SUBLETHAL EFFECTS OF NITRITE AND SELENATE ON TWO SPECIES OF FRESHWATER FISH

THESIS

Presented to the Graduate Council of the
North Texas State University in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Donald E. Watenpaugh, B.S.

Denton, Texas

August, 1984

Watenpaugh, Donald E., Sublethal Effects of Nitrite and Selenate on Two Species of Freshwater Fish. Master of Science (Biology), August, 1984, 127 pp., 13 tables, 13 figures, bibliography, 140 titles.

Physiological and behavioral effects of exposure of fathead minnows (Pimephales promelas) and channel catfish (Ictalurus punctatus) to selenate-selenium (Se) and nitrite were investigated. Routine oxygen consumption of minnows was unaffected by acute exposure to Se. Effects may have been masked by physiological compensatory responses of dosed minnows. Se exposures of fathead minnows significantly reduced their upper thermal tolerance. No avoidance of lethal Se concentrations was observed in minnows experiencing Se concentration gradients.

Nitrite exposure reduced routine oxygen consumption rates of fathead minnows. Acute exposure of channel catfish to nitrite increased blood methemoglobin levels. Nitrite exposure significantly reduced thermal tolerance, hypoxic resistance, and swimming performance of catfish. Correlations between decreased physiological performance and elevated methemoglobin were observed in each test of nitrite's effects on catfish.

TABLE OF CONTENTS

Page

LIST OF TABLES iv

LIST OF ILLUSTRATIONS. vi

Chapter

I. INTRODUCTION. 1

II. THE EFFECT OF SELENIUM ON THE ROUTINE METABOLIC RATE OF FATHEAD MINNOWS (Pimephales promelas). 8

III. SELENIUM TOXICITY AND THERMAL TOLERANCE IN THE FATHEAD MINNOW (Pimephales promelas) 18

IV. EVALUATION OF FATHEAD MINNOW AVOIDANCE OF SELENIUM 29

V. THE EFFECT OF NITRITE ON THE ROUTINE METABOLIC RATE OF FATHEAD MINNOWS (Pimephales promelas). 46

VI. NITRITE TOXICITY, METHEMOGLOBIN FORMATION AND THERMAL TOLERANCE IN CHANNEL CATFISH (Ictalurus punctatus). 56

VII. HYPOXIC RESISTANCE OF NITRITE-EXPOSED CHANNEL CATFISH (Ictalurus punctatus). 75

VIII. SWIMMING PERFORMANCE OF CHANNEL CATFISH (Ictalurus punctatus) AFTER NITRITE INTOXICATION 91

IX. CONCLUSION. 110

BIBLIOGRAPHY 115

LIST OF TABLES

Table		Page
I.	Absolute and Relative Mortality of Fathead Minnows During 24 h Exposures to Various Concentrations of Selenate-Se	11
II.	Mean Weight Specific Oxygen Consumption Rates of Fathead Minnows From Various 24 h Selenate-Se Exposures	14
III.	Dunnett's Multiple Range Grouping of Mean Critical Thermal Maxima of Fathead Minnows From Various 24 h Selenate-Se Exposures . . .	25
IV.	Water Quality of Water Used in Preference/ Avoidance Tests	34
V.	Evaluation of Fathead Minnow Avoidance of Selenate-Se: Trial One Observations And Chi-Square Values	37
VI.	Evaluation of Fathead Minnow Avoidance of Selenate-Se: Trial Two Observations And Chi-Square Values	38
VII.	Evaluation of Fathead Minnow Avoidance of Selenate-Se: Trial Three Observations And Chi-Square Values	39
VIII.	Evaluation of Fathead Minnow Avoidance of Selenate-Se: Trial Four Observations And Chi-Square Values	40
IX.	Absolute and Relative Mortality of Fathead Minnows During 24 h Exposure to Various Concentrations of Nitrite and During Subsequent Respirometry. Relative Values Appear as Percentages in Parentheses.	51
X.	Mean Weight Specific Oxygen Consumption Rates of Fathead Minnows From Various 24 h Nitrite-N Exposures. Standard Deviations Appear in Parentheses.	52

LIST OF TABLES--Continued

Table		Page
XI.	Results of Duncan's Multiple Range Testing of Percent MHb and CTM Means After Exposures to the Various Nitrite Concentrations	61
XII.	Duncan's Multiple Range Test Grouping of Mean Percent Methemoglobin and Hypoxic Resistance Time of Channel Catfish From Various 24 h Nitrite Exposures. Standard Deviations Appear in Parentheses	80
XIII.	Duncan's Multiple Range Test Groupings of Mean Percent Methemoglobin and Swim Time of Channel Catfish From Various 24 h Nitrite Exposures. Standard Deviations Appear in Parentheses.	101

LIST OF ILLUSTRATIONS

Figure | Page

1. Critical Thermal Maxima of Fathead Minnows Plotted Against the Square of their 24 h Selenate-Se Exposure Concentrations 23

2. System Utilized to Evaluate Fathead Minnow Avoidance of Selenium 32

3. Percent Methemoglobin of Channel Catfish from Various 24 h Nitrite Exposures 62

4. Critical Thermal Maxima of Channel Catfish Exposed to Various Nitrite Concentrations for 24 h 64

5. Critical Thermal Maxima (^{o}C) of Channel Catfish Plotted Against their Percent Methemoglobin 67

6. Percent Methemoglobin of Channel Catfish from Various 24 h Nitrite Exposures 81

7. Hypoxic Resistance Time of Channel Catfish from Various 24 h Nitrite Exposures 83

8. Hypoxic Resistance Time of Channel Catfish Plotted Against their Percent Methemoglobin . 86

9. Apparatus Utilized to Evaluate the Effect of Nitrite Exposure on Channel Catfish Prolonged Swimming Performance 94

10. Paddlewheel Employed to Measure Current Speed in Channels of the Swimming Performance Apparatus 94

11. Percent Methemoglobin of Channel Catfish from Various 24 h Nitrite Exposures 98

12. The Base Ten Logarithm of Channel Catfish Swim Time (In Minutes) Plotted Against 24 h Nitrite Exposure 102

13. Prolonged Swim Time (In Minutes) of Channel Catfish Plotted Against Their Percent Methemoglobin 105

"Lethal levels (of pollutants) are to be considered only as the boundaries of the zone within which the real work goes on."

F.E.J. Fry, 1971

CHAPTER I

INTRODUCTION

Materials released to the environment by activities of an increasing human population can have effects ranging from lethal to beneficial on the earth's biota; these materials, however, often create physiochemical circumstances that stress homeostatic capabilities of organisms, and thus threaten their ability to survive and/or reproduce successfully. Most toxicological research has involved determination of lethal levels of contaminants to particular organisms (Buikema et al., 1982). To more fully understand our impact on the environment and to more effectively derive protective limitations on its use, characterization of sublethal effects of pollutants that may influence the fitness of organisms is necessary (Sullivan et al., 1978; Larrick et al., 1978; Lemly, 1982; Beitinger and Freeman, 1983; Sprague, 1971). Several different methodologies have emerged as potential quantifiers of sublethal toxicity in fish (Anderson, 1971; Wedemeyer and McLeay, 1981). This series of laboratory studies employed some of these techniques to separately deal with sublethal effects of two inorganic chemicals on the behavior and physiology of two species of freshwater fish.

Selenium

Although often grouped with the heavy metals in the environmental toxicology literature, selenium is a group VIA non-metal with geochemical and biochemical properties similar to sulfur (Callahan et al., 1979; Stadtman, 1979). It occurs in four oxidation states: -2 (selenide - Se^{-2}), 0 (elemental selenium), +4 (selenite - SeO_3^{-2}), and +6 (selenate - SeO_4^{-2}). The geochemistry of selenium involves a typical sedimentary cycle (N.A.S., 1976). Sediments in receiving waters of seleniferous effluent or atmospheric outfall may retain and/or recycle anthropogenic Se in aquatic systems (Cherry et al., 1979; Adams and Johnson, 1981). Selenite and selenate predominate in normoxic conditions, but hydrogen selenide and organoselenides may exist in reducing, low pH environments (Cutter, 1982). Because selenate is more water soluble and less reactive with metal oxides than selenite, the selenate oxyanion probably represents the primary bioavailable form of selenium under most conditions (Callahan et al., 1979; Shamberger, 1983).

Selenium was first recognized as a naturally occurring toxin in the 1930's when livestock became ill or died after grazing on plants that bioconcentrated selenium from soils containing relatively high concentrations of the element (Nassos et al., 1980; Shamberger, 1983). Now selenium is

known to be an essential micronutrient to many organisms, including fish, yet the difference between required and detrimental levels may be small (Poston et al., 1976; Stadtman, 1979). Contamination of the environment with various forms of selenium occurs via fertilizers containing the element as an impurity, refinement of ores and metals containing selenides, manufacture of electronic equipment, and mining, processing, and combustion of fossil fuels, particularly low grade coal (Pakalla et al., 1972; Ward et al., 1981; Hodson et al., 1980; Nassos et al., 1980). Although substantial data exist regarding the dynamics and biochemistry of selenium in terrestrial ecosystems, its fate in aquatic and marine systems and effects on their biota remain less known (Halter et al., 1980).

Sandholm et al. (1973) proposed that fish take up selenium primarily in their diet, but this remains to be conclusively demonstrated (Cardwell et al., 1979). Selenium is a cumulative toxicant, concentrating primarily in the spleen, heart, liver, and kidneys of aquatic vertebrates (Adams, 1976; Cardwell et al., 1979; Lemly, 1982). Listed among 129 priority pollutants by the E.P.A., selenium is probably more dangerous to fish populations than to human populations ingesting Se-exposed fish, because fish skeletal muscle shows little tendency to bioconcentrate the element (Callahan et al., 1979). Its deleterious effects most often result from chronic, sublethal exposures, and are

probably expressed through indiscriminate selenoprotein formation and resulting cytotoxicity (Stadtman, 1979; Sorensen et al., 1982a). Documented secondary effects of selenium intoxication in vertebrates include hepatic necrosis, nephritis, anemia, hemorrhage, dermatitis, edema, and various development aberrations (Cumbie and Van Horn, 1979; Sorensen et al., 1982a; Sorensen and Bauer, 1983; Shamberger, 1983). In the single investigation of selenium effects on fish behavior, Wier and Hine (1970) found that sublethal exposures alter conditioned behavior in goldfish.

Adams and Johnson (1981) and Taylor (1982) have reviewed the toxicity of selenium to aquatic organisms. Reported acute (96-h LC_{50}) toxic values for various forms of selenium to freshwater fish range from 0.1-mg Se/l for rainbow trout (Salmo gairdneri) to 40 mg/l for carp (Cyprinus carpio) (Adams and Johnson, 1981; Sato et al., 1980). Adams (1976) and Lemly (1982), however, have demonstrated that uptake, tissue partitioning, and depuration of selenium in fish may not completely equilibrate for as many as 120 days, and time independent LC_{50} values may be significantly below acute values. In general, selenite-Se is more toxic to aquatic organisms than selenate, and organoselenium compounds have rather variable toxicity (Cardwell et al., 1979; Niimi and LaHam, 1975). The much studied relationship between selenium and mercury in organisms is highly variable in fish, ranging from

synergistic toxicity to mutual amelioration of toxic effects (Huckabee and Griffith, 1974; Kim et al., 1977; Klavercamp et al., 1983). This work will investigate effects of selenate-Se on respiration rate, thermal tolerance, and preference/avoidance behavioral responses of fathead minnows (Pimephales promelas).

Nitrite

Nitrite is a monovalent anion consisting of two oxygen atoms covalently bonded to a single nitrogen, with the single negative charge resonating between the two relatively electronegative oxygen atoms. In aqueous solution, a dynamic, pH-dependent equilibrium exists between the anion and monoprotic nitrous acid (pK_a = 3.35):

$$NO_2^- + H^+ \rightleftharpoons HNO_{2(aq)}$$

(Keenan et al., 1978).

Under near-neutral pH conditions in aquatic (and terrestrial) systems, the large majority of nitrite occurs in the charged form, and is a transient intermediate in the nitrification pathway of the nitrogen cycle. In this pathway bacteria of the genus Nitrosomonas oxidize ammonia to produce nitrite, and Nitrobactor oxidize nitrite to nitrate (Curtis, 1979).

Although potentially toxic accumulations of nitrite seldom occur in natural, undisturbed aquatic systems (Mortonson and Brooks, 1980), circumstances capable of

causing chemical or biological imbalance in the nitrification process may result in nitrite concentrations hazardous to aquatic vertebrates (Wedemeyer and Yasutake, 1978). Such situations include: Sewage treatment plant discharge sites, nitrogenous cropland or feedlot runoff, intensive aquacultural systems, and laboratory holding tanks (Westin, 1974; Tucker et al., 1979; Russo et al., 1981). In lotic systems receiving high BOD effluent, ammonia and carbon loading at the discharge site often induce concurrent nitrite maxima and hypoxic conditions downstream (Sawyer, 1960). Excretion of ammonia by fish in holding tanks or isolated pools may similarly result in high levels of nitrite, making it a recognized problem in intensive aquaculture of fish (Wedemeyer and Yasutake, 1978; Schwedler and Tucker, 1983).

Although the toxic mechanisms of nitrite in vertebrates are not completely understood, known effects include methemoglobin formation, inhibition of enzymes with exposed amine and sulfhydryl groups, and oxidation of cellular membrane lipids (Mensi et al., 1982; Margiocco et al., 1983). Of these acute effects, methemoglobin formation has been studied extensively. Nitrite oxidizes the iron moiety of hemoglobin from a ferrous (Fe^{+2}) to a ferric (Fe^{+3}) state (Bodansky, 1951). The reaction is autocatalytic, so its rate increases steadily (in the presence of excess nitrite) until limited by hemoglobin concentration when

approximately 66% of hemoglobin is converted to methemoglobin (Rodkey, 1976). Methemoglobin cannot bind oxygen, and thus decreases the oxygen-carrying capacity of blood. Vertebrates afflicted with methemoglobinemia may suffer from tissue hypoxia and metabolic acidosis (Bodansky, 1951), yet enzymatic systems to reverse methemoglobin formation exist in many fish and other vertebrates (Huey and Beitinger, 1982a; Freeman et al., 1983).

Acute nitrite toxicity in fish varies within and between species, and is generally inversely related to pH, hardness, and chloride levels (Patrick et al., 1979; Tomasso et al., 1979; Wedemeyer and Yasutake, 1978; Russo et al., 1981). Reported nitrite 96-h LC_{50} values for freshwater fish range from less than 1 to about 70-mg NO_2-N/l (Freeman et al., 1983; Palachek and Tomasso, 1984): Salmonids and channel catfish appear to be particularly susceptible to nitrite toxicity, while fathead minnows are rather tolerant. This series of studies will deal with the effects of nitrite exposure on fathead minnow respiration rate and on channel catfish thermal tolerance, hypoxic resistance, and swimming performance.

CHAPTER II

THE EFFECT OF SELENIUM ON THE ROUTINE METABOLIC RATE OF FATHEAD MINNOWS (*Pimephales promelas*)

Introduction

Several authors have identified respiration rate as a potential indicator of sublethal stress in organisms exposed to toxic substances (Sprague, 1971; Anderson, 1971; Waldichuk, 1979; Hughes, 1981). Changes in standard, routine, and/or active respiration rates of ectotherms resulting from exposure to harmful chemicals indicate some abnormality or adaptive response in at least one of the biochemical pathways or physiological processes governing metabolic rate in the whole organism. Metabolic rate, often estimated indirectly by oxygen consumption, may fluctuate with changes in rates of uptake, internal transport, or tissue utilization of oxygen. The ecological implications of an alteration in metabolic rate depend on the nature and magnitude of the change and the persistence of the toxic effect. Studies of metabolic rate effects, therefore, can provide clues to a chemical's mode(s) of toxicity, in addition to revealing an important sublethal effect.

This investigation attempted to determine if selenium affects routine metabolic rate of fathead minnows, as measured indirectly by whole animal oxygen consumption. Increasing anthropogenic release of selenium into the environment via coal processing and combustion, as well as various other industrial processes, has brought about increased concern regarding the fate of selenium in the environment and its toxicity to aquatic organisms in particular (Gutenmann et al., 1976). Andren et al., (1975) calculated that coal burning alone discharges $2.5 \cdot 10^6$ kg Se/yr to surface waters and the atmosphere. Selenium is known to bioconcentrate in living tissue (Cardwell et al., 1979). Studies of acute and chronic toxicity of selenium to aquatic organisms have been reviewed by Adams and Johnson (1976) and Taylor (1982).

Materials and Methods

Fathead minnows (Pimephales promelas) were obtained from a local fish hatchery and maintained in a recirculating "living stream" (Frigid Units, Inc.). Holding water was aerated and charcoal filtered continuously, and consisted of deionized water reconstituted into hard water as per U.S. EPA (1975). Fish were held at $21 \pm 1^{\circ}C$ ($\bar{X} \pm s$) for at least 5 weeks prior to experimentation and experienced a 16L:8D photoperiod cycle throughout holding and testing. At no time did the holding water contain any

detectable selenium. Fish were fed ground catfish chow at 1% body weight per day until 3 days prior to experimentation.

Acute (24 h), static exposures of fathead minnows to selenium were conducted in aerated, reconstituted hard water at 21 ± 1°C ($\bar{X}$ ± s) (U.S. EPA, 1975). Nominal doses of 0, 15, 40, and 60-mg Se/l occurred in 30 l all glass aquaria. All exposures were replicated except the 60-mg Se/l dose, with 20 to 25 fish per replicate. Higher selenate-Se exposures run concurrent with these yielded a 24-h LC_{50} of 82-mg Se/l (95% fiducial limits of 76 to 89 mg/l) for fathead minnows at this combination of temperature and water chemistry (Table I). Because only 8% mortality was observed at the 60-mg Se/l nominal dose, the concentration range used in this study was considered sublethal. Actual selenium concentrations of water samples collected at the beginning and end of 24 h exposures were measured (to ± 0.5 mg Se/l) by flame atomic absorption spectrophotometry (Rann and Hambly, 1965; Perkin-Elmer, 1976).

Routine respiration rates were determined from consumption of oxygen by individual fish in 300 ml BOD bottles closed for 120 min (2 h) at a temperature of 21.7 ± 1.0°C ($\bar{X}$ ± s) (Anderson et al., 1980). In 2 h, the typical test fish depleted dissolved oxygen in a bottle to an easily measurable but unstressful level. BOD bottles were filled

TABLE I

ABSOLUTE AND RELATIVE MORTALITY OF FATHEAD MINNOWS DURING 24 h EXPOSURES TO VARIOUS CONCENTRATIONS OF SELENATE-Se

Mg Se/l $\bar{X}$	$\pm$ s	Number Exposed	Number Dead	Relative Mortality (%)
0.0	0.0	40	0	0.0
15.4	0.4	20	0	0.0
16.0	0.6	20	0	0.0
40.0	0.3	13	1	7.7
40.2	0.5	10	0	0.0
40.4	0.4	20	0	0.0
58.8	1.6	24	2	8.3
70.5	4.9	23	11	47.8
97.3	0.0	24	14	58.3
100.8	1.7	22	20	90.9

with water from the fish's exposure aquarium. Dissolved oxygen determinations were made to $\pm$ 0.05 mg O_2/l with a Weston and Stack model 330 dissolved oxygen analyzer equipped with a model 33 BOD probe. The oxygen analyzer was calibrated by comparison to the azide modification of Winkler's iodometric method for dissolved oxygen measurement (A.P.H.A., 1975). Respirometry of fish in bottles took place in a quiet, dimly lit setting. Bottles without fish were treated similarly to quantify meter drift and/or microbial oxygen consumption. Weight and standard length of each fish were measured after respirometry to the nearest 0.01 g and 1 mm, respectively.

Weight-specific routine metabolic rates ($\dot{M}O_2$) were calculated by dividing oxygen consumption of each fish by its weight and incubation time in hours (2 h in all cases). Because oxygen concentration of "fishless" bottles decreased an average of 0.24-mg O_2/l ($\pm$ s of 0.14), this amount was subtracted from the measured oxygen consumption of each fish.

Although deviations from normality were observed in distribution of respiration rates of fish from two of the exposure groups (Shapiro and Wilk's test, $p<0.05$), all variances were similar (Bartlett's test, $\alpha=0.001$), and the non-normal distributions were not considered threatening to the parametric analysis of variance (ANOVA) used to statistically evaluate these data (Zar, 1974). Statistical

Analysis System procedures were employed for all tests (S.A.S., 1982).

Results

ANOVA detected no difference in mean weight-specific oxygen consumption of fathead minnows from the various exposure groups ($F = 1.10$, $p>0.35$). Mean weight-specific oxygen consumption of control minnows and those from the highest Se exposure were only slightly higher than those of the two intermediate exposures (Table II). Respiration rates of all groups tested were rather variable: coefficients of variation ranged from 0.20 to 0.29. Casual (unquantified) observation of fathead minnows in BOD bottles indicated that undosed fish were somewhat more active than minnows exposed to selenate, yet seemed to have lower operculation rates.

Dissolved oxygen in BOD bottles containing fish dropped from 8.62 ± 0.28 ($\bar{X} \pm s$) to 5.93 ± 1.11 mg/l overall, and weight specific oxygen consumption of all groups combined averaged 0.45 ± 0.11 mg $O_2 \cdot g^{-1} h^{-1}$. Only four minnow mortalities were observed: two (of 24) died during exposure to the highest measured selenium concentration (58.8-mg Se/l), and two more from that exposure died during respirometry.

Mean weights were similar for all groups (ANOVA:$F = 2.33$, $p>0.05$), yet minnows from the highest Se exposure had

TABLE II

MEAN WEIGHT SPECIFIC OXYGEN CONSUMPTION RATES OF FATHEAD MINNOWS FROM VARIOUS 24 h SELENATE-Se EXPOSURES

Selenate-Se (mg/l)		% of		wt. (g)		$\dot{M}O_2$ ($mgO_2 \cdot g^{-1} \cdot h^{-1}$)		
$\bar{X}$	$\pm$ s	24 h LC_{50}	n	$\bar{X}$	$\pm$ s	$\bar{X}$	$\pm$ s	C.V.
0.0	0.0	0.0	20	0.90	0.21	0.49	0.14	0.29
15.7	0.6	19.1	20	0.83	0.19	0.44	0.11	0.25
40.3	0.4	49.1	20	0.90	0.13	0.43	0.09	0.22
58.8	1.6	71.7	10	1.00	0.14	0.46	0.09	0.20

a significantly higher mean condition factor than all other groups, as indicated by ANOVA ($F = 5.39$, $p<0.01$) and Duncan's multiple range test. Overall, test fish had mean ($\pm$ s) weights and condition factors (using standard length) of 0.89 $\pm$ 0.18 g and 1.33 $\pm$ 0.17 mm, respectively.

Discussion

Although the variability in these data would probably statistically overcome a minor effect of selenium exposure on fathead minnow respiration rate, the similarity of means from the various exposure groups implies that no such net effect exists. Also, there is no reason to suspect that the relatively high mean condition factor of minnows from the highest exposure masked a significant alteration in respiration rate. The high mean condition factor could have been due to edema and fluid accumulation resulting from selenium poisoning; other researchers have noted such an effect in Se-exposed teleosts (Halter et al., 1980; Niimi and LaHam, 1975; Sorensen et al., 1982a; Shamberger, 1983). $\dot{M}O_2$ values reported here for fathead minnows are comparable to those reported by Anderson et al. (1980) for red shiners (Notropis lutrensis) of similar size.

Other researchers have investigated effects of various substances on metabolic rates of fish both by use of whole animal oxygen consumption (as in this study) or by using operculation rate, heart rate, gill diffusing capacity, and

isolated gill oxygen consumption (Thurberg and Dawson, 1974; Thomas and Rice, 1975; Cairns et al., 1981; Hughes, 1981). Thomas and Rice (1975) found that operculation rate of pink salmon fry (Oncorhynchus gorbuscha) was a sensitive indicator of sublethal stress resulting from exposure to water soluble components of crude oil, as the rate increased significantly during 24 h exposure to 20% of the 96-h LC_{50} of the pollutant. Cairns et al. (1981) proposed that the frequency and amplitude of fish ventilatory behavior could be used as an indicator of sublethal stress in continuous biomonitoring of potentially toxic effluents that elicit this response. Utilizing isolated dogfish (Scyliorhynus canicula) gills, Tort et al. (1982) demonstrated that zinc can inhibit tissue oxygen uptake, and Hughes (1981) has correlated inhibition of respiration by zinc and nickel with concomitant decreases in gill surface area, increases in diffusion distance, and tissue hypoxia.

Whole animal oxygen consumption may be altered at the gas exchange surface, in the circulatory system, or at the tissues, and is directly proportional to activity in ectotherms (White, 1982a). Although selenium exposure results in multiple histopathologies in both blood and internal tissues of fish, no effects on gill epithelia have been identified (Ellis et al., 1937; Sorenson et al., 1982 a, b, and c; Cardwell et al., 1976). Toxicological effects of selenium on fish blood result in symptoms of general

profound anemia (Ellis et al., 1937; Sorensen and Bauer, 1983).

The lack of a net measurable effect of selenium intoxication on oxygen uptake of fathead minnows could be due to various combinations of counterbalancing physiological and behavioral effects. The relatively high operculation rates and low activity levels noted in Se-exposed fathead minnows correspond to the hyperventilation observed in calves incapacitated by the hematologic effects of selenite-Se (Shamberger, 1983) and the depressed filtering activity of Se-exposed Daphnia (Reading and Buikema, 1980). Expression of sublethal selenium toxicity to fathead minnows through changes in respiration rate, therefore, may not occur because intoxicated minnows compensate for decreased blood oxygen transport by increasing gill ventilation and perfusion and reducing all other activity to a minimum. Nevertheless, because routine respiration rate determinations do not in themselves regulate or take account of activity, they are probably at best insensitive indicators of changes in metabolic rate induced by toxicants, and would thus be better substituted with standard or active metabolic rate determinations when possible.

CHAPTER III

SELENIUM TOXICITY AND THERMAL TOLERANCE IN THE FATHEAD MINNOW (*Pimephales promelas*)

Introduction

Two or more abiotic factors may additively or synergistically affect an organism when they simultaneously attain stressful levels (Fry, 1971; Paladino et al., 1980). Temperature is known to influence the toxicity of many substances (Cairns, et al., 1975), and toxic chemicals, in turn, can affect thermal tolerance (Hutchison, 1976; Wedemeyer and McLeay, 1981). Ellis et al. (1937) reported a marked increase in selenium toxicity to channel catfish with increased temperature. Substances known to decrease thermal tolerance of certain fish include nickel, arsenic, and dieldrin (Becker and Wolford, 1980; Paladino and Spotila, 1978; Silbergeld, 1973). The goal of this laboratory study was to assess the influence of acute, sublethal selenium exposure on the thermal tolerance of fathead minnows.

As coal-fired power generation and coal processing increase in the southern states, selenium may become an environmental hazard, since low-grade coal often contains selenium in excess of 2 mg/l (Adams and Johnson, 1981).

Surface waters receiving runoff from coal mines or effluent from power plants and coal processing facilities risk prolonged contamination with selenium, as the element is released during coal processing and combustion, yet is incompletely removed by industrial waste treatment (Andren et al., 1975). Because aquatic systems in southern latitudes commonly reach temperatures of 30-35°C in summer (Tucker et al., 1979), and in many cases receive thermal discharge from power generating stations, possible selenium toxicity/temperature interactions need to be addressed.

Materials and Methods

Fathead minnows (Pimephales promelas, wet weight 1.0 ± 0.2 g, $\bar{X}$ ± s) obtained from a local fish hatchery were held at 21 ± 1°C ($\bar{X}$ ± s) for 5 weeks prior to experimentation. A recirculating "living stream" (Frigid Units, Inc.) containing aerated hard water reconstituted from deionized water was used to hold fish (U.S. EPA, 1975). Photoperiod was maintained at 16L:8D throughout, and fish were fed ground catfish chow at 1% wet body weight per day until 2 days prior to testing.

Acute (24 h), static exposures of fathead minnows to selenium took place in aerated, reconstituted hard water at 21 ± 1°C ($\bar{X}$ ± s) (U.S. EPA, 1975). Nominal concentrations of 0, 15, 40, and 60-mg selenate-Se/l were employed in 30 l glass aquaria. All exposures were replicated except the

60-mg Se/l dose, with 20 to 25 fish per trial. Higher selenate-Se exposures run concurrent with these yielded a 24-h LC_{50} of 82-mg Se/l (95% fiducial limits of 76 to 89 mg/l) for fathead minnows. Because only 8% mortality was observed from the 60-mg Se/l nominal dose and 0% at lower concentrations, the concentration range used in this study was considered sublethal. Actual selenium concentrations of water samples collected at the beginning and end of exposures were measured by flame atomic absorption spectrophotometry (Rann and Hambly, 1965; Perkin-Elmer, 1976).

Critical thermal maxima (CTM) were determined to evaluate the thermal tolerance of fathead minnows (Cowles and Bogert, 1944). Both a method and a parameter, the CTM is defined as "the arithmetic mean of the collective thermal points at which locomotory activity becomes disorganized and the animal loses its ability to escape from conditions that will promptly lead to its death when heated from a previous acclimation temperature at a constant rate just fast enough to allow deep body temperature to follow environmental temperature without a significant time lag" (Cox, 1974). As recommended by Becker and Genoway (1979) and Hutchison (1976), temperature was raised at $0.3^{\circ}C$/min and the endpoint criterion was final loss of equilibrium.

Ten fish from a particular exposure were tested simultaneously in a 60-l aquarium (base = 30 cm x 75 cm) containing 47 l of E.P.A. hard water. Fish were segregated

in two adjacent rows of five 10-cm x 10-cm plastic mesh enclosures. The water was aerated prior to and during CTM trials, and was changed between trials. Dissolved oxygen was measured to the nearest 0.05 mg/l before and after each CTM trial with a Weston and Stack D.O. analyzer (Rexnord Instrument Co.). Temperature was raised from 20.9 $\pm$ 0.1^{o}C ($\bar{X} \pm s$) at 0.3^{o}C/min by two Haake E-52 circulating thermoregulators, and monitored to the nearest 0.01^{o}C with a Digitec HT-5810 digital thermometer.

Least squares regression analysis statistically evaluated the effect of selenium exposure on CTM. Normality and homoscedasticity of the data were tested with Shapiro and Wilk's W statistic and Bartlett's test, respectively (Zar, 1974). Since critical thermal maxima of three of the exposure groups were not normally distributed, Kruskal-Wallis and Dunnett's multiple range tests were employed to further hornswoggle the relationship between CTM and selenate dose. Statistical Analysis System procedures performed all tests (S.A.S., 1982).

Results

Measured selenium exposures were ($\bar{X} \pm s$):0.0 $\pm$ 0.0 mg Se/l for controls, 15.7 $\pm$ 0.6 for the 15 mg/l nominal exposures, 40.2 $\pm$ 0.4 for the 40 mg/l trials, and 58.8 $\pm$ 1.6 mg/l for the single 60 mg/l dose. Coefficients of variation of Se concentrations ranged from 0 to 4%. Percent

recovery of selenium from spiked samples ranged from 100 to 105%. CTM endpoint behavior of fathead minnows was similar to that described by Hutchison (1976), Cheetham et al. (1976), and Becker and Genoway (1979) for other fish species and was consistent among control and dosed minnows. As a fish's endpoint approached, increased locomotory activity accompanied by periodic losses of equilibrium gave way to a series of violent, spasmodic outbursts that culminated with ineffectual quivering and final, complete loss of equilibrium.

Acute sublethal selenate-Se exposure resulted in a highly significant reduction of fathead minnow thermal tolerance, as described by the least squares regression model: $CTM = 35.20 - 0.0016\ (mg\ Se/l)^2$; $p<0.001$, $r^2 = 0.50$, $n = 70$ (Figure 1), where CTM is inversely proportional to the square of the selenate-Se concentration. Kruskal-Wallis test found that mean critical thermal maxima of fish from the various exposures were indeed different ($X^2 = 35.49$, $p<0.001$). Dunnett's multiple range test grouped the mean control CTM with that of fish from the lowest selenate exposure (15.7 mg Se/l), but distinguished CTM means of fish from the two higher exposures from the control mean (Table III). Individual fathead minnow critical thermal maxima ranged from 22.90 to 36.25°C, and mean CTM of the group exposed to 72% of their 24-h LC_{50} for selenate-Se was almost 6°C below the control mean.

Fig. 1--Critical thermal maxima of fathead minnows plotted against the square of their 24 h selenate-Se exposure concentrations. Means, ± one standard deviation, and sample size (parenthesis) are given for each group.

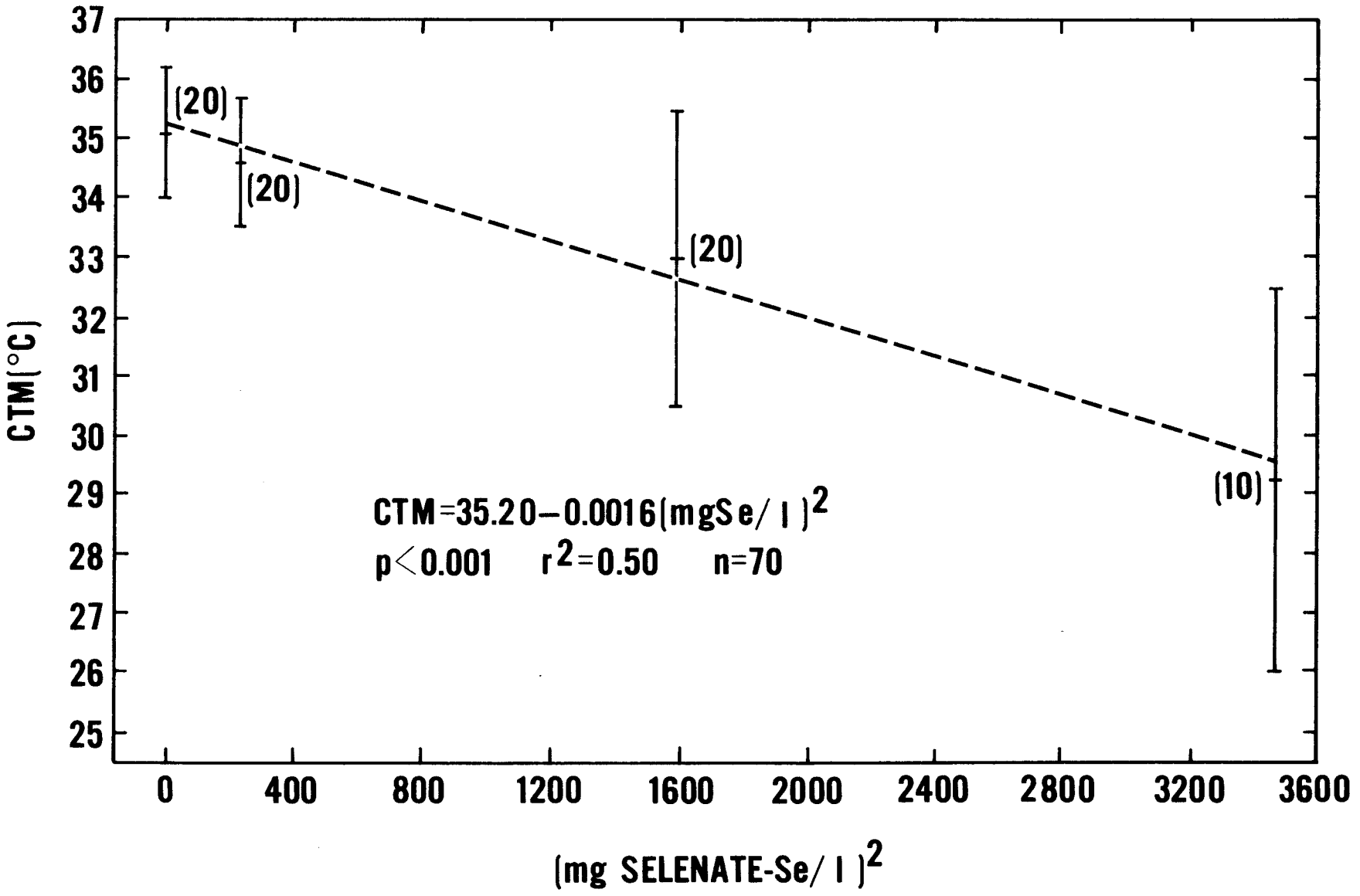

37
36
35
34
33
32
31
30
29
28
27
26
25
CTM(°C)
(20)
(20)
(20)
(10)
CTM=35.20−0.0016(mgSe/l)2
p<0.001 r2=0.50 n=70
0
400
800
1200
1600
2000
2400
2800
3200
3600
(mg SELENATE-Se/l)2

TABLE III

DUNNETT'S MULTIPLE RANGE GROUPING OF MEAN CRITICAL THERMAL MAXIMA OF FATHEAD MINNOWS FROM VARIOUS 24 h SELENATE-Se EXPOSURES

Selenate-Se (mg/l) $\bar{X} \pm s$	Critical Thermal Maxima (^{o}C) (n)	$\bar{X} \pm s$	Dunnett's Grouping
0.0 ± 0.0	(20)	35.12 ± 1.15	A
15.7 ± 0.6	(20)	34.65 ± 1.09	A
40.2 ± 0.4	(20)	33.02 ± 2.44	B
58.8 ± 1.6	(10)	29.21 ± 3.30	B

Discussion

Thermal tolerance of fish has been determined as either incipient lethal temperature or critical thermal maximum (Fry, 1971). Although the former methodology probably provides a more physiologically and ecologically meaningful index of thermal tolerance for individual organisms and species, the utility and expedience of CTM for intra- and inter- specific comparisons of thermal tolerances of poikilotherms has been recognized by many researchers (Hutchison, 1976; Becker and Genoway, 1979; Paladino et al., 1980; Wedemeyer and McLeay, 1981). As in this investigation, this laboratory and others have used CTM methodology to determine if various chemicals affect fish thermal tolerance (Paladino and Spotila, 1978; Schubauer et al., 1980; Takle et al., 1983). The mean CTM of control fathead minnows in this study corresponds roughly to lethal temperatures reported by Brungs in 1971.

These results demonstrate that sublethal selenium exposure decreases the ability of fathead minnows to cope with thermal stress resulting from an acute, steady increase in temperature. Neither the lethal effects of high temperatures nor the mode(s) of selenium toxicity are completely understood (Prosser, 1973; Shamberger, 1983). For their effects to be additive or synergistic, as these data indicate, toxic selenium body burden and thermal stress

probably act through related physiological pathways to decrease fathead minnow CTM.

Many histopathologic and physiologic effects of selenium poisoning (and deficiency) have been described in vertebrates (Stadtman, 1974; Stadtman, 1979; Shamberger, 1983). In particular, multiple hematologic disorders result from both acute and chronic ingestion and environmental exposure in birds, mammals, and fish (Sorensen and Bauer, 1983). In fish, blood pathology associated with chronic selenium exposure includes: malformed erythrocytes, and reduced hematocrit, hemoglobin concentration, erythrocyte count, and erythrocyte volume (Ellis et al., 1937; Sorensen and Bauer, 1983). All of these conditions are symptomatic of anemia, and thus reduce the oxygen-carrying capacity of the blood (White, 1982b).

Elevated temperatures increase the metabolic rate of ectotherms and thus increase tissue oxygen demand. Both Fry (1971) and Prosser (1973) have postulated that tissue hypoxia exacerbates the lethal effects of heat stress. Selenium-induced reduction in fathead minnow CTM, then, may be explained in part by the hematologic effects of selenium coupled with the metabolic demands of thermal loading.

Although this research utilized acute exposures and Se body burdens were not determined, the results indicate a potential for loss of thermal tolerance of fish after chronic sublethal selenium exposure resulting in body

burdens similar to those produced by these acute exposures. Prolonged exposure to selenium from a coal-fired power plant has been implicated as the cause of anemia, edema, tissue necrosis, and death of various fish species in an east Texas reservoir (Sorensen et al., 1982a, b, and c; Sorensen and Bauer, 1983), and other chronic and acute exposures of fresh-water teleosts have induced multiple cardiovascular and respiratory histopathologies capable of reducing temperature tolerance, thereby limiting thermal niche (Ellis et al., 1937; Cardwell et al., 1976; Hodson et al., 1980; Cumbie and Van Horn, 1979). Temperature/ toxicity relationships for selenium in aquatic systems should be more completely described, and the resulting information used in management of southern watersheds receiving seleniferous effluent.

CHAPTER IV

EVALUATION OF FATHEAD MINNOW AVOIDANCE OF SELENIUM

Introduction

Behavioral responses of organisms to environmental pollutants are important to impact assessment and natural resource management because alterations of behavior resulting from detection of or exposure to sublethal levels of a toxic substance may influence an individual's fitness or choice of habitat, and thus the structure of populations and ecosystems (Anderson, 1971; Sprague, 1971; Olla et al., 1980). In particular, avoidance of effluents or specific chemicals by fish has been investigated by many researchers; the preference/avoidance behavior literature has recently been reviewed by Beitinger and Freeman (1983) and Giattina and Garton (1983). Although avoidance of pollutants by fish has been observed in nature, it is likely that threshold avoidance levels are higher and more variable in the environment than in the laboratory due to the lack of directive and/or masking factors in the laboratory that might override avoidance behavior in nature (Sprague, 1971; Beitinger and Magnuson, 1976; Weber et al., 1981; Giattina et al., 1981). Nevertheless, laboratory evaluations of avoidance behavior can determine if the potential

for behavioral mitigation of environmental stress exists.

Selenium is an increasingly common environmental contaminant because of the increased mining, processing, and combustion of fossil fuels, particularly low grade coal, in which the element is concentrated (Andren et al., 1975; Gutenmann et al., 1976). The cumulative toxicity of selenium to fish and other organisms has been documented in the laboratory and the environment (Adams and Johnson, 1981; Taylor 1982; Shamberger, 1983). Although known to be a required micronutrient in most organisms, selenium has also been shown to bioconcentrate readily (Stadtman, 1974; Callahan et al., 1979; Lemly, 1982). Chronic exposures may be lethal or physiologically debilitating (Halter et al., 1980; Sorensen et al., 1982a). This study sought to determine if fathead minnows avoid acute and chronic lethal levels of selenium in the laboratory.

Materials and Methods

Fathead minnows (Pimephales promelas, wet wt. 0.9 $\pm$ 0.2 g, $\bar{X} \pm s$) procured from a local fish hatchery were maintained in a recirculating "living stream" containing hard water reconstituted from deionized water (U.S. EPA, 1975). Fish were held at 17 $\pm$ 2^{o}C ($\bar{X} \pm s$) for at least ten days prior to experimentation, and were fed ground catfish chow at 1% body weight per day.

A counter-current apparatus similar to those of Jones (1947), Sprague (1964), and Cherry et al. (1977) was employed to evaluate fathead minnow preference/avoidance behavior (Figure 2). The apparatus permitted simultaneous observation of ten visually isolated fish. Visual and spatial segregation prevented intraspecific behavioral interactions that may have masked the reactions of individual fish to chemical sensory stimuli. A blind around the plexiglas gradient tank allowed viewing of test fish from either the north or south end with minimal disturbance.

The flow-through system utilized 11 l/min of aerated, charcoal-filtered tap water diluted with deionized water. Water quality determinations performed for each trial include: temperature, dissolved oxygen, pH, alkalinity, hardness, and conductivity (Table IV) (A.P.H.A., 1975). A stock sodium selenate solution was mixed into and diluted by the water flowing through one side (north or south) of the system. The selenium-contaminated water then entered one end of each fish chamber in the gradient tank, while uncontaminated water entered the other end of each chamber, producing a relatively steep toxicant gradient over the central drains of the chambers, as recommended by Larrick et al. (1978) and Giattina et al. (1982). Flow rates out of the central drains were equilibrated with a Gilmont variable area fluid flowmeter.

Fig. 2--System utilized to evaluate fathead minnow avoidance of selenium. All dimensions are in centimeters.

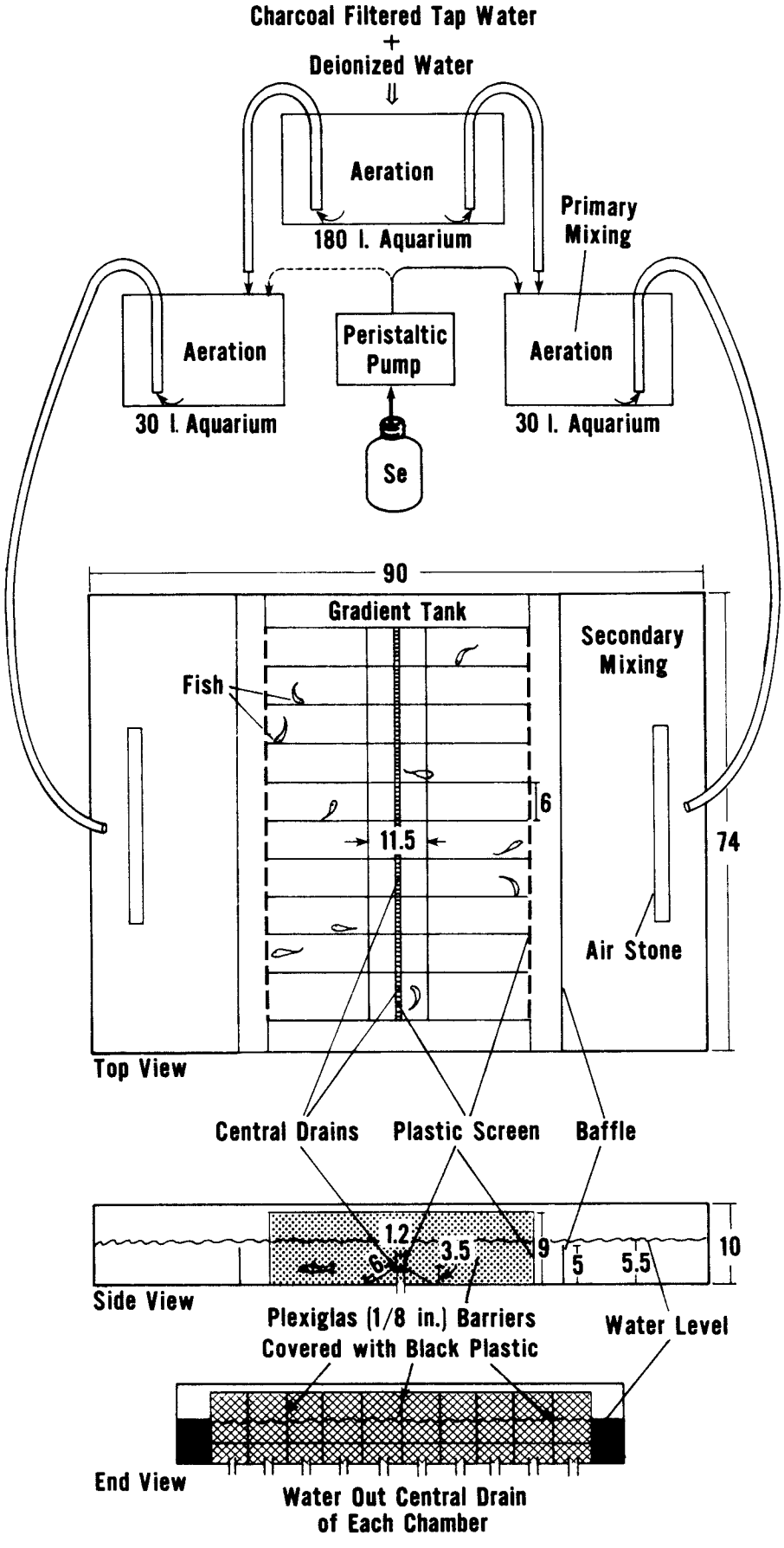

Charcoal Filtered Tap Water
+
Deionized Water
Aeration
180 l. Aquarium
Primary Mixing
Aeration
30 l. Aquarium
Peristaltic Pump
Se
Aeration
30 l. Aquarium
90
Gradient Tank
Secondary Mixing
Fish
6
11.5
74
Air Stone
Top View
Central Drains
Plastic Screen
Baffle
1.2
3.5
6
9
5
5.5
10
Side View
Plexiglas (1/8 in.) Barriers
Covered with Black Plastic
Water Level
End View
Water Out Central Drain
of Each Chamber

TABLE IV

WATER QUALITY OF WATER USED IN PREFERENCE/AVOIDANCE TESTS

Parameter	$\bar{X} \pm$ S.D.
Temperature	$16 \pm 1\ ^{o}C$
Dissolved oxygen	8.7 ± 0.1 mg0_2/l
pH	7.7 ± 0.2
Alkalinity	45 ± 7 mg $CaCo_3$/l
Hardness	51 ± 7 mg $CaCo_3$/l
Conductivity	136 ± 22 µmhos

After introduction of one fish to each of the ten chambers, experimental protocol included a 5 min "familiarization" period. Thirty minutes of control observations then followed. Upon completion of control observations, the peristaltic pump delivering sodium selenate to one side or the other of the system was activated, and 5 min were allowed for stabilization of the selenium gradient in each chamber. Fish were observed again for 30 min. During the two observation periods (control and "two-choice"), the position of each fish (on one end or the other of its chamber) was recorded every 2 min, producing 15 observations per fish for each period. After the second observation period, the trial was concluded with collection of water samples for Se analysis from each end of the ten chambers.

Four independent trials were conducted with ten different fish for each trial. The range of selenate-Se concentrations delivered to the two-choice system was chosen to represent levels of selenium acutely and chronically lethal to fathead minnows. As lethal concentrations from 0.6 to 12 mg Se/l have been reported (Halter et al., 1980; Adams and Johnson, 1981), target concentrations of 1 and 10 mg selenate-Se/l were employed. Selenate-selenium was utilized in these tests because it is probably the most dangerous form environmentally (Callahan et al., 1979). Selenium concentrations in water samples were measured by flame atomic absorption spectrophotometry (Rann and Hambly,

1965; Perkin-Elmer, 1976).

Behavioral data were analyzed with chi-square techniques (Zar, 1974). Control and "two-choice" observations were compared to the null hypothesis of random fish movement between ends of a chamber to reveal statistically significant side preference and/or response to the selenium gradient. In addition, heterogeneity chi-square analyses were performed on the pooled data from each observation period to see if fish responded similarly during observation. Yates continuity correction was used in all chi-square analyses (Zar, 1974).

Results

After a brief post-introductory period of inactivity, fish generally began swimming about their chambers, freely crossing the central drain, yet rarely lingering over it. While some fish became more quiescent as the trial progressed, most remained active for the duration of the experiment.

Control period observations indicated most fish had no intrinsic preference for either end of their chamber. Eight fish of 40 (20%) were observed significantly more often in one end of their chamber during the control period: two from trial one, three from trial two, and three from trial four (Tables V, VI, VII, and VIII). Of those eight fish, three preferred the north end, five the south.

TABLE V

EVALUATION OF FATHEAD MINNOW AVOIDANCE OF SELENATE-Se: TRIAL ONE OBSERVATIONS AND CHI-SQUARE VALUES

Fish	Control Period			0.3 ± 0.1 mg Se/l in South End		
	N	S	X^2	N	S	X^2
1	10	5	1.07	10	5	1.07
2	13 ←	2	6.67**	6	9	0.27
3	8	7	0.00	6	9	0.27
4	11	4	2.40	6	9	0.27
5	8	7	0.00	8	7	0.00
6	8	7	0.00	4	11	2.40
7	9	6	0.27	8	7	0.00
8	11	4	2.40	14 ←	1	9.60**
9	5	10	1.07	6	9	0.27
10	1 →	14	9.60**	4	11	2.40
Sums	84	66	23.53	72	78	16.55
Pooled X^2 (n=150, v=1) = 1.93				Pooled X^2 = 0.17		
Heterogeneity X^2 (n=150, v=9) = 21.60*				Heterogeneity X^2 = 16.38		

* $p < 0.05$; Arrow indicates direction of Preference/avoidance response

** $p < 0.01$

TABLE VI

EVALUATION OF FATHEAD MINNOW AVOIDANCE OF SELENATE-Se: TRIAL TWO OBSERVATIONS AND CHI-SQUARE VALUES

Fish	Control Period			0.4 ± 0.1 mg Se/l in North End		
	N	S	X^2	N	S	X^2
1	12 ←	3	4.27*	3 →	12	4.27*
2	8	7	0.00	4	11	2.40
3	3 →	12	4.27*	9	6	0.27
4	5	10	1.07	15 ←	0	13.07**
5	10	5	1.07	7	8	0.00
6	3 →	12	4.27*	4	11	2.40
7	7	8	0.00	6	9	0.27
8	5	10	1.07	10	5	1.07
9	6	9	0.27	6	9	0.27
10	6	9	0.27	7	8	0.00
Sums	65	85	16.83	71	79	24.02
Pooled X^2 (n=150, v=1) = 2.41				Pooled X^2 = 0.33		
Heterogeneity X^2 (n=150, v=9) = 14.42				Heterogeneity X^2 = 23.69**		

* $p < 0.05$; Arrow indicates direction of Preference/avoidance response.

** $p < 0.01$

TABLE VII

EVALUATION OF FATHEAD MINNOW AVOIDANCE OF SELENATE-Se: TRIAL THREE OBSERVATIONS AND CHI-SQUARE VALUES

Fish	Control Period			6.7 ± 0.4 mg Se/l in South End		
	N	S	X^2	N	S	X^2
1	9	6	0.27	7	8	0.00
2	6	9	0.27	7	8	0.00
3	10	5	1.07	7	8	0.00
4	7	8	0.00	6	9	0.27
5	9	6	0.27	6	9	0.27
6	6	9	0.27	9	6	0.27
7	6	9	0.27	11	4	2.40
8	6	9	0.27	13 ←	2	6.67**
9	9	6	0.27	9	6	0.27
10	8	7	0.00	5	10	1.07
Sums	76	74	2.96	80	70	11.22
Pooled X^2 (n=150, v=1) = 0.01				Pooled X^2 = 0.54		
Heterogeneity X^2 (n=150, v=9) = 2.95				Heterogeneity X^2 = 10.68		

* $p < 0.05$; Arrow indicates direction of Preference/avoidance response.

** $p < 0.01$

TABLE VIII

EVALUATION OF FATHEAD MINNOW AVOIDANCE OF SELENATE-Se: TRIAL FOUR OBSERVATIONS AND CHI-SQUARE VALUES

Fish	Control Period			11.2 ± 0.4 mg Se/l in North End		
	N	S	X^2	N	S	X^2
1	10	5	1.07	6	9	0.27
2	7	8	0.00	11	4	2.40
3	3 →	12	4.27*	10	5	1.07
4	6	9	0.27	8	7	0.00
5	5	10	1.07	3 →	12	4.27*
6	8	7	0.00	13 ←	2	6.67**
7	8	7	0.00	12 ←	3	4.27*
8	7	8	0.00	2 →	13	6.67**
9	13 ←	2	6.67**	5	10	1.07
10	1 →	14	9.60**	2 →	13	6.67**
Sums	68	82	22.95	72	78	33.36
Pooled X^2 (n=150, v=1) = 1.13				Pooled X^2 = 0.17		
Heterogeneity X^2 (n=150, v=9) = 21.82**				Heterogeneity X^2 = 33.19**		

* $p < 0.05$; Arrow indicates direction of Preference/avoidance response.

** $p < 0.01$

Chi-square analyses of the pooled control data for each trial showed no net response of fish to the apparatus itself during any control period. Heterogeneity chi-square analysis of each trial's control period revealed significantly dissimilar behavior among fish in trials one and four. Total control observations per end of the gradient tank after pooling all trials were 293 north and 307 south.

Selenium concentrations measured in the contaminated ends of chambers at the conclusion of each trial's two-choice observation period were ($\bar{X} \pm s$) 0.3 $\pm$ 0.1 mg Se/l for trial one, 0.4 $\pm$ 0.1 for trial two, 6.7 $\pm$ 0.4 for trial three, and 11.2 $\pm$ 0.4 for trial four. No measurable selenium "leaked" across the gradient, as none was detected in any of the water samples collected from uncontaminated ends of the fish chambers. Percent recovery of selenium from spiked samples ranged from 100 to 105%.

During the two-choice observation periods, nine of 40 (22.5%) fish spent significantly greater time in one end of their chambers. Only two of those nine had exhibited side preference during their control period: Fish one of trial two (Table VI) spent more time in the end of its chamber uncontaminated with selenium after residing in the opposite end during control observations, and fish ten of trial four (Table VIII) was observed 13 of 15 times in the uncontaminated end, for which it had shown highly significant preference during control observations. Six of the

nine fish that demonstrated significant side selection during two-choice observations spent less time on the selenium-contaminated ends of their chambers; three of those six experienced the highest selenium gradient tested, yet no pooled chi-square statistic from any of the four two-choice data sets approached statistical significance. In total, fish were observed on the selenium-contaminated end 291 times out of 600, or 48.5% of the time they were under observation in the two-choice situation. According to heterogeneity chi-square analyses of two-choice observations for each trial, highly significant dissimilarity in behavior existed among fish participating in trials two and four. Only in trial four did heterogenous behavior occur during both control and two-choice observations.

Discussion

Although fish experiencing the highest selenate concentration gradient (trial four, Table VIII) displayed more variable behavior than any other group (heterogeneity X^2 = 33.19), the significant side selections exhibited during two-choice observations in trial four and overall did not uniformly coincide with the established selenium gradient. Furthermore, no group of fish displayed any net side selection during control or two-choice observations, and heterogenous behavior was noted in four of the eight observation periods. In spite of the variability in behavior

among individual fish, the group as a whole responded randomly to both the apparatus and the selenium gradient, indicating that fathead minnows are not attracted to or repelled by lethal concentrations of sodium selenate in the conditions of these tests. Once set up and operating, this system provided a rapid, objective, and experimentally flexible means for simultaneous evaluation of the preference/avoidance behavior of a reasonable number of small fish.

Behavioral responses of fish to chemicals imply that those chemicals act as directive factors (Larrick et al., 1978). This would be the case, however, only if the response results from direct sensory detection of the substance (Fry, 1971). A fish may "sense" a substance indirectly through detectable changes in water quality or internal physiological consequences of uptake of the substance. Possible responses to perception of the chemical included kineses (orientation movements) and/or taxes (directed locomotion), although the distinction between the two may be somewhat artificial (Fry, 1971). An appropriate (adaptive) response to perception of a toxic substance would be to avoid the substance and thus minimize exposure to it, yet the ability of a fish to sense a chemical and respond appropriately stems from natural selection for those abilities in that species ancestral history (Olla, et al., 1980).

Natural occurrences of potentially toxic levels of selenium in aquatic systems are rare, being limited in this country to low-order drainages from localities with seleniferous geology in the Great Plains and Rocky Mountain foothills (Shamberger, 1983). Because selective pressure to sense and avoid dangerous concentrations of selenium has, for all practical purposes, been nonexistent, fish would not be expected to have those abilities. Also, because selenium probably exerts its toxic effects through cumulative, indiscriminate substitution of sulfur in enzymes, no immediately detectable physiological consequences of selenium uptake would cue a fish to avoid dangerous concentrations (Stadtman, 1974).

Fish avoidance of pollutants would minimize exposure to potentially harmful conditions, yet also implies temporary or permanent changes in local ecosystem structure and loss of fishery habitat. Lack of an avoidance response could result in mortality or sublethal effects and reductions in fitness. Because zero discharge of effluents into aquatic systems is at present unrealistic, studies of lethality and sublethal effects, such as avoidance behavior, must be employed to adequately predict site-specific effects of pollutant outfalls and to derive standards for protection of receiving waters (Larrick et al., 1978). This study demonstrated that fathead minnows do not avoid potentially lethal concentrations of selenate-Se in the laboratory and

carries the assumption that toxic yet sublethal levels would also not elicit a response in the environment.

CHAPTER V

THE EFFECT OF NITRITE ON THE ROUTINE METABOLIC RATE OF FATHEAD MINNOWS (*Pimephales promelas*)

Introduction

Dissolved nitrite in aquatic systems is potentially toxic to many organisms (Huey, 1982). Aquacultural systems or receiving waters of nitrogenous effluent may accumulate relatively high nitrite-nitrogen concentrations due to imbalances in the nitrification pathway of the nitrogen cycle (Sawyer, 1960; Westin, 1974).

In fish, nitrite toxicity is relatively variable compared to other chemicals: reported 96-h LC_{50} values range from less than 1 to about 70 mg NO_2-N/l (Freeman et al., 1983; Palachek and Tomasso, 1984). Increases in pH, chloride, and hardness levels have each been shown to decrease nitrite toxicity in various freshwater fish species (Perrone and Meade, 1977; Tomasso et al., 1979; Russo et al., 1981). Demonstrated mechanisms of acute nitrite toxicity in vertebrates include methemoglobin formation, inhibition of enzymes with exposed amine and sulfhydryl groups, and oxidation of cellular membrane lipids (Mensi et al., 1982; Margiocco et al., 1983).

In addition to serving as a sublethal bioassay tool, respirometry may provide indirect insight to the mode(s) of toxicity of a chemical (Hughes, 1981). Several researchers have utilized various measures of metabolic rate to ascertain sublethal stress in fish. Suspended solids, crude oil, zinc, copper and cadmium directly or indirectly affect metabolic rate of some fish (Neumann et al., 1982; Thomas and Rice, 1975; Skidmore, 1970; Sellers et al., 1974; Thurberg and Dawson, 1974). The conversion of hemoglobin to methemoglobin by nitrite in fish may reduce their oxygen uptake capability, and thereby depress their metabolic rate. This study attempted to determine if nitrite influences the routine weight specific oxygen consumption of fathead minnows, a fish species this laboratory found to be particularly tolerant of nitrite exposure.

Materials and Methods

Fathead minnows (Pimephales promelas) were obtained from a local fish hatchery and maintained in a recirculating "living stream" (Frigid Units, Inc.). Holding water was aerated and charcoal filtered continuously, and consisted of deionized water reconstituted into hard water as per U.S. EPA (1975). Fish were held at $20 \pm 1^{o}C$ ($\bar{X} \pm s$) for at least 3 weeks prior to experimentation and experienced a 16L:8D photoperiod cycle throughout holding and testing. At no time did the holding water contain greater

than 0.1 mg NO_2-N/l. Fish were fed ground catfish chow at 1% body weight per day until 3 days prior to experimentation.

Acute (24 h), static exposures of fathead minnows to nitrite were conducted in aerated, reconstituted hard water at 19 $\pm$ 1°C ($\bar{X} \pm s$) in 30 l all glass aquaria (U.S. EPA, 1975). Palachek and Tomasso (1984) found that 24 h nitrite lethality to fathead minnows is highly variable and inversely related to weight: 24-h LC_{50}'s ranged from 46 mg NO_2-N/l for 1.1 g fish to over 300 mg/l for 0.3 g fatheads. Minnows used in this study weighed 0.60 $\pm$ 0.15 g ($\bar{X} \pm s$), and nominal doses of 0, 9, 18, and 27 mg NO_2-N/l were employed. All exposures were replicated, with 15 to 20 fish per replicate. Actual nitrite concentrations of water samples collected at the beginning and end of 24 h exposures were measured (to $\pm$ 0.1 mg NO_2-N/l) by the azo-dye colorimetric technique (A.P.H.A., 1975).

Routine respiration rates were determined from consumption of oxygen by individual fish in 300 ml BOD bottles closed for 90 min (1.5 h) at a temperature of 19.2 $\pm$ 0.2°C ($\bar{X} \pm s$) (Anderson et al., 1980). In 90 min the typical test fish depleted dissolved oxygen in a bottle to an easily measurable but unstressful level. BOD bottles were filled with water from the fish's exposure aquarium. Dissolved oxygen determinations were made to $\pm$ 0.1 mg O_2/l with a Yellow Springs Instruments dissolved oxygen meter

equipped with a stirring probe. The D.O. meter was calibrated by comparison to the azide modification of Winkler's iodometric method for dissolved oxygen measurement (A.P.H.A., 1975). Respirometry of fish in bottles took place in a quiet, dimly lit setting. Bottles without fish were treated similarly to quantify meter drift and/or microbial oxygen consumption. Weight of each fish was measured after respirometry to the nearest 0.01 g.

Weight-specific routine respiration rates were calculated by dividing oxygen consumption of each fish by its weight and incubation time in hours (1.5 hours in all cases). Because oxygen concentration of "fishless" bottles decreased an average of 0.05 mg O_2/l $\pm$ s of 0.05), this amount was subtracted from the measured oxygen consumption of each fish.

Because deviations from normality were observed in distribution of respiration rates of fish from two of the exposure groups (Shapiro and Wilk's test, $p<0.01$), Kruskal-Wallis test (chi-square approximation) and Dunnett's test were employed to compare mean weight specific oxygen consumption rates of minnows from the four exposure groups (Zar, 1974). Statistical Analysis System procedures conducted all tests (S.A.S., 1982).

Results

Some fathead minnows died both during exposure to nitrite and during subsequent respirometry at all nominal doses (Table IX), and some were obviously stressed upon commencement or completion of respirometry. Observed symptoms considered indicators of stress included darkened coloration, rapid operculation rate, and loss of equilibrium. No control fish displayed such behavior, and only two minnows from the lowest nitrite dose appeared stressed. Cursory observation of minnows during respirometry revealed minimal activity in most cases, and severly stressed fish were somewhat less active. Overall, dissolved oxygen in BOD bottles containing fish dropped from 8.6 ± 0.1 mg O_2/l to 7.7 ± 0.4 ($\bar{X} \pm s$). The lowest terminal D.O. measurement observed was 6.6 mg O_2/l.

Mean weights of minnows from the various nominal dose groups were not identical, nor were they significantly different (ANOVA: $F = 1.26$, $p>0.29$). Kruskal-Wallis test found differences between mean weight specific oxygen consumption of fathead minnows from the various groups to be highly significant ($X^2 = 22.41$, $p<0.001$). Mean routine respiration rates of minnows from the two highest NO_2-N exposures were significantly lower than controls (Dunnett's test, $\alpha = 0.05$; Table X).

TABLE IX

ABSOLUTE AND RELATIVE MORTALITY OF FATHEAD MINNOWS DURING 24 h EXPOSURE TO VARIOUS CONCENTRATIONS OF NITRITE AND DURING SUBSEQUENT RESPIROMETRY. RELATIVE VALUES APPEAR AS PERCENTAGES IN PARENTHESES.

mg NO_2-N/l $\bar{X}$ $\pm$ s	Number Exposed	Mortality	Number Utilized In Respirometry	Mortality
0.0 0.0	30	0 (0.0)	30	0 (0.0)
9.3 0.1	31	1 (3.2)	30	3 (10.0)
18.3 0.1	35	4 (11.4)	29	3 (10.3)
27.7 0.3	35	6 (17.1)	28	6 (21.4)

TABLE X

MEAN WEIGHT SPECIFIC OXYGEN CONSUMPTION RATES OF FATHEAD MINNOWS FROM VARIOUS 24 h NITRITE-N EXPOSURES. STANDARD DEVIATIONS APPEAR IN PARENTHESES.

mg NO_2-N/l	n	wt. (g)	$\dot{M}O_2$ ($mgO_2 \cdot g^{-1} \cdot h^{-1}$)	Dunnett's Grouping
0.0 (0.0)	30	0.64 (0.16)	0.32 (0.08)	A
9.3 (0.1)	27	0.60 (0.12)	0.31 (0.05)	A
18.3 (0.1)	26	0.57 (0.15)	0.24 (0.06)	B
27.7 (0.3)	22	0.59 (0.16)	0.25 (0.06)	B

Discussion

Fathead minnow mortality was 3% at the lowest nominal exposure, yet a preliminary exposure of P. promelas to a nitrite-N concentration approximately nine times greater (82.2 mg NO_2-N/l) under similar conditions yielded only 31% mortality. Palachek and Tomasso (1984) reported a 24-h LC_{50} in excess of 150 mg NO_2-N/l for minnows of the size class used in this study. Twenty four h exposures up to 27 mg NO_2-N/l, therefore, were considered sublethal to fathead minnows. Nevertheless, the slight but nonsignificant increase in mean respiration rate at the highest nominal nitrite dose may be due to selection by the experimental procedure for minnows capable of surviving relatively high nitrite concentrations. Although routine respiration rate determinations detected a sublethal effect of nitrite on P. promelas, such determinations are usually insensitive indicators of changes in metabolic rate induced by chemicals because activity is unregulated. Effects of chemicals on metabolic rate of organisms should be evaluated with standard or active weight specific oxygen consumption when possible (Anderson et al., 1980).

The effect of any chemical on oxygen consumption of an organism may occur at the site of oxygen uptake, in the circulatory system, or in the internal tissues. Zinc is known to decrease oxygen uptake of fish by disruption of the gill epithelia, and nickel and cadmium operate in a

similar fashion (Skidmore, 1970; Sellers et al., 1974; Hughes, 1981; Thurberg and Dawson, 1974). Organic compounds, particularly naphthalene, are often metabolized within an organism, and so increase energetic costs and oxygen consumption (Lee et al., 1972; Thomas and Rice, 1975).

Fathead minnows are extremely tolerant of nitrite exposure: their LC_{50}'s are the highest yet reported for any aquatic organism (Huey, 1982; Palachek and Tomasso, 1984). The reduction in weight-specific oxygen consumption of fathead minnows exposed to 18.3 and 27.7 mg NO_2-N/l represents saturation of whatever mechanism(s) P. promelas possess to prevent or ameliorate nitrite intoxication. Although chronic sublethal nitrite exposure has resulted in minor gill damage in steelhead trout (Salmo gairdneri), and several poorly understood modes of nitrite toxicity exist in vertebrates, the acute toxic effects of nitrite on respiration rate are probably founded in the circulatory system, where nitrite renders hemoglobin useless as an oxygen-carrying pigment by oxidizing it to methemoglobin (Wedemeyer and Yasutake, 1978; Margiocco et al., 1983; Bodansky, 1951).

Most vertebrates, including many freshwater fish, possess enzymatic systems to reduce methemoglobin back to hemoglobin (Freeman et al., 1983). Although methemoglobin-reductase activity has not been studied in P. promelas, a

highly active reductase system and/or other biochemical mechanisms to ameliorate primary and secondary toxic effects of nitrite could explain their relative resistance to its toxicity. Alternatively, fathead minnows may not take up nitrite as readily as other more susceptible species, or may depurate NO_2^- more rapidly. If fatheads possess MHb-reductase activity, the concentrations of nitrite-N used in this investigation may significantly decrease oxygen uptake capacity of their blood by oxidizing Hb to MHb faster than the enzyme system can compensate.

No reported correlations exist between habitat preference of various fish species and their relative susceptibility to nitrite toxicity. Size/toxicity relationships have been described in fathead minnows and rainbow trout, with smaller individuals being less sensitive to nitrite exposure. This study demonstrated that environmentally unrealistic exposure of fathead minnows to nitrite reduced their weight specific oxygen consumption by about 22%. Continued study of physiological aspects of nitrite toxicity in tolerant species may reveal valuable information regarding its toxic effects in more sensitive species.

CHAPTER VI

NITRITE TOXICITY, METHEMOGLOBIN FORMATION AND THERMAL TOLERANCE IN CHANNEL CATFISH (Ictalurus punctatus)

Introduction

Nitrite, an intermediate in the nitrogen cycle, may become toxic to aquatic organisms owing to either chemical or biological imbalances in the nitrification process (Wedemeyer and Yasutake, 1978). Such imbalances have been observed in intensive aquacultural systems, laboratory holding tanks, and natural systems receiving high BOD effluent or runoff (Tucker et al., 1979; Westin, 1974; Russo, et al., 1981; Sawyer, 1960).

Nitrite lethality varies within and between fish species: 96-h LC_{50} values range from 0.3 to about 70 mg/l NO_2-N (Huey, 1982; Freeman et al., 1983). While nitrite may have multiple modes of toxicity in fishes (Margiocco et al., 1983), the best understood mechanism is nitrite-induced methemoglobinemia. Nitrite oxidizes the iron moiety of the oxygen-carrying pigment hemoglobin from a ferrous (Fe^{+2}) to a ferric (Fe^{+3}) state; the resulting methemoglobin cannot bind oxygen (Bodansky, 1951). Methemoglobinemia may eventually result in tissue hypoxia and death (Huey et al., 1980).

Acute nitrite toxicity in fish is inversely related to pH, chloride, and hardness levels (Patrick et al., 1979; Tomasso et al., 1979; Wedemeyer and Yasutake, 1978; Russo et al., 1981; Schwedler and Tucker, 1983). Other environmental variables, such as dissolved oxygen and temperature, may influence the toxicity of nitrite; similarly, nitrite may affect the ability of fish to tolerate limiting levels of various abiotic factors. The objectives of this study were to quantify the effect of acute nitrite exposure on the thermal tolerance of channel catfish, and to investigate relationships between changes in tolerance and methemoglobinemia.

Materials and Methods

Channel catfish fingerlings, Ictalurus punctatus, (wet wt. 10.7 $\pm$ 2.0g, $\bar{X} \pm s$) were obtained from a local fish hatchery and acclimated to 20^{o}C in a "living stream" (Frigid Units, Inc.) containing hard water reconstituted from distilled water (U.S. EPA, 1975). Since chloride inhibits nitrite-induced methemoglobin formation in catfish (Tomasso et al., 1979), 3 g/l Cl^- (as NaCl) was added to the holding water. The catfish were fed trout chow daily until 4 days before testing. Photoperiod was maintained at 16L:8D throughout holding and testing.

Nitrite exposures were acute (24h), static exposures in aerated, E.P.A. reconstituted hard water at 20 $\pm$ 0.5^{o}C.

Nominal chloride levels were 4.8 mg/l Cl^-, and nominal test concentrations of nitrite-N were 0.00, 0.30, 0.60, 1.00, and 1.40 mg/l. Actual nitrite concentrations were measured at the beginning and end of each exposure by the azo-dye method (A.P.H.A., 1980). Percent recovery of nitrite from spiked samples ranged from 97 to 102%. Eleven or 12 fish per trial were exposed to nitrite in 30-l glass aquaria. All selected nominal nitrite concentrations were duplicated except 0.30 mg/l.

Critical thermal maxima (Cowles and Bogert, 1944) were determined to evaluate the thermal tolerance of channel catfish. Procedural recommendations of Becker and Genoway (1979) were followed, including a 0.3°C/min rate of temperature increase and use of final loss of equilibrium as the endpoint criterion.

Ten fish from a nitrite exposure were tested simultaneously in a 60-l aquarium (base = 30 cm x 75 cm) containing 47 l of E.P.A. hard water. The water was aerated prior to and during CTM trials, and was changed after each trial. Dissolved oxygen was measured to the nearest 0.1 mg/l before and after each run with a Yellow Springs Instruments dissolved oxygen meter. A total of 9 trials (i.e., 90 fish) were conducted. Fish were segregated in two adjacent rows of five 10-cm x 10-cm plastic mesh enclosures. Temperature was raised at 0.3°C/min by two Haake E-52 circulating thermoregulators, and monitored to the

nearest 0.01°C with a Digitec HT-5810 digital thermometer. Two observers corroborated the endpoint of each fish.

After a fish reached its critical thermal maximum (CTM), a sample of its blood was collected from the caudal peduncle and subjected to hemoglobin and methemoglobin assays (Hainline, 1958; and Evelyn and Malloy, 1938, respectively). Percent methemoglobin (%MHb) was calculated as

$$\frac{\text{g Methemoglobin}}{\text{g total Hemoglobin}} \cdot 100\%.$$

Shapiro and Wilk's W statistic (α=0.01) tested for normal distributions of CTM, %MHb, and arcsine-transformed %MHb values within exposure groups, and Barlett's test for homoscedasticity (α=0.001) was utilized to detect dissimilar variances (Zar, 1974).

Although some of the groups exhibited non-normal distributions, arcsine transformation did not normalize deviate %MHb groups; untransformed values were therefore retained. The few observed deviations from normality were not considered threatening to the robustness of least squares regression or parametric analysis of variance (ANOVA).

Least squares regression analyses were performed to identify possible linear relationships between (1) percent methemoglobin and nitrite exposure; (2) CTM and nitrite exposure; and (3) CTM and %MHb. Analyses of variance

(ANOVA) and Duncan's multiple range tests were conducted to test for differences among exposure groups. Statistical Analysis System procedures were utilized for all tests (S.A.S., 1982).

Results

Highly significant relationships were found between nitrite exposure concentration, percent methemoglobin in blood, and upper thermal tolerance of channel catfish. The percentage of total hemoglobin converted to methemoglobin increased with increased ambient nitrite (Figure 3). The best fit linear equation was %MHb = 46.78 (mg/l NO_2-N) + 5.77 ($p<0.001$, $r^2 = 0.73$, $n = 82$). ANOVA confirmed significant differences in %MHb levels among our exposure groups ($F = 37.19$, $p<0.001$), and multiple range testing revealed four statistically distinct groupings of %MHb means (=0.05) with a single overlap at the lowest nitrite exposure concentration and segregation of means from the two highest exposures (Table XI).

Increased acute nitrite exposure was found to significantly decrease thermal tolerance as evaluated by CTM methodology (Figure 4). Least squares regression generated the following relationship: CTM = -1.44 (mg/l NO_2-N) + 38.12 ($p<0.001$, $r^2 = 0.51$, $n = 89$). ANOVA substantiated the above relationship ($F = 17.02$, $p<0.001$), and multiple range testing delineated four distinct groups, isolating

TABLE XI

RESULTS OF DUNCAN'S MULTIPLE RANGE TESTING OF PERCENT MHb AND CTM MEANS AFTER EXPOSURES TO THE VARIOUS NITRITE CONCENTRATIONS

NO_2-N (mg/l) $\bar{X}$	$\pm$ s	Methemoglobin % (n)	$\bar{X}$	$\pm$ s	Duncan's Grouping		Critical Thermal Maximum (^{o}C) (n)	$\bar{X}$	$\pm$ s	Duncan's Grouping	
0.00	0.01	(20)	9.0	14.5	A		(20)	38.02	0.39	A	
0.34	0.01	(10)	20.5	6.8	A	B	(10)	37.69	0.25	A	B
0.59	0.02	(20)	29.6	10.7		B	(20)	37.26	0.57	C	B
1.01	0.02	(7)	55.7	22.6	C		(10)	37.12	0.45	C	B
1.04	0.03	(10)	49.8	19.5	C		(10)	36.79	0.47	C	
1.41	0.09	(5)	72.7	12.9	D		(9)	35.91	0.93	D	
1.37	0.08	(10)	75.0	11.4	D		(10)	35.85	1.48	D	

Fig. 3--Percent methemoglobin of channel catfish from various 24 h nitrite exposures. Means, ± one standard deviation, and sample size (parenthesis) are given for each group.

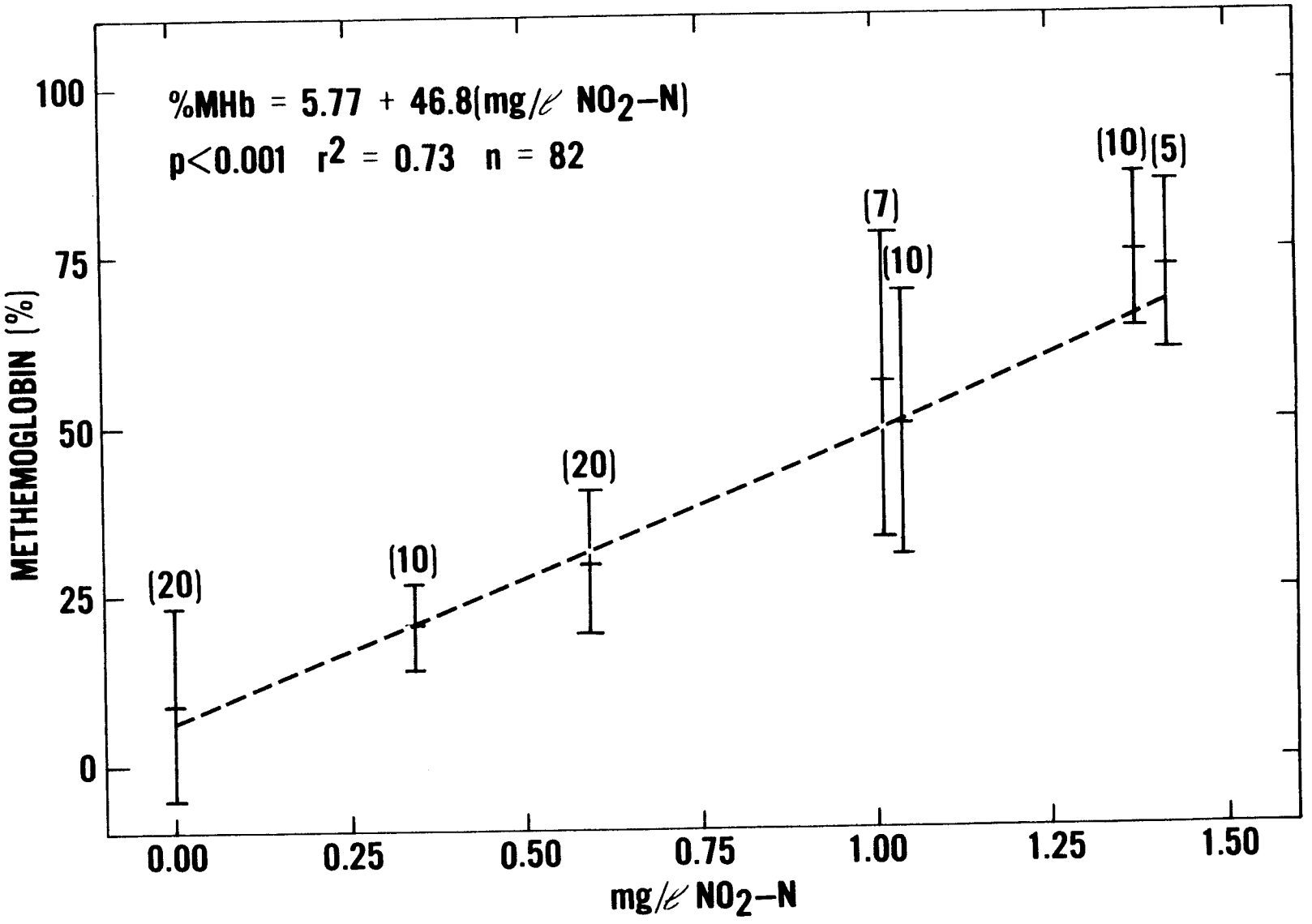
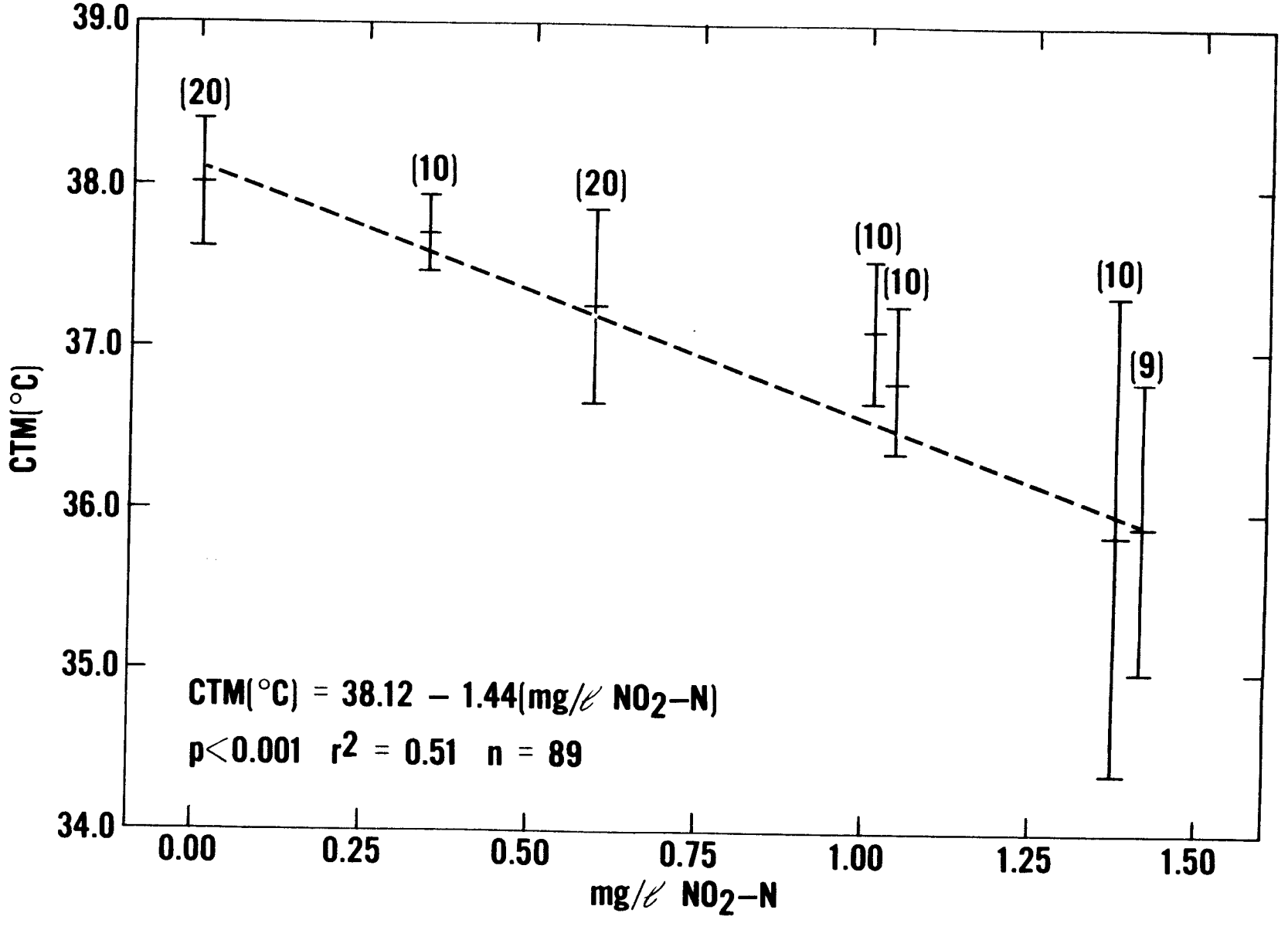

%MHb = 5.77 + 46.8(mg/ℓ NO2–N)
p<0.001 r2 = 0.73 n = 82
METHEMOGLOBIN (%)
100
75
50
25
0
0.00
0.25
0.50
0.75
1.00
1.25
1.50
mg/ℓ NO2–N
(20)
(10)
(20)
(7)
(10)
(10)
(5)

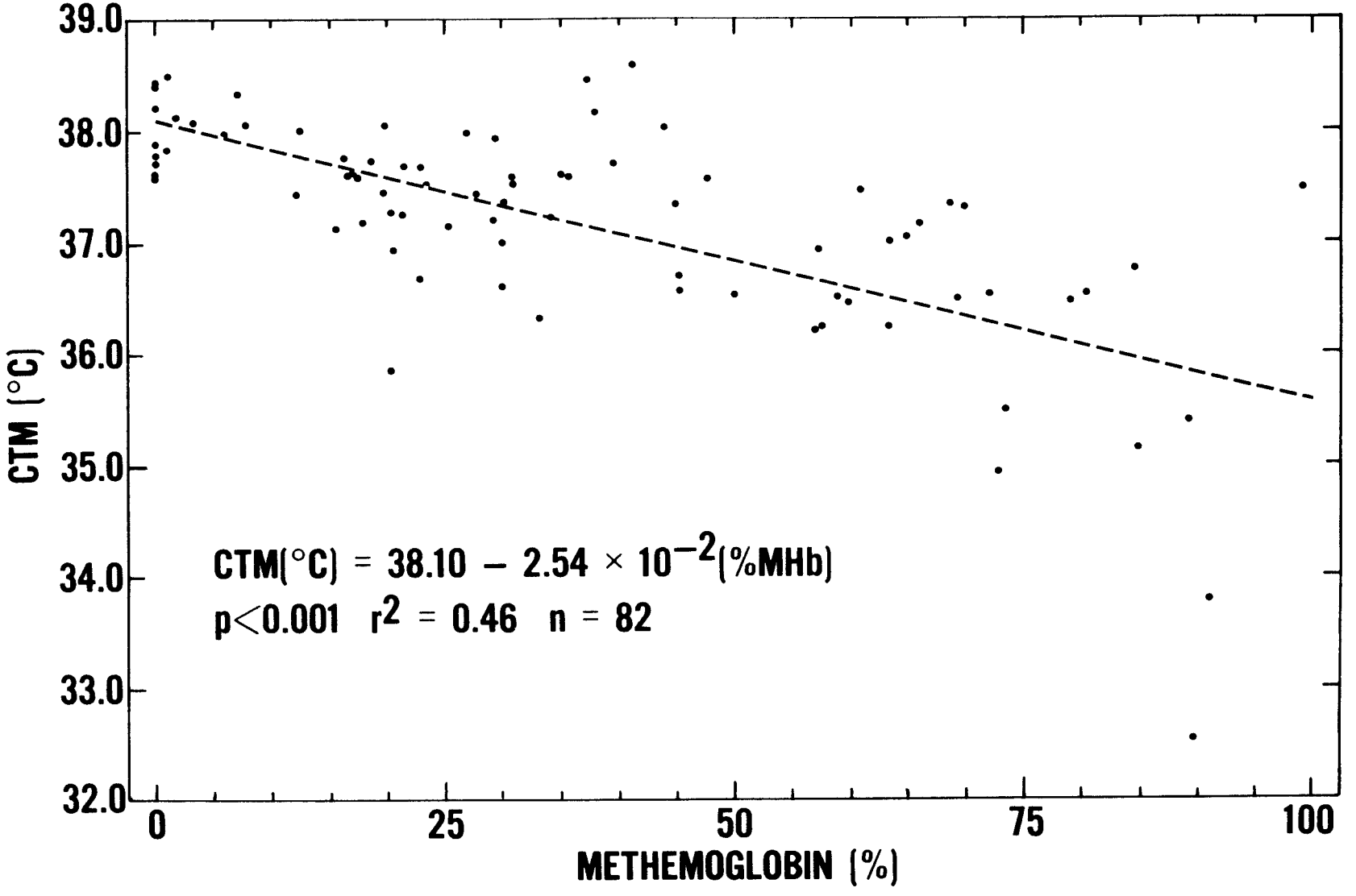
Fig. 4--Critical thermal maxima of channel catfish exposed to various nitrite concentrations for 24 h. Means, ± one standard deviation, and sample size (parenthesis) are given for each group.


39.0
38.0
37.0
36.0
35.0
34.0
CTM(°C)
(20)
(10)
(20)
(10)
(10)
(10)
(9)
CTM(°C) = 38.12 – 1.44(mg/ℓ NO2–N)
p<0.001 r2 = 0.51 n = 89
0.00
0.25
0.50
0.75
1.00
1.25
1.50
mg/ℓ NO2–N

CTM means from the two highest exposure groups (Table XI). Both CTM variability and the negative effect of nitrite on thermal tolerance increase markedly at the highest nitrite exposures. In general, fish from the higher nitrite exposures showed increased locomotor activity earlier in the CTM determination, but endpoint behavior was similar to that reported by Cheetham et al. (1976) for channel catfish and was consistent among fish and exposure groups, including the controls. Individual critical thermal maxima ranged from 32.54 to 38.64^{o}C. A single fish from the highest exposure (1.41 mg/l NO_2-N) displayed atypical endpoint behavior at about 27^{o}C and died immediately thereafter. This early endpoint and unusally rapid death were attributed to the toxic effects of nitrite and not thermal stress. Data from this fish were excluded from all analyses.

Least squares regression also revealed a significant inverse relationship between %MHb and CTM: CTM = -0.0254 (%MHb) + 38.10 ($p<0.001$, $r^2 = 0.46$, $n = 82$) (Figure 5). Dissolved oxygen decreased from 8.0 $\pm$ 0.3 to 5.8 $\pm$ 0.2 mg/l ($\bar{X} \pm s$) as temperature increased during CTM trials. Hemoglobin levels in test fish equalled 7.66 $\pm$ 1.30 g Hb/100 ml of blood ($\bar{X} \pm s$).

Fig. 5--Critical thermal maxima of channel catfish plotted against their percent methemoglobin.


39.0
38.0
37.0
36.0
35.0
34.0
33.0
32.0
CTM (°C)
0
25
50
75
100
METHEMOGLOBIN (%)
CTM(°C) = 38.10 – 2.54 × 10⁻²(%MHb)
p<0.001 r² = 0.46 n = 82

Discussion

The utility of the critical thermal maximum technique to delineate the effects of a chemical on the thermal tolerance of fish has been recognized by several researchers (Paladino et al., 1980; Becker and Wolford, 1980; Schubauer et al., 1980; Wedemeyer and McLeay, 1981; Takle et al., 1983). Perhaps the first investigation of this type was conducted by Silbergeld in 1973. Etheostoma nigrum pretreated with 2.3 ug/l of dieldrin had significantly higher mortality rates when heated at $1^{o}C/h$ than untreated fish. Paladino and Spotila (1978) determined that arsenic depresses the thermal tolerance of muskellunge fry (Esox masquinongy) with CTM methodology. Becker and Wolford (1980) reported similar results regarding the effect of nickel on CTM of rainbow trout (Salmo gairdneri). Not all chemicals affect the temperature tolerance of fish. Schubauer et al. (1980) reported that recommended concentrations of the antibiotic tetracycline hydrochloride did not change the CTM of Notropis cornutus. Also, an aquatic herbicide, aquathol-K, had no significant effect on CTM in Notropis lutrensis, even at a concentration many times its recommended use (Takle et al., 1983).

Critical thermal maximum quantified the effect of nitrite on thermal tolerance of channel catfish. The mean CTM in our control group was more than $2.0^{o}C$ higher than that of catfish in the highest nitrite exposure. The mean

CTM for controls, 38.02°C, is virtually identical to the 38.0°C mean CTM determined by Reutter and Herdendorf (1976) for channel catfish; however, both of these values are considerably higher than the 35.5°C CTM reported by Cheetham et al. (1976). Differences in rates of heating and/or fish size, as discussed by Becker and Genoway 1979), may have contributed to this variation.

My %MHb values are comparable to those reported for NO_2-exposed channel catfish (Tomasso et al., 1979; Huey et al., 1980; Schwedler and Tucker, 1983). Blood samples from seven of the test fish yielded %MHb values greater than 100%, and were thus excluded from analyses involving %MHb. Some variability in the %MHb data is attributable to procedural difficulties. During the time (several minutes) between collection of blood from the first few fish to reach their CTM and initiation of analysis of that blood, some separation of blood components inevitably occurred. Consequently, measures were undertaken to minimize this problem.

Although most research regarding the mode of nitrite toxicity has addressed its ability to oxidize hemoglobin to methemoglobin and thereby decrease the oxygen carrying capacity of blood, other toxic effects exist (Margiocco et al., 1983). Conversion of nitrite to carcinogenic nitrosamines in the low pH conditions of mammalian stomachs is well documented; the acute toxicological effects of nitrite

on aquatic vertebrates, however, are not carcinogenic in origin. Nitrite is known to adversely affect microsomes and membranes of some intracellular organelles (Inoue, 1978; Shertzer and Duthu, 1979; Mensi et al., 1982), and bioaccumulates in the livers and brains of rainbow trout (Margiocco et al., 1983). Methemoglobin formation, however, undoubtedly contributes to the overall toxicity of nitrite in fish, probably by inducing tissue hypoxia and thus limiting aerobic capacity (Huey et al., 1980). Differing primary modes of toxicity and/or differences in anaerobic tolerance may explain some of the intra- and interspecific variability observed in nitrite lethality determinations.

My results show that nitrite can compromise the ability of channel catfish to accommodate thermal stress in the laboratory, and indicate that this effect is correlated with NO_2-induced methemoglobinemia. Elevated temperature increases the metabolic rate of ectotherms, and thus their energetic requirements. Higher energetic demands may be supplied by aerobic or anaerobic metabolism. Methemoglobinemia decreases oxygen transport to tissues, and thus reduces their supply of oxygen. Vertebrate organ systems dependent on aerobic metabolism, such as the nervous system, are first affected by the decreased oxygen-carrying capacity of blood with high methemoglobin levels.

Prosser (1973) proposed that thermal stress first affects the central nervous system, perhaps by reducing neuronal membrane integrity, and noted that hypoxia may exponentiate that effect. Tissue hypoxia combined with heat loading could accelerate the onset of nervous system dysfunction, as manifested by the vestibular failure and spasmodic convulsions that characterize the CTM endpoint.

Blood is preferentially shunted to organs requiring oxygen, such as the brain, when vertebrates experience hypoxia (White, 1982b). This may explain the bioconcentration of nitrite in the brains of rainbow trout noted by Margiocco et al. (1983). If blood acts to transport nitrite to tissues, then the effective hypoxia resulting from nitrite exposure would cause preferential transport of nitrite to oxygen sensitive tissues and bioconcentration of nitrite in those tissues. Such bioconcentration could further exacerbate cytotoxic effects of nitrite on membranes and microsomes, and thus interfere with normal central nervous system function. In addition, the increased gill ventilation brought on by hypoxia and/or high temperature probably increases uptake of environmental nitrite (Gerald and Cech, 1970). Burggren and Cameron (1980) found that anaerobically produced lactate sequestered in tissues causes tissue acidosis in channel catfish exposed to environmental hypoxia. Tissue acidosis may influence nitrite toxicity in catfish.

Internal mechanisms exist for amelioration of nitrite toxicity. Tucker and Schwedler (1983) have demonstrated that channel catfish can partially acclimate to nitrite, perhaps via induction of the methemoglobin-reductase enzyme system identified in channel catfish by Huey and Beitinger (1982a). Huey et al. (1984) found that recovery of channel catfish from NO_2-induced methemoglobinemia occurred more rapidly at 30°C than at 10 or 20°C, suggesting that the optimum temperature for MHb-reductase approximates the channel catfish final temperature preferendum of 30°C (Cheetham et al., 1976).

Nevertheless, nitrite's toxicity to channel catfish in commercial culture ponds can influence productivity and economic returns by killing or stressing fish (Schwedler and Tucker, 1983). Tucker et al. (1979) reported temperatures up to 35°C in such ponds, and higher temperatures undoubtedly occur in the southern United States. Temperature and other abiotic factors may directly or indirectly influence the toxic effects of nitrite. Increased temperature reduces dissolved oxygen in any aquatic system and low oxygen levels are often associated with low environmental pH (Wetzel, 1975). Russo et al. (1981) and Huey and Beitinger (1982b) have determined that reduced pH increases nitrite toxicity to fish, and low dissolved oxygen may also. High feeding rates in culture ponds can both reduce dissolved oxygen and increase nitrite (Tucker et al.

1979; Hollerman and Boyd, 1980). Aeration can alleviate dissolved oxygen problems, yet it may also increase nitrite-N concentrations (Hollerman and Boyd, 1980). As total ammonia nitrogen concentrations may rise concurrent with nitrite, their joint toxicity may pose an additional hazard, although the toxicity of ammonia to fish is directly related to pH (Tomasso et al., 1980; Tucker et al., 1979; Hollerman and Boyd, 1980).

Major abiotic factors must be considered in assessing the risk of nitrite toxicity in intensive culture of fish. This laboratory investigation demonstrated that acute, sub-lethal exposure to nitrite can reduce the thermal tolerance of channel catfish by at least $2^{o}C$. The potential exists, therefore, for high environmental temperatures to stress or kill nitrite-intoxicated fish.

CHAPTER VII

HYPOXIC RESISTANCE OF NITRITE-EXPOSED CHANNEL CATFISH (*Ictalurus punctatus*)

Introduction

The toxic effects of dissolved nitrite on fish are interspecifically variable and not completely understood, yet nitrite seems to be particularly toxic to the commonly cultured salmonids and channel catfish (Smith and Williams, 1974; Margiocco et al., 1983; Huey et al., 1980). Among other less defined modes of toxicity, nitrite autocatalytically oxidizes hemoglobin to methemoglobin, a form incapable of binding oxygen (Rodkey, 1976). The blood of vertebrates afflicted with methemoglobinemia has reduced oxygen carrying capacity (Bodansky, 1951).

In addition to being the preeminent physiological limiting factor for fish in practically all aquatic systems (Fry, 1971), dissolved oxygen is of paramount importance to productivity and economic returns from intensive aquaculture of fish (Smart, 1981). Increased fish mortality and decreased overall productivity in southern channel catfish culture ponds have been related to oxygen depletion resulting from high stocking rates, feeding rates, and/or temperatures (Tucker et al., 1979; Lovell, 1979). Potentially

toxic substances such as ammonia, carbon dioxide, and nitrite may accumulate to stressful levels before, during, or after periods of oxygen depletion (Hollerman and Boyd, 1980). Elevated temperature simultaneously decreases dissolved oxygen in aquatic systems and increases biological oxygen demand. Other abiotic factors are known to interact additively or synergistically in their deleterious effects on fish: nitrite intoxication has been shown to reduce the thermal tolerance of channel catfish (Chapter VI). Nitrite's hematologic effects may decrease the ability of fish to survive low dissolved oxygen levels. This laboratory study explored that hypothesis in channel catfish.

Materials and Methods

Channel catfish fingerlings, Ictalurus punctatus, (wet wt. = 9.3 $\pm$ 2.1 g, $\bar{X} \pm s$) obtained from a local fish hatchery were acclimated to and held at 30 $\pm$ 0.5°C ($\bar{X} \pm s$) for a minimum of 4 weeks. Channel catfish were fed catfish chow daily until 2 days prior to experimentation. Two Haake E-52 circulating thermoregulators controlled temperature in a 450 l "living stream" (Frigid Units, Inc.) that continuously circulated holding water through charcoal filters. Aerated hard water reconstituted from deionized water was utilized for acclimation and holding (U.S. EPA, 1975). Since chloride has been shown to inhibit nitrite toxicity in channel catfish in addition to reducing disease, 5 g/l

Cl^- as NaCl was added (Tomasso et al., 1979). Nitrite-N levels never exceeded 0.1 mg NO_2-N/l during holding.

Acute (24 h) exposure of channel catfish to nitrite occurred in all glass aquaria containing 30 l of aerated, reconstituted hard water. Aquaria were partially immersed in a thermoregulated water bath to maintain temperature at 30 $\pm$ 0.1°C ($\bar{X} \pm s$). Nominal exposure concentrations of 0.0, 0.5, 1.0, and 1.5 mg NO_2-N/l were chosen to produce a graded yet sublethal increase in methemoglobin concentration of channel catfish blood (Huey et al., 1980). Nitrite was measured with the azo-dye method (A.P.H.A., 1975). Nominal nitrite concentrations were maintained throughout the 24 h exposure period by addition of reagent grade sodium nitrite or deionized water, as necessary. Five fish were used in each exposure, and all exposures were duplicated.

Hypoxic resistance times of channel catfish were determined in 30°C E.P.A. hard water deoxygenated by nitrogen gas stripping to 0.75 $\pm$ 1.0 mg O_2/l ($\bar{X} \pm s$). Removal of oxygen from reconstituted water was accomplished in a 170 l aquarium with a counter-current stripper similar to that described by Petrosky and Magnuson (1973). Actual exposure of catfish to hypoxic conditions occurred in a 60 l aquarium filled with 35 l of water siphoned from the 170 l stripping tank. Fish were segregated in five 10-cm x 10-cm plastic mesh enclosures during hypoxic resistance trials,

and were denied access to the water's surface by a 6.2 mm thick piece of plexiglas over their enclosures. The end-point criterion for a particular fish was cessation of opercular movements. At that point, a fish was removed from its enclosure, and blood collected from its severed caudal peduncle was analyzed for hemoglobin and methemoglobin concentrations (Hainline, 1958; Evelyn and Malloy 1938). Dissolved oxygen was measured before and after hypoxic resistance trials with the azide modification of Winkler's iodometric method (A.P.H.A., 1975).

Preliminary statistical analyses revealed that hypoxic resistance times of some groups of nitrite-exposed catfish were not normally distributed (Shapiro and Wilk's test, $\alpha=0.05$). Percent methemoglobin (% MHb) of groups, however, displayed normal distributions, and Bartlett's test indicated data were homoscedastic ($\alpha=0.001$). Relationships between nitrite exposure, hypoxic resistance time, and % MHb of channel catfish were described with simple linear regression, and the effects of nitrite on resistance time and % MHb were further ascertained by analysis of variance (ANOVA) and Duncan's multiple range test. The robustness of these parametric procedures tolerates moderate deviations from normality (Zar, 1974). Statistical Analysis System procedures conducted all tests (S.A.S., 1982).

Results

Nitrite exposure significantly increased percent methemoglobin of channel catfish according to the linear relationship seen in Figure 6. ANOVA confirmed the existence of highly significant differences among % MHb means ($F = 90.69$, $p<0.001$), and each % MHb mean was statistically distinct (Duncan's test, $\alpha=0.05$, Table XII).

After introduction to hypoxic water, catfish generally settled to the bottom and became motionless, except for a large unquantified increase in opercular rate and stroke volume over that noted prior to hypoxic exposure. A few fish exhibited short yet increasingly frequent bouts of activity during hypoxic exposure; more often, catfish remained largely immobile through most of the trial. Most fish progressed from immobility to loss of equilibrium followed by a steady decline in amplitude of opercular movements, occasional coughing, erratic locomotor activity, and finally cessation of operculation. Dissolved oxygen at the conclusion of trials averaged 0.65 ± 0.14 mg O_2/l ($\bar{X} \pm s$).

Exposure to nitrite resulted in a highly significant reduction in hypoxic resistance time (in minutes) of channel catfish, as described by the best-fit equation: time = 166 - 94 (mg NO_2-N/l); $p<0.001$, $r^2 = 0.42$, $n = 40$ (Figure 7). ANOVA substantiated this conclusion ($F = 9.75$, $p<0.001$), and mean hypoxic resistance times of fish from the two high exposures were distinguished from the control

TABLE XII

DUNCAN'S MULTIPLE RANGE TEST GROUPINGS OF MEAN PERCENT METHEMOGLOBIN AND HYPOXIC RESISTANCE TIME OF CHANNEL CATFISH FROM VARIOUS 24 h NITRITE EXPOSURES. STANDARD DEVIATIONS APPEAR IN PARENTHESES.

mg NO_2/-N/l	n	% MHb	Grouping	Time (min)	Grouping
0.0	10	13.0 (4.3)	A	173.3 (86.5)	A
0.5	10	43.6 (8.1)	B	117.6 (69.9)	A
1.0	10	65.3 (8.5)	C	52.3 (42.3)	B
1.5	10	78.5 (14.3)	D	37.8 (43.7)	B

Fig. 6--Percent methemoglobin of channel catfish from various 24 h nitrite exposures. Means, ± one standard deviation, and sample size (parenthesis) are given for each group.

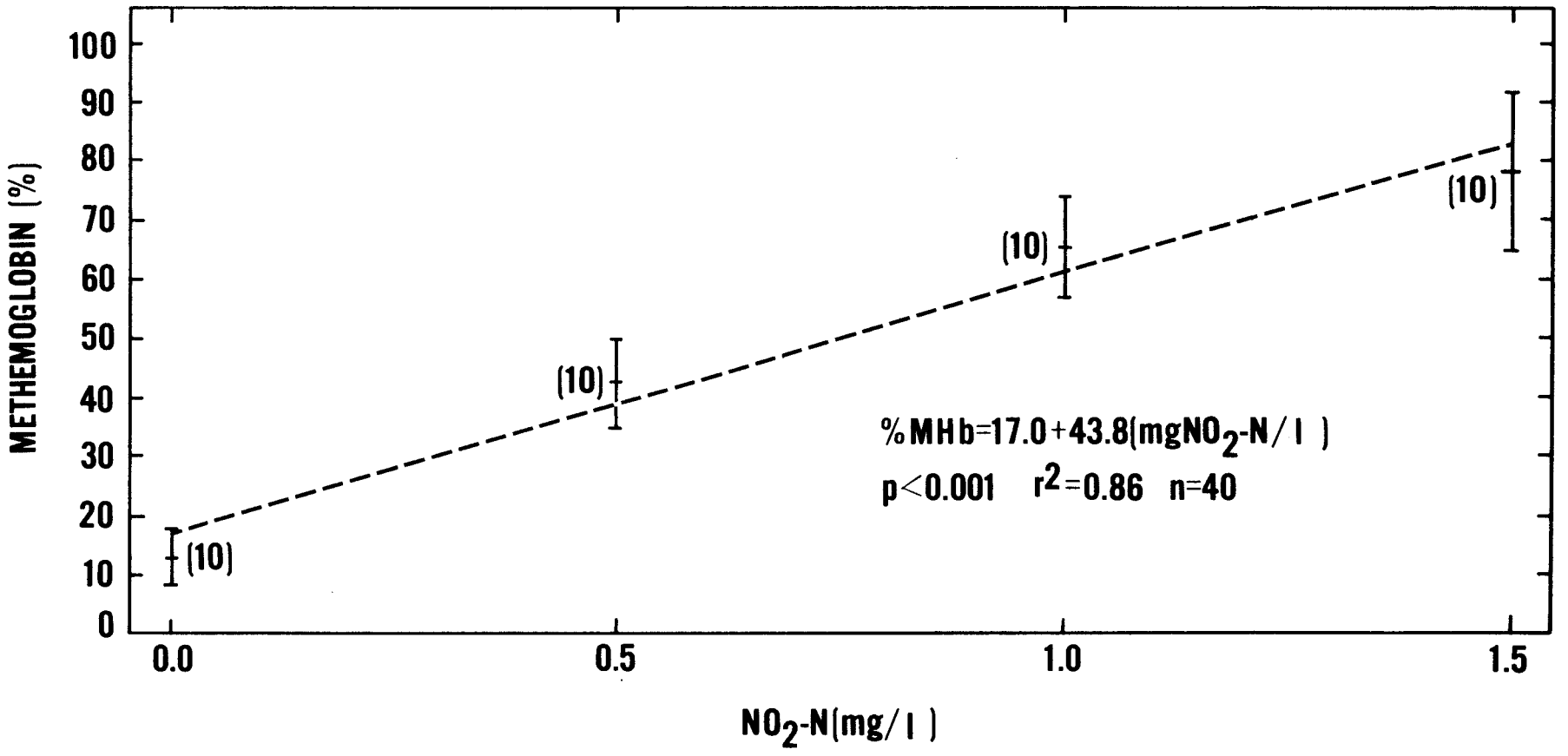
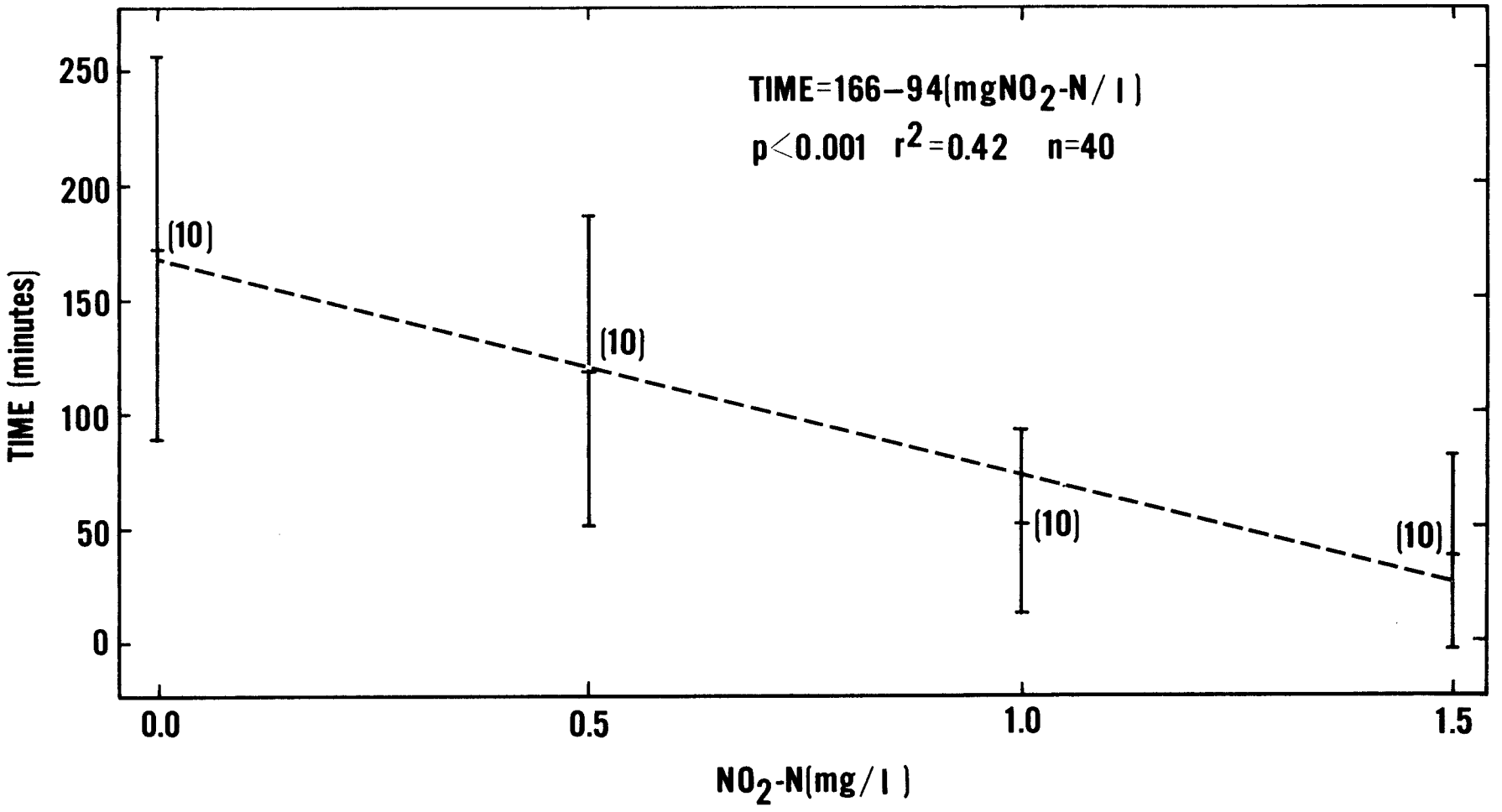

METHEMOGLOBIN (%)
100
90
80
70
60
50
40
30
20
10
0
0.0
0.5
1.0
1.5
NO2-N(mg/l)
(10)
(10)
(10)
(10)
%MHb=17.0+43.8(mgNO2-N/l)
p<0.001 r2=0.86 n=40

Fig. 7--Hypoxic resistance time of channel catfish from various 24 h nitrite exposures. Means, ± one standard deviation, and sample size (parenthesis) are given for each group.

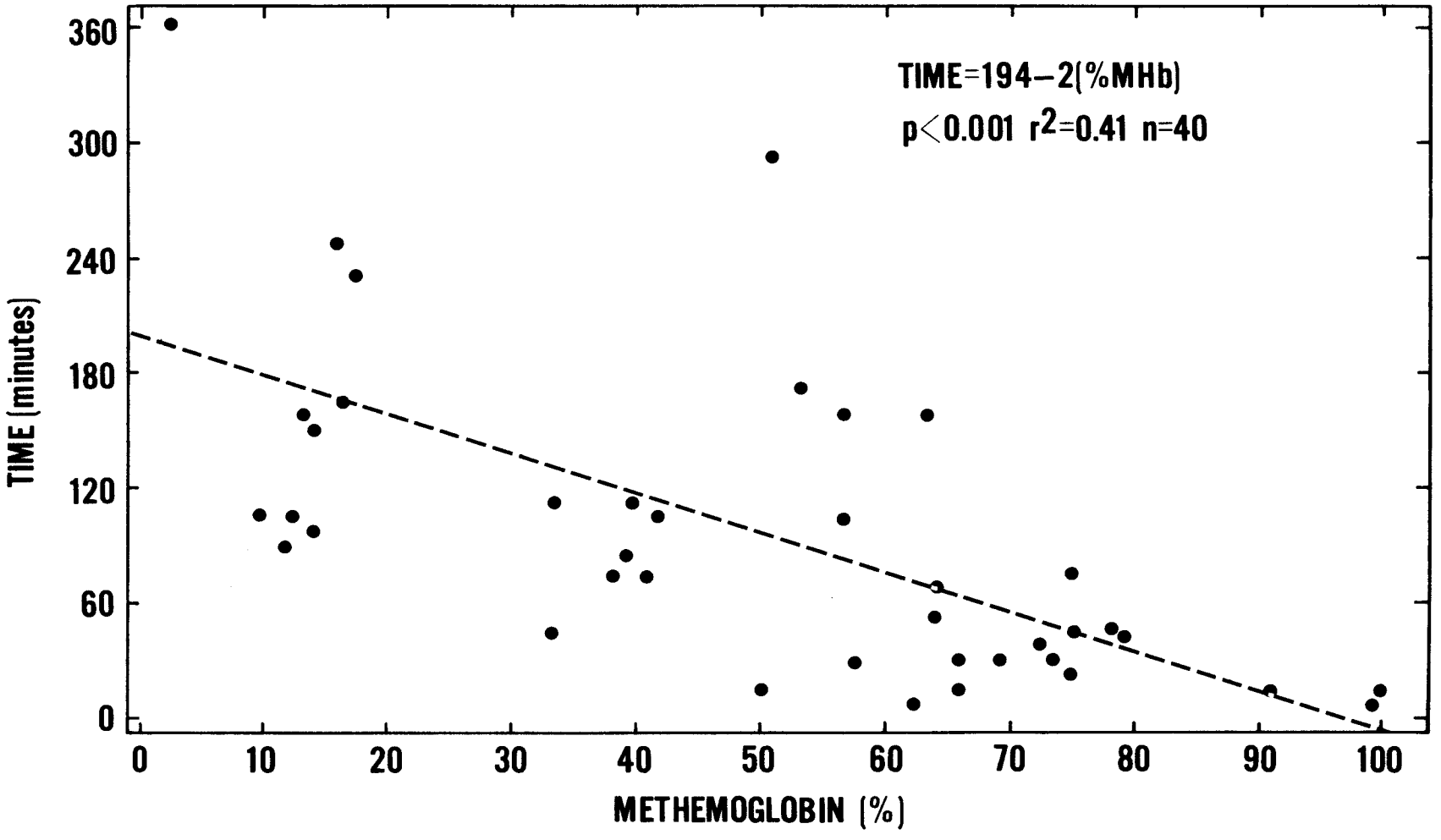

TIME=166−94(mgNO2-N/l)
p<0.001 r2=0.42 n=40
(10)
(10)
(10)
(10)
TIME (minutes)
250
200
150
100
50
0
0.0
0.5
1.0
1.5
NO2-N(mg/l)

and 0.5 mg NO_2-N/l group means by Duncan's test (Table XII). Hypoxic resistance times of the ten control fish ranged from 92 to 362 min, with a mean of 173 min. Each nominal dose group contained one exceptionally tolerant fish: a catfish from the second 0.5 mg NO_2-N/l trial survived 293 min in hypoxic water, and one fish from each of the higher exposures lasted 157 min. A fish from the first 1.5 mg NO_2-N/l exposure exhibited the shortest observed time to death of 9 min. Mean resistance times equalled 118, 52, and 38 min for channel catfish exposed to nitrite concentrations of 0.5, 1.0, and 1.5 mg NO_2-N/l, respectively (Table XII). Correlation of hypoxic resistance time with %MHb produced the relationship: time = 194 - 2 (% MHb); $p<0.001$, $r^2 = 0.41$, n=40 (Figure 8), which implies partial dependence of time to death on % MHb, and not the converse.

Discussion

Channel catfish behavior during hypoxic exposure in this study was similar to that observed by Caillouet (1968), and supports Scott and Roger's (1981) suggestion that channel catfish are "oxygen conformers" in that they physiologically and biochemically accomodate environmental hypoxia by devoting all available energy to maintenance of a minimal activity level, i.e., a "wait it out" approach. Channel catfish characteristically inhabit the depths of

Fig. 8--Hypoxic resistance time of channel catfish plotted against their percent methemoglobin.


TIME=194−2(%MHb)
p<0.001 r2=0.41 n=40
TIME (minutes)
360
300
240
180
120
60
0
0
10
20
30
40
50
60
70
80
90
100
METHEMOGLOBIN (%)

oxygenated rivers or lakes, and so only infrequently cope with hypoxic conditions in nature (Miller and Robison, 1973; Burggren and Cameron, 1980).

Previous laboratory exposures of channel catfish to acute hypoxia, however, have revealed several adaptive responses. Gerald and Cech (1970) noted a fourfold increase in gill ventilation, due primarily to increased opercular stroke volume, similar to that observed (but not quantified) in similar sized catfish during hypoxic exposures in this investigation. They proposed that gill perfusion also increased, but the percentage of oxygen extracted by the gills from hypoxic water decreased up to two-thirds below normoxic percent extraction. Burggren and Cameron (1980) found that larger channel catfish maintained oxygen uptake at control levels during moderate hypoxic exposure (3.5 mg O_2/l), yet partial utilization of anaerobically derived energy occurred in more severe hypoxia. The behavioral and physiological responses of channel catfish appear to facilitate endurance of hypoxia by maintenance of standard metabolism and hyperventilatory activity with both aerobic and anaerobically derived energy.

Although some researchers have proposed that non-glycolytic anaerobiosis occurs in certain freshwater fish, it has not been identified in catfish (Burton, 1971). The organic byproduct of anaerobic glycolysis, lactate, accumulates in the blood and tissues of channel catfish in

hypoxic water, eventually causing a general metabolic acidosis (Burggren and Cameron, 1980; Scott and Rogers, 1981). Caillouet (1968) suggested that acidification of the central nervous system induces loss of equilibrium in hypoxic catfish. Death from hypoxic exposure probably results from progressive deoxygenation and lactic acidification of critical tissues until metabolism becomes impossible.

Nitrite-induced methemoglobin formation has been previously observed in salmonids and channel catfish; the NO_2-methemoglobin dose-response relationship, seen in Figure 6, compares well with other reports (Tomasso et al., 1979; Huey et al., 1980). Methemoglobinemia decreases the oxygen carrying capacity of blood by making less hemoglobin available for oxygen transport and by shifting the oxygen dissociation curve to the left (Bodansky, 1951). The latter effect may be confounded in hypoxic catfish: decreased blood pH and elevated pCO_2 both shift the curve right (Bohr shift). Nevertheless, reduced delivery of oxygen in an organism will accelerate tissue depletion of oxygen and anaerobic production of lactate. The depression of hypoxic resistance of channel catfish exposed to nitrite (Figure 7), therefore, may at least partially be explained by the hematologic effects of nitrite.

The general variability observed in hypoxic resistance of both control and nitrite-dosed channel catfish may be due to intraspecific differences in aerobic and anaerobic

capacity. Scott and Rogers (1981) noted highly variable plasma lactate levels after 24 h 1.5 mg O_2/l exposure of I. puncatus, and Caillouet (1968) reported similar results for lethal exposures.

Dissolved oxygen is the primary limiting factor in aquaculture of channel catfish (Lovell, 1979). Maintenance of dissolved oxygen above 3 mg O_2/l allows maximal food conversion efficiency in catfish, yet high feeding rates, overstocking of culture ponds, high temperature and/or cloudy weather can lower dissolved oxygen to 1 mg O_2/l or less (Smart, 1981; Tucker et al., 1979; Romaire and Boyd, 1979). Nitrite-N in aquatic systems can increase before, during, or after periods of oxygen depletion (Sawyer, 1960). This research demonstrated that acute sublethal exposure of channel catfish to nitrite reduces their ability to survive subsequent low oxygen exposure, and raises the possibility that the toxic effects of nitrite on catfish could increase mortality or aggrevate stress associated with environmental hypoxic conditions.

CHAPTER VIII

SWIMMING PERFORMANCE OF CHANNEL CATFISH (*Ictalurus punctatus*) AFTER NITRITE INTOXICATION

Introduction

Nitrite may accumulate in natural aquatic or aquacultural systems when nitrification is disrupted (Sawyer, 1960). Such circumstances may produce concentrations of nitrite toxic to fish. Salmonids and channel catfish, two fish commonly cultured commercially, are especially susceptible to nitrite toxicity (Russo et al., 1974; Huey et al., 1980). Toxic effects of nitrite include oxidation of hemoglobin to methemoglobin, a form incapable of binding oxygen (Tomasso et al., 1979).

Several investigators have identified swimming performance as a potentially sensitive indicator of sublethal stress in fish (Sprague, 1971; Waldichuk, 1979; Wedemeyer and McLeay, 1981; Schneider and Connors, 1982). Farlinger and Beamish (1977) subdivided swimming performance of fish into three general categories: sustained swimming utilizes only aerobic metabolism and may continue indefinitely, prolonged swimming depends on both aerobic and anaerobic metabolism and cannot be sustained indefinitely, and burst swimming lasts seconds and thus uses largely anaerobic

metabolism. Tests of prolonged swimming are generally considered most useful in sublethal stress assessment, as they draw on both major biochemical energy sources (Brett, 1964). The purpose of this investigation was to determine if nitrite exposure affects the prolonged swimming performance of channel catfish, and to delineate the extent to which methemoglobinemia resulting from nitrite exposure correlates with performance.

Materials and Methods

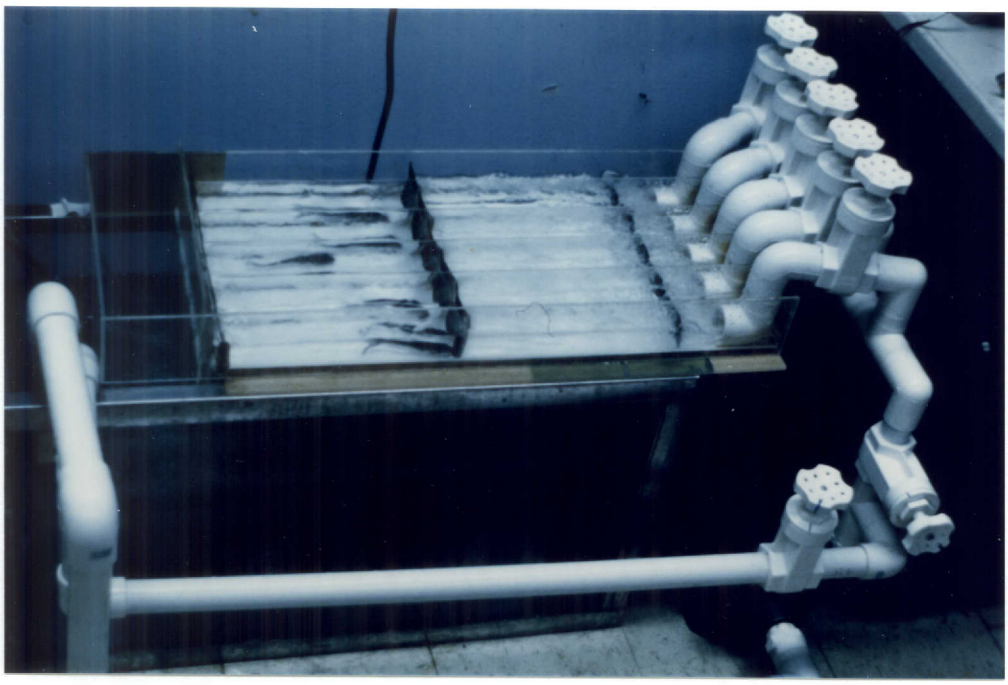
Channel catfish fingerlings (Ictalurus punctatus, wet wt. = 12.6 ± 2.6 g, S.L. = 105 ± 8 mm, $\bar{X}$ ± s) obtained from a local fish hatchery were acclimated to and held at 30 ± 0.5°C ($\bar{X}$ ± s) for a minimum of 4 weeks. Channel catfish were fed catfish chow daily until 2 days prior to experimentation. Two Haake E-52 circulating thermoregulators controlled temperature in a 450 l "living stream" (Frigid Units, Inc.) that continuously circulated holding water through charcoal filters. Aerated hard water reconstituted from deionized water was utilized for acclimation and holding (U.S. EPA, 1975). As chloride has been shown to inhibit nitrite toxicity in channel catfish in addition to reducing disease, 5 g/l Cl^-, as NaCl, was also added (Tomasso et al., 1979). Nitrite-N levels never exceeded 0.1 mg NO_2-N/l during holding.

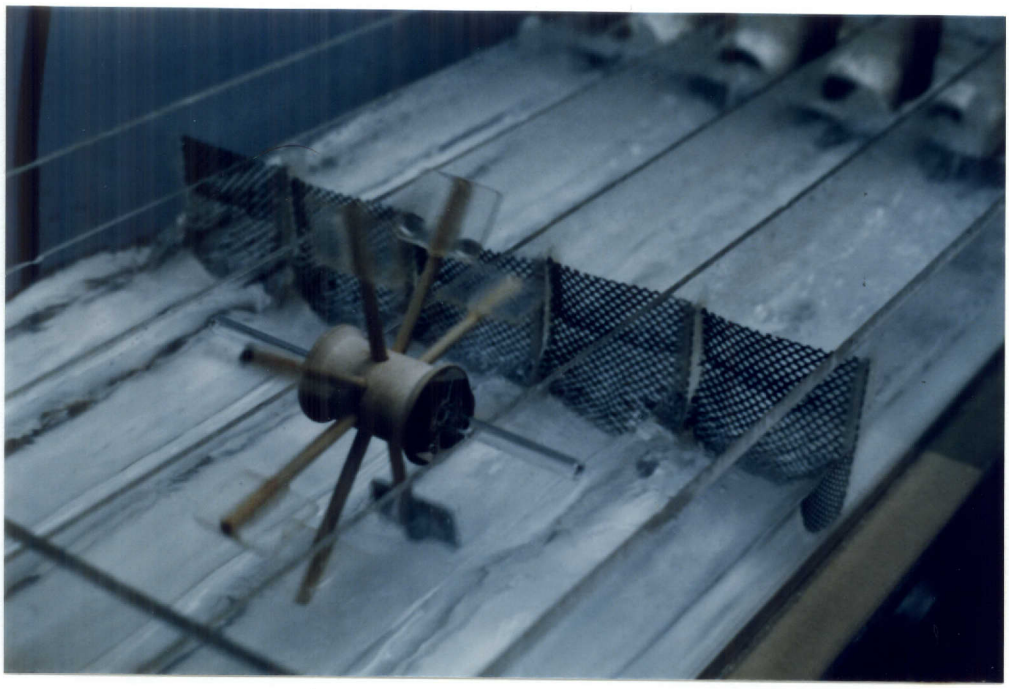
Acute (24 h) exposure of channel catfish to nitrite occurred in all glass aquaria containing 30 l of aerated, reconstituted, hard water. Aquaria were partially immersed in a thermoregulated water bath to maintain temperature at $30 \pm 0.1^{o}C$ ($\bar{X} \pm s$). Nominal exposure concentrations of 0.0, 0.5, 1.0, and 1.5 mg NO_2-N/l were chosen to produce a graded yet sublethal increase in methemoglobin concentration of channel catfish blood (Huey et al., 1980). Nitrite was measured with the azo-dye method (A.P.H.A., 1975). Nominal nitrite concentrations were maintained throughout the 24 h exposure period by addition of reagent grade sodium nitrite or deionized water, as necessary. Five fish with visibly similar standard lengths were withdrawn from holding for each exposure, and all exposures were duplicated.

Swimming performance of channel catfish was evaluated in a recirculating system designed to produce identical current flow through five adjacent channels (Figure 9). The channels, constructed of plexiglas, were 75 cm long, 6.5 cm wide, and had curved bottoms. Plastic mesh baffles moderated water flow in the channels. During swimming performance tests, individual fish were segregated in the final 30 cm section of each channel by a plastic mesh baffle at the front and an electrified grid at the rear. The grid was charged during swimming performance trials with 6V D.C. from a Grass S9 stimulator. A 1.5 horsepower 220V

Fig. 9--Apparatus utilized to evaluate the effect of nitrite exposure on channel catfish prolonged swimming performance.

Fig. 10--Paddlewheel employed to measure current speed in channels of the swimming performance apparatus.





A.C. centrifugal pump moved water from a 170 l reservoir tank to the swim channels, which drained back into the tank. Water flow into channels was controlled with P.V.C. valves in the plumbing at the head of each channel. A single plexiglas vane behind the channels controlled outflow from them. Water depth in channels was maintained at 6 cm.

Current flow rate was measured in cm/sec before and after swimming performance trials using a paddlewheel with a circumference of 50 cm (Figure 10). Revolutions per minute of the paddlewheel were counted during its placement in a particular channel, and current flow rate in that channel calculated as: cm/sec = rpm · 50/60.

Upon completion of their 24 h exposure to nitrite, five test fish were introduced one each to the swimming enclosures of the apparatus, through which water flowed at 12.5 $\pm$ 0.5 cm/sec ($\bar{X} \pm s$), or about 1.2 body lengths per second. Five minutes were then allowed for fish to become familiar with their channels and establish positive rheotaxis. During the next 5 min, current flow was increased at 1 min intervals to about 90% of the pump's maximal output, which produced a current flow rate of 37.3 $\pm$ 2.5 cm/sec, or approximately 3.5 body lengths per second, in each of the channels. When a fish no longer avoided the electrified grid at the rear of its channel, swim time since introduction was recorded and the fish was removed from its

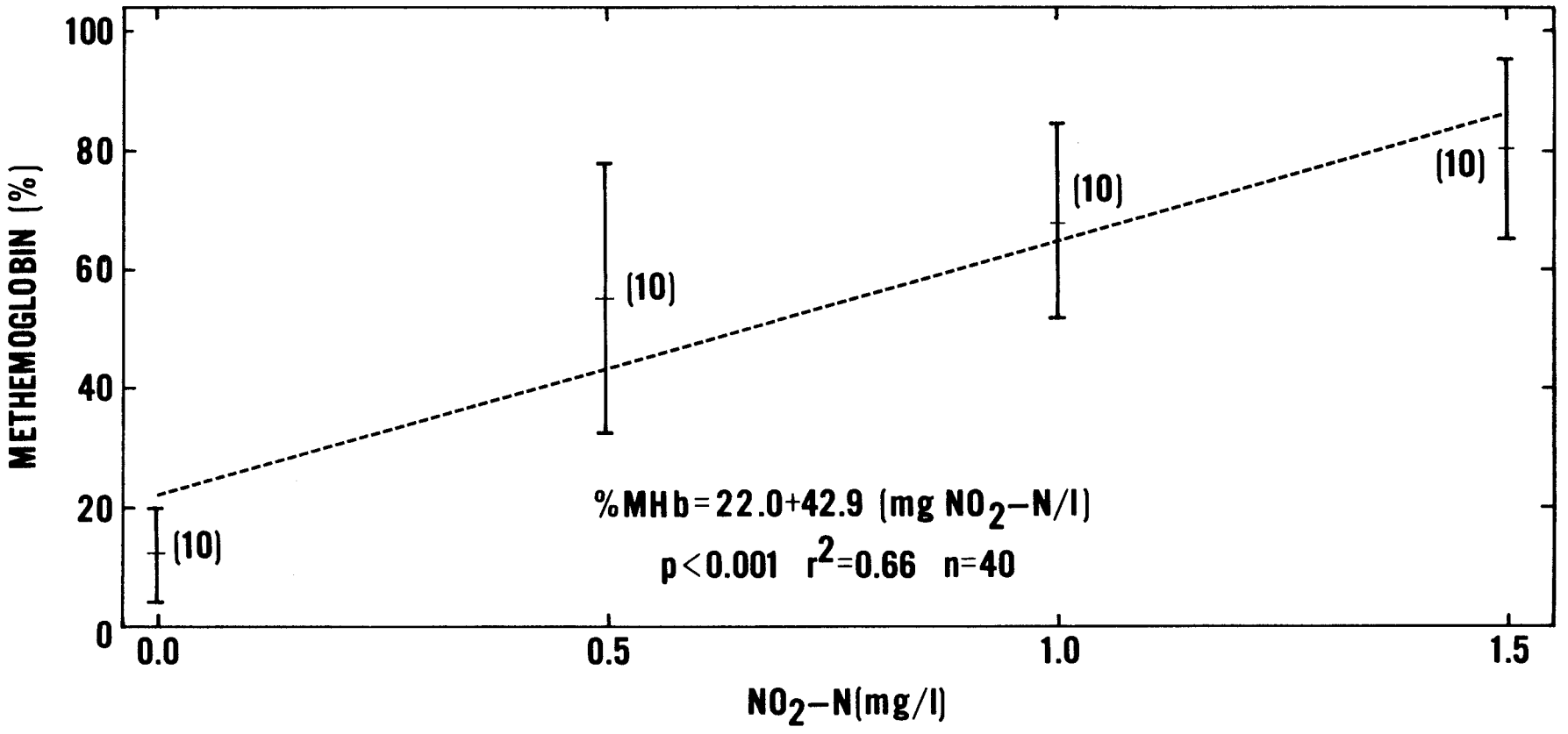
channel. Blood collected from the caudal peduncle was then analyzed for hemoglobin and methemoglobin with methods of Hainline (1958) and Evelyn and Malloy (1938), respectively. Dissolved oxygen ($\pm$ 0.05 mg O_2/l) and temperature ($\pm$ 1^{o}C) of water in the apparatus were measured before and after each trial with a Weston and Stack model 330 D.O. analyzer (Rexnord Instrument Co.).

The relationships between nitrite exposure, percent methemoglobin (% MHb), and swimming performance of channel catfish were described with least squares linear regression. Because Shapiro and Wilk's W statistic indicated data were normally distributed (α=0.01), and Bartlett's test found sufficient homoscedasticity for parametric testing (α=0.001), differences in mean swim time and % MHb of the various exposure groups were further substantiated with ANOVA and Duncan's multiple range test (Zar, 1974). All analyses were conducted with Statistical Analysis System procedures (S.A.S., 1982).

Results

Percent methemoglobin of nitrite-exposed channel catfish exhibited highly significant elevation over controls, as seen in the relationship modeled in Figure 11. ANOVA supported this conclusion ($F = 32.85$, $p<0.001$), and Duncan's multiple range test grouped mean % MHb of controls separately from all exposures, and distinguished between

Fig. 11--Percent methemoglobin of channel catfish from various 24 h nitrite exposures. Means, ± one standard deviation, and sample size (parenthesis) are given for each group.


METHEMOGLOBIN (%)
100
80
60
40
20
0
0.0
0.5
1.0
1.5
NO2–N(mg/l)
(10)
(10)
(10)
(10)
%MHb=22.0+42.9 (mg NO2–N/l)
p<0.001 r2=0.66 n=40

mean % MHb of catfish from the lowest and highest exposures (Table XIII).

Exposure of channel catfish to water-borne nitrite significantly reduced their prolonged swimming performance, as best described by the linear regression of the base ten logarithm of swim time (in minutes) on nitrite exposure concentration: Log_{10} (swim time) = 1.42 - 0.38 (mg NO_2-N/l); $p<0.001$, $r^2 = 0.42$, $n = 40$ (Figure 12). ANOVA of untransformed mean swim times indicated highly significant differences did exist ($F = 7.58$, $p<0.001$), yet only the control mean stood independent in Duncan's test (Table XIII).

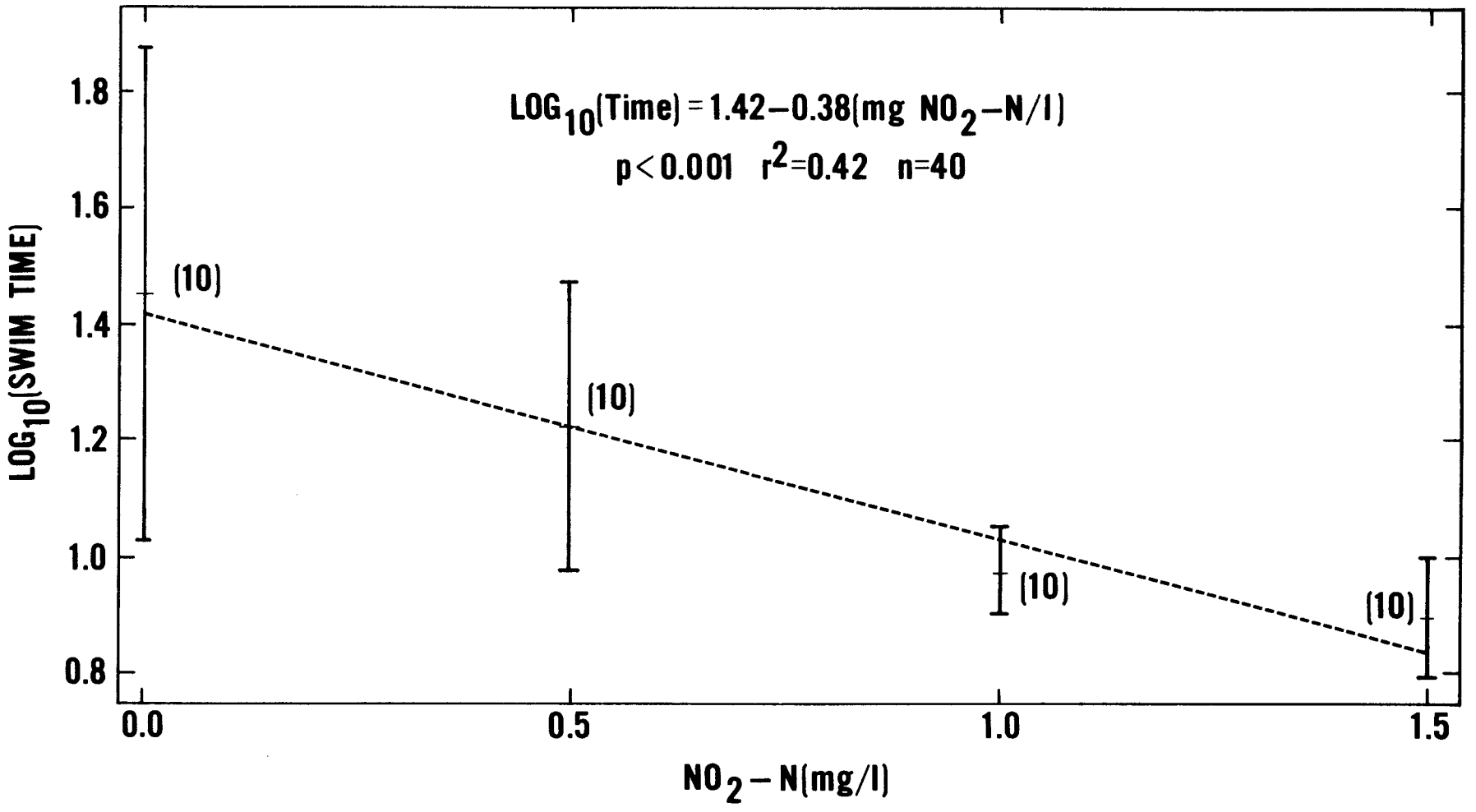
Fish usually encountered the electrified grid at the rear of their channels soon after introduction to the apparatus, and avoided it thereafter until they approached exhaustion. Tail beat frequency was generally constant, yet catfish occasionally used "burst and coast" swimming, especially as they neared the test endpoint. A single fish from the 1.5 mg NO_2-N/l exposure could not tolerate any increase in current flow rate above the initial 12.5 cm/sec., and no catfish from either of the highest exposures swam against the maximum flow rate (37.3 cm/sec) for more than 1 min (11 minutes post-introduction). Although four unexposed catfish exhibited a similar inability to perform against currents exceeding three body lengths/sec., four other control fish swam for more than 1 h after introduc-

TABLE XIII

DUNCAN'S MULTIPLE RANGE TEST GROUPINGS OF MEAN PERCENT METHEMOGLOBIN AND SWIM TIME OF CHANNEL CATFISH FROM VARIOUS 24 h NITRITE EXPOSURES. STANDARD DEVIATIONS APPEAR IN PARENTHESES.

mg NO_2-N/l	n	% MHb	Grouping	Time (min)	Grouping
0.0	10	13 (7)	A	40 (30)	A
0.5	10	54 (23)	B	20 (15)	B
1.0	10	69 (15)	B C	9 (1)	B
1.5	10	80 (15)	C	8 (2)	B

Fig. 12--The base ten logarithm of channel catfish swim time (in minutes) plotted against 24 h nitrite exposure. Means, ± standard deviation, and sample size (parenthesis) are given for each group.


$LOG_{10}(Time) = 1.42 - 0.38(mg\ NO_2 - N/l)$
$p < 0.001\ \ r^2 = 0.42\ \ n = 40$
$LOG_{10}(SWIM\ TIME)$
1.8
1.6
1.4
1.2
1.0
0.8
(10)
(10)
(10)
(10)
0.0
0.5
1.0
1.5
$NO_2 - N(mg/l)$

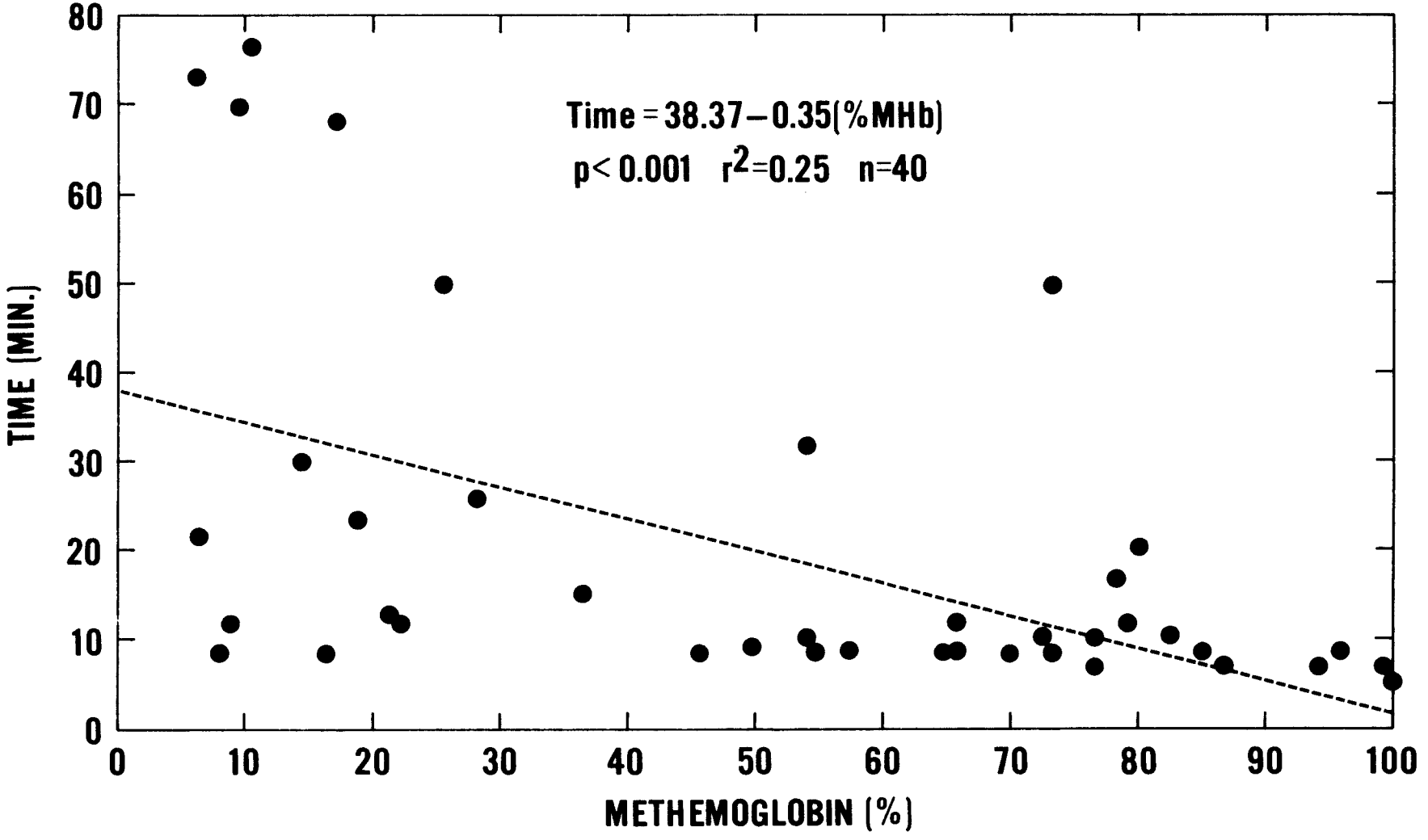
tion. The longest post-introduction time recorded was 77 min, and the control mean was 40 $\pm$ 30 min ($\bar{X} \pm s$). Channel catfish from the 0.5 mg NO_2-N/l exposure demonstrated performance intermediate between controls and higher exposures. No fish died prior to blood collection. Water temperature during swimming performance trials averaged 28 $\pm$ 1°C, and dissolved oxygen ranged from 7.5 to 7.9 mg O_2/l.

Catfish swimming performance was also inversely correlated with percent methemoglobin (Figure 13). Notable departures from the presented best-fit linear model include a fish from the low exposure which swam for 57 min with 76.8% MHb, and control fish that swam for rather short periods with characteristically low % MHb levels. No significant correlations were found between swim time and total hemoglobin, standard length, weight, condition factor, or swim channel (α=0.05).

Discussion

Most investigations of fish swimming performance have dealt with physiological responses to major abiotic factors during forced activity, yet many have also sought to quantify sublethal stress in fish exposed to toxic substances. Lemke and Mount (1963) found that concentrations of ABS detergent damaging to gills did not affect bluegill swimming ability. Macleod and Smith (1966), however, showed that sublethal levels of pulpwood fiber reduce fathead min-

Fig. 13--Prolonged swim time (minutes) of channel catfish plotted against their percent methemoglobin.


Time = 38.37 – 0.35(%MHb)
p< 0.001 r2=0.25 n=40
TIME (MIN.)
METHEMOGLOBIN (%)
0
10
20
30
40
50
60
70
80
90
100

now swimming performance, and critical swim speed of rainbow trout is decreased by prior exposure to copper or low pH (Waiwood and Beamish, 1978). Similarly, the insecticide fenitrothion reduced critical swim speed of brook trout (Peterson, 1974). Prolonged swimming performance of fish has been determined most often as critical swim speed, yet fatigue time at a set speed and various other methods have also been employed (Wedemeyer and McLeay, 1981). Critical swim speed involves incremental increases in current flow until fish become exhausted (Brett, 1964). Although standardization of methodology to evaluate fish swimming performance is desirable for comparative purposes, variations in fish size and hypotheses in question make a standardized testing format unlikely.

No research to date has quantified the swimming performance of I. punctatus. The prolonged swimming ability of unexposed channel catfish in this study was rather variable (C.V. = 75%), and was slightly lower than that recorded for other similar sized non-scombroid fish (Blake, 1983). This observation relates well to the relatively inactive existence of channel catfish in lentic depths (Miller and Robison, 1973). Although mean percent methemoglobin of control catfish was somewhat high in this investigation, the overall relationship between methemoglobin formation and acute nitrite exposure reported here is similar to past reports (Tomasso et al., 1979; Huey et al.,

1980; Huey et al., 1984).

The reduction in prolonged swimming performance of nitrite-exposed channel catfish is probably due in part to methemoglobin formation. Methemoglobin in blood reduces oxygen transport to tissues by making less hemoglobin available for oxygen transport and by shifting the oxygen dissociation curve to the left (Bodansky, 1951). The latter effect increases the affinity of the remaining hemoglobin for oxygen, thereby making it less able to unload oxygen to the tissues.

Channel catfish with larger percentages of their hemoglobin oxidized to methemoglobin by nitrite must rely more heavily on anaerobic glycolysis than aerobic metabolism to fuel locomotor activity. Glycolysis, in addition to being only a short term energy source for vertebrates, produces lactate as an end product. Muscle glycolysis still eventually requires oxygen to metabolize the toxic lactate after it is transported to the liver (Bartholomew, 1982). The limitations on aerobic capacity of channel catfish with nitrite-induced methemoglobinemia, therefore, should severely limit prolonged swimming performance. The statistically significant but low correlation coefficient of the model relating performance to % MHb, however, implies that other factors are also influencing catfish swimming performance (Figure 13). Intraspecific variation in aerobic and anaerobic capacity may be among those factors: both

Caillouet (1968) and Scott and Rogers (1981) have noted high variability of blood glucose and lactate of channel catfish after exposure to hypoxia.

In conclusion, prolonged swimming performance as measured by fatigue time proved to be a senstive yet imprecise indicator of sublethal nitrite toxicity in channel catfish. In spite of the variable performance of fingerlings swimming 3.5 standard body lengths per second, the potential for relatively low nitrite concentrations to reduce scope for activity of channel catfish is apparent.

CHAPTER IX

CONCLUSION

This series of seven investigations revealed that sublethal exposures of selenate-selenium and nitrite can influence some aspects of the physiology of the two freshwater fish species studied. The only statistically significant effect of acutely exposing fathead minnows to selenium was on upper thermal tolerance: mean critical thermal maximum (CTM) of fish exposed to 72% of their 24 h selenate-Se LC_{50} was more than $6^{o}C$ below the control mean, and fathead minnow CTM was inversely related to the square of their Se exposure concentration (Chapter III). Similar sublethal selenate exposures had no effect on routine weight specific oxygen uptake rates of fathead minnows, yet observation of minnows during closed-bottle respirometry indicated exposed fish may have been behaviorally and physiologically compensating for the deleterious hematologic effects of selenium. Dosed minnows may have maintained oxygen uptake rates similar to unexposed fish by decreasing general activity while increasing gill ventilation and perfusion (Chapter II). In evaluating fathead minnow reactions to selenate-Se concentration gradients, fish were found to respond randomly to both the gradient and the

apparatus used to test preference/avoidance behavior. It was concluded, therefore, that fathead minnows do not avoid selenium concentrations reported to be lethal (Chapter IV).

Weight specific oxygen consumption of fathead minnows decreased to 76% of control rates after acute sublethal exposure to nitrite; these data imply that a relatively high concentration of nitrite surpassed a threshold below which this nitrite-tolerant species can effectively allay its toxicity (Chapter V). In accordance with previous studies, 24 h exposures of channel catfish, a nitrite-sensitive species, produced a graded increase in the percentage of hemoglobin oxidized to methemoglobin, a form incapable of transporting oxygen in blood. Various post-exposure tests of physiological performance were indicative of nitrite-induced stress complicated by intraspecific variation in aerobic and anaerobic capacity: Channel catfish thermal tolerance (as CTM) was reduced more than $2^{o}C$ by nitrite exposure (Chapter VI), mean resistance time of dosed fish experiencing lethal hypoxia decreased to 22% of the control mean (Chapter VII), and a highly significant negative semilogarithmic relationship was observed between prolonged swimming performance and nitrite exposure concentration (Chapter VIII).

The sensitivity and precision of a sublethal bioassay technique will depend on the species-specific primary and secondary toxic effects of the substance in question as

well as the variation in expression of those effects between individuals of the species being dosed. In addition to questions regarding environmental significance, these factors should be given thorough consideration to appropriately assign bioassay methodology for specific species/chemical combinations to be investigated. Metabolic rate determinations can be informative indicators of physiological stress if activity is constant among control and exposed organisms. Combined determinations of a chemical's effects at standard and active levels of metabolism may reveal alterations in scope for activity. Routine oxygen uptake, however, is necessarily the least sensitive metabolic indicator of chemically induced stress because activity, and thus oxygen demand, can vary between individuals and exposure groups.

Critical thermal maximum may provide a sensitive measure of the effects of a chemical on thermal tolerance if: 1. The species being utilized responds to a steady acute increase in temperature with predictable, repeatable endpoint behaviors; 2. The behaviors themselves are not altered by the chemical; and 3. The variation in response of the species to thermal stress alone will not mask the effects of the chemical, if any, on thermal tolerance. Because channel catfish seem to have varying physiological performance characteristics, and because the intraspecific effects of nitrite can be variable, no sublethal evaluation

of nitrite's whole animal effects in I. punctatus will be extremely precise. Nevertheless, both hypoxic resistance and swimming performance decreased substantially after exposure of channel catfish to the lowest nominal nitrite concentration used in this research, and so are relatively sensitive indicators of sublethal effects.

Although evaluations of avoidance behavior differ from other tests discussed here in that avoidance behavior deals primarily with sensory perception of a chemical and not systemic dysfunction caused by it, such evaluations can also provide valuable information. Their sensitivity and environmental applicability, however, are clouded (relative to purely physiological bioassay) by directive and masking factors that may obscure avoidance behavior in nature.

Interesting and/or pertinent possibilities for future research include:

1. Comparison of selenium partitioning and speciation in tissues of fish from acute and chronic exposures.
2. Comparison of selenium partitioning and speciation in tissues of fish from dietary and water-borne exposures.
3. Effects of selenium content of living prey on prey selection by aquatic predators.
4. pH/selenium toxicity interactions in aquatic organisms.
5. Effects of selenium or nitrite on aerobic scope of fish.

6. Effects of selenium or nitrite on social dominance interactions.

7. NO_2^-/NH_3 toxicity interactions in aquatic organisms.

8. Investigate relationship between acclimation of channel catfish to nitrite and induction of methemoglobin reductase production.

9. Evaluate avoidance of nitrite by NO_2^--tolerant and sensitive fish species.

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