook salmon after exposure to supersaturation. In some affected fish there was darkening of the skin due presumably to the loss of melanophore reactivity to light. On examination of the ocular lesion in the severe form, the globe of the eye was prolapsed outward from a point adjacent to the optic disk (Plate 6) 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 ocular lesion detected was that of an emphysematous space within a vascular space of the choroid body of the eye (Plate 7). Another microscopic lesion, more advanced and presumably the precursor lesion to globe prolapse, was a dissection 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

Plate 6. A cross section of the head through the eyes of a 3-month-old rainbow trout exposed for 52 days in supersaturated water at 115 percent TDGS, showing a macroscopic lesion of severe unilateral exophthalmia (arrow). 18X

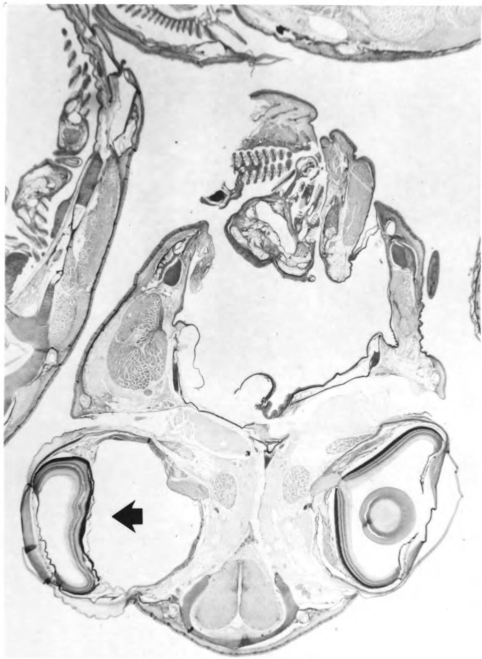


Plate 6

Plate 7. Detail of the eye of a 3-month-old rainbow trout exposed for 49 days in supersaturated water at 115 percent TDGS, showing a lesion of the early exophthalmia manifested by the presence of an air space in a vessel of the choroid gland (arrow). 200X

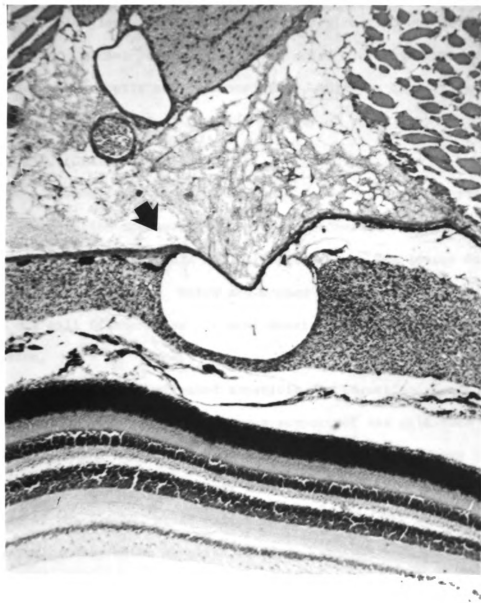


Plate 7

disk which served as the only point of remaining attachment (Plate 8) and minor degenerative lesions of the optic nerve were also observed. In the normal eye (Plate 9) 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 vacuolization of the optic nerve, musculature, adjacent connective tissue and glandular tissue was evident (Plate 10).

4. Gill Lesions

A lesion found in every moribund fish, both after chronic and acute supersaturation treatment, was gas displacement of the blood from the afferent arteriole within the gill filaments. The lesion was never recorded in controls. Marsh and Gorham (1905) described gas bubbles in the gill filaments as the most constant and significant macroscopic lesion.

The gas emboli affected arteriole was identified by its anatomical association with the cartilagenous support of the gill arch (Plates 11, 12, 13 and 14) while 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. 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 (Plate 15) while the control sections did not demonstrate such lesions (Plate 16). The findings suggested that the origin of gas was from the heart with build-up in the afferent side of the gill arches. The lesions were histopathologically similar in both acute and chronic treatment fish.

Plate 8. Detail of the eye of a 3-month-old rainbow trout exposed for 52 days in supersaturated water at 115 percent TDGS, showing the precursor lesion to ocular prolapse (arrows) in GBD induced exophthalmia. 200X

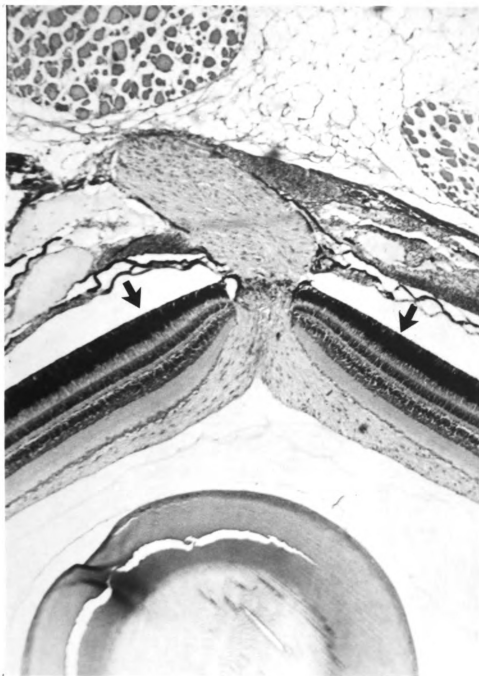


Plate 8

Plate 9. Detail of the eye of a 3-month-old rainbow trout from the control group, showing the normal appearance of the optic nerve, choroid and retina. 200X

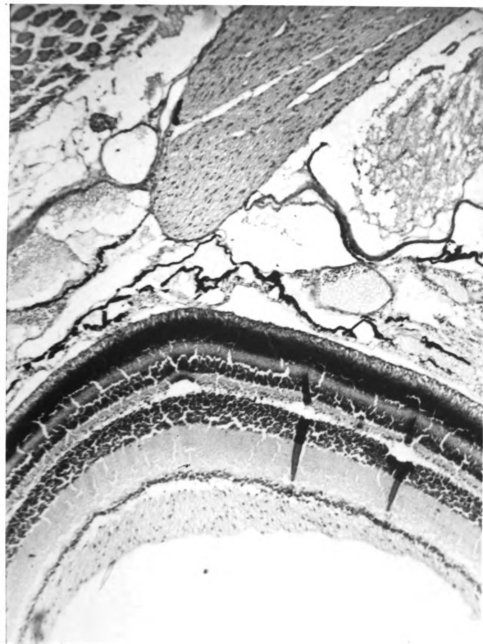


Plate 9

Plate 10. Detail of the eye of a 3-month-old rainbow trout exposed for 52 days in supersaturated water at 115 percent TDGS, showing the degenerative vacuolization of the optic nerve, adjacent connective and muscle tissue in GBD induced exophthalmia (arrow). 200X



Plate 10

Plate 11. A cross section of the gill filaments of a 3-month-old rainbow trout exposed for 53 days in supersaturated water at 115 percent TDGS, showing the gas displacement of the blood from the afferent arterioles (straight arrows) and normal blood content of the efferente arterioles (curved arrows). 200X

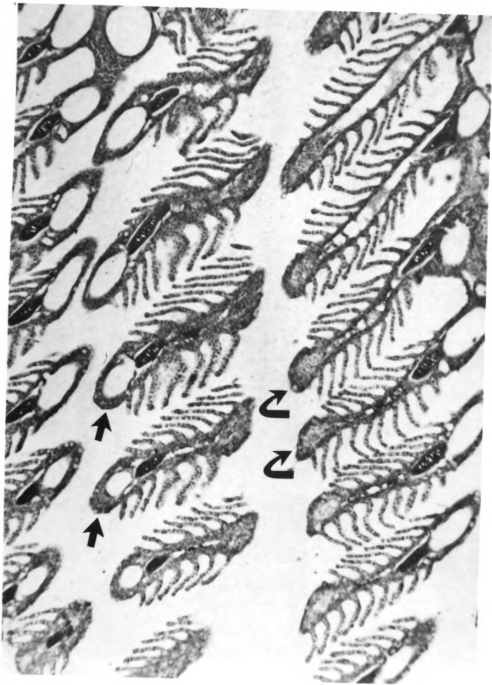


Plate 11

Plate 12. A cross section of the gill filaments of a 3-month-old rainbow trout from the control group, showing the appearance of normal afferent (straight arrows) and efferent arterioles (curved arrows). 200X



Plate 12

Plate 13. Detail of the gill filaments of a 3-month-old rainbow trout exposed for 53 days in supersaturated water at 115 percent TDGS, showing gas displacement of the blood from the afferent arterioles (arrow). 500X



Plate 13

Plate 14. Detail of the gill filaments of a 3-month-old rainbow trout from the control group, showing the appearance of normal afferent arterioles (arrow). 500X



Plate 14

Plate 15. A longitudinal section of the gill arch of a 3-month-old rainbow trout exposed for 53 days in supersaturated water at 155 percent TDGS, showing the ventral aorta with evidence for gas displacement of blood (arrows) close to the origin of the afferent branchial arteries. 200X

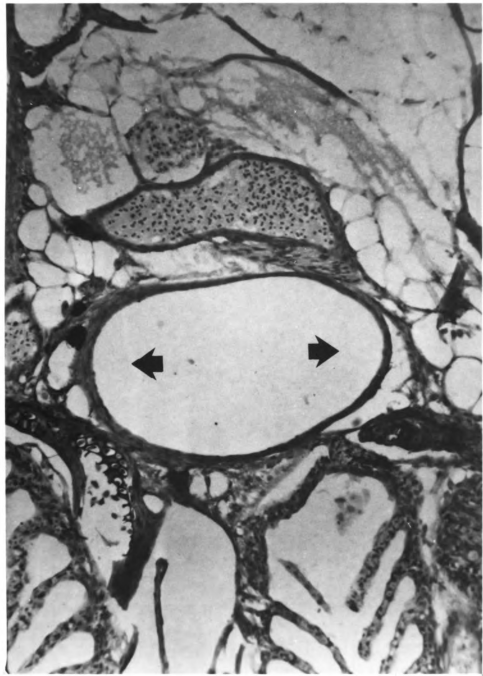


Plate 15

Plate 16. A longitudinal section of the gill arch of a 3-month-old rainbow trout from the control group, showing the normal appearance of gill structure and ventral aortic vasculature without gas-blood embolization (arrow). 200X



Plate 16

5. Additional Lesions

In two fish exposed to acute supersaturation conditions, an emphysematous space was apparently fixed within the skull (Plate 17). The spaces surrounded the medulla oblongata and appeared to "compress" the brain. The suspect lesion was visible on sagittal and on cross section in one fish. The spaces were apparently continuous with semi-circular canals, and, because it is not possible to prove the presence of gas after fixation, may have been an artifactual because of 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 (Plate 18).

Additional lesions were observed in 70 individual fish from the chronic treatment groups. They consisted of various gill lesions, extraordinary liver glycogen accumulation, fatty vacuolization of the liver, possible mineralization of scattered kidney tubules and suspected lymphoid hyperplasia of the thymus gland associated with gill alterations. All of these lesions were randomly distributed among treatment and control groups with no frequency association with a particular supersaturation exposure. Gill lesions, seen in 21 fish on gross and/or microscopical examination, consisted predominantly of epithelia hyperplasia. This change was segmental or nodular in two cases, but most often diffuse and associated with accumulation of mucous. 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.

Plate 17. A sagittal section of the brain of a 3.5-week-old rainbow trout exposed to acute GBD, (TDGS _ 130%) showing dilated semi-circular canal surrounding the medulla oblongata and brain stem (arrows). 80X



Plate 17

Plate 18. A sagittal section of the brain of a 3.5-week-old rainbow trout from the control group, showing the semicircular canal space (arrow), medula oblongata and brain stem. 80X



Plate 18

SUMMARY AND CONCLUSION

Following the acute case study, the rapid fixation technique for histopathological examination was shown to be useful for laboratory diagnosis of GBD since gas emboli were better preserved than with standard techniques. Therefore, the hypothesis that temperature and time may influence fixation was accepted at the conclusion of Experiments 1 and 2.

After evaluating the results of the chronic exposure of rainbow trout fry to supersaturated water, it was logical to conclude that the ratio of the saturation levels of oxygen and nitrogen as well as total dissolved gases are critical in inducing GBD.

Tables with statistical analyses correlating gas saturation levels with mortality are not presented since the gas levels varied within treatment groups; in addition, control group nitrogen saturation levels were above 100 percent.

Nonetheless, these tests suggest that gas supersaturated water may produce GBD in fish especially when both nitrogen and oxygen saturation levels are above 100 percent, but it remains unclear exactly what nitrogen level would have had substantial lethal effect in this experiment assuming oxygen levels were over 100 percent. Levels of nitrogen saturation up to 117% did not produce GBD in rainbow trout fry when oxygen saturation levels were below 100 percent. During the course of the test,

mortality increased drastically when oxygen levels were elevated above saturation. The final percent saturation of oxygen achieved with the change in waterflow was presumably the result of the difference in partial pressure of two gases, N_2 and O_2 , and their solubilities in water. It is suggested that the mortality occurred (Figure 1) as an exacerbation of an acute and pathologic gas embolization rather than as a result of a chronic disease process. Therefore, the hypothesis tested in Experiment 3, which suggested a basic difference in pathogenesis between acutely lethal and chronic GBD, was rejected. The detected lesions in chronic supersaturation exposure, besides the exophthalmia, were similar in acute GBD or were unrelated to treatment, since they were equally frequent in control fish.

In summary, the data indicate that an undefined physiological factor, triggered by environmental factors and probably oxygen supersaturation in combination with nitrogen excess in the blood stream, appeared to cause the gas bubble formation and gas embolization.

A gas bubble disease hypothesis which is logical (Bell and Farrel 1972) hypothesizes that violent oxygen unloading, characteristic of some fish hemoglobin under conditions in which blood is gas saturated, forms small oxygen emboli. These emboli build to form larger emboli under conditions of high total dissolved gas pressure.

Laboratory investigations have shown that fish probably only respond to the level of oxygen in the water and blood (Eddy 1971) rather than carbon dioxide levels as do mammals. Therefore, fish respond to excess of oxygen levels by slowing the respiratory and circulatory efforts even though their tissues may be accumulating lactic and carbonic acids. This metabolic process produces a decrease in the blood pH Bohr effect) which

is enhanced by the increase in the partial pressure of carbon dioxide (Root effect). This process could precipitate a violent oxygen unloading from red blood cells and would form the oxygen emboli. This process may occur even faster during physical stress, as in feeding or handling, since the increase in muscular activity can raise the partial pressure of carbon dioxide in venous blood from 2.5 to up to 8 mmHg (Holeton 1971a, b; Randall 1975).

Histopathologic lesions were located in the gill arches, choroid gland of the eye, brain and peripheral tissues. Since each of these areas are associated with acid secreting tissue and may foster accelerated gas exchange from hemoglobin, the lesion distribution suggests that oxygen unloading may play a role.

Well water supply is often supersaturated with gases. A practice currently used in Michigan fish hatcheries to remedy nitrogen supersaturation is to increase aeration. However, high oxygen levels in the field have not been assessed with regard to GBD. This research preliminarily reports that water supersaturated with nitrogen may induce GBD in fish if the levels of oxygen are above 100 percent. It may be recommended that, in situations where maintenance of oxygen below saturation levels is possible when nitrogen supersaturation is present, reduced oxygen saturation may be a method to reduce mortality.

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LITERATURE CITED

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