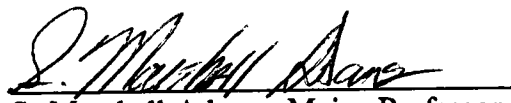


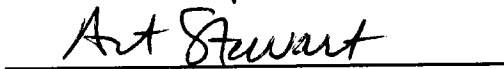
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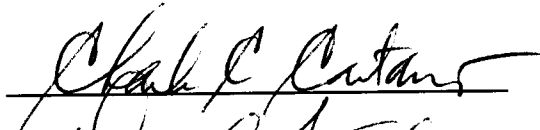
I am submitting herewith a dissertation written by Kenneth D. Ham entitled "Metabolic Responses to Hypoxic Challenge as Indicators of Sublethal Environmental Stress in Fish Populations." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Ecology.


S. Marshall Adams, Major Professor

We have read this dissertation
and recommend its acceptance:









Accepted for the Council


Associate Vice Chancellor
and Dean of The Graduate School

**METABOLIC RESPONSES TO HYPOXIC CHALLENGE AS
INDICATORS OF SUBLETHAL ENVIRONMENTAL STRESS IN
FISH POPULATIONS**

A Dissertation

Presented for the

DOCTOR OF PHILOSOPHY

DEGREE

THE UNIVERSITY OF TENNESSEE, KNOXVILLE

KENNETH D. HAM

May 1994

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ABSTRACT

The effects of different environmental histories on metabolic processes in sunfish were determined by comparing the responses of individuals during hypoxic challenge tests. Patterns of metabolic responses to hypoxic challenge detected the effects of prior exposure conditions on physiological processes in these fish. Prior exposure conditions altered the relationship of routine metabolic rate (RMR) and dissolved oxygen concentration (DO) during progressive hypoxia under identical conditions in an automated respirometer. Exposure of bluegill, *Lepomis macrochirus*, to treatment combinations of temperature and food availability in laboratory tanks for two weeks caused significant differences in multiple response parameters representing various aspects of the RMR:DO relationship. Exposure differences in pH (5 vs 7) failed to cause significant differences in response among groups. A multivariate response model improved the ability to detect and visualize even complex differences among groups. In a second experiment, the mean critical oxygen concentrations (COC) for redbreast sunfish, *Lepomis auritus*, collected from polluted field sites, were significantly lower than for individuals collected from a reference site. Differentiation of polluted and reference sites by COC was consistent with other indicators of fish and aquatic system health measured previously, but polluted sites were not ranked consistently among themselves by these indicators. In a third experiment, only the routine metabolic rate differed among groups of cold-acclimated bluegill (15°C) held at test conditions (25°C) in laboratory tanks for differing times prior to testing. Metabolic responses to hypoxia were able to reveal metabolic differences caused by prior exposure conditions in the laboratory or at field sites. Metabolic response parameters indicated significant differences consistent with the presence or absence of a stressor, industrial pollution, among field sites.

TABLE OF CONTENTS

CHAPTER	PAGE
PART 1: GENERAL INTRODUCTION AND OVERVIEW	
1. LITERATURE REVIEW	2
2. RESPONSES TO HYPOXIA AS STRESS INDICATORS	7
3. OBJECTIVES.....	9
LITERATURE CITED.....	11
PART 2: THE EFFECT OF ENVIRONMENTAL FACTORS ON METABOLIC RESPONSES OF FISH TO HYPOXIA	
1. INTRODUCTION	15
Stress and Metabolism.....	15
Hypoxic Challenge Tests.....	15
Testing at Standard Conditions	16
Objectives	17
2. MATERIALS AND METHODS	18
Experimental Subjects	18
Treatment Exposures	18
Respirometry	19
Apparatus.....	19
Respirometry Procedures.....	22
Analysis of Metabolic Responses.....	24
Statistical Procedures.....	28
3. RESULTS.....	31
Comparison of Group Responses	31
RMR:DO Curves	31
Regression Coefficients and COC.....	32
Additional Measures of Metabolism	35
Treatment Factor Effects on Weight	39
4. DISCUSSION.....	40

CHAPTER	PAGE
LITERATURE CITED	49

PART 3: FIELD TRIAL OF METABOLIC INDICATORS OF STRESS

1. INTRODUCTION	55
2. MATERIALS AND METHODS	57
Sampling Sites	57
Experimental Subjects	58
Collection and Holding	58
Respirometry	59
Analysis of Metabolic Responses	59
Indicators of Aquatic System Health	60
3. RESULTS	62
Comparison of Group Responses	62
RMR:DO Curves	62
Response Parameters	62
Indicators of Fish and Aquatic System Health	63
Comparison of COC with Other Parameters	65
4. DISCUSSION	66
LITERATURE CITED	69

PART 4: EFFECT OF HOLDING PERIODS ON METABOLIC RESPONSES TO HYPOXIA

1. INTRODUCTION	73
2. MATERIALS AND METHODS	75
Experimental Subjects	75
Experimental Design	75
Respirometry	76
Analysis of Metabolic Responses	77

CHAPTER	PAGE
3. RESULTS.....	78
Comparison of Group Responses	78
RMR:DO Curves	78
Response Parameters	78
Weight Loss	79
4. DISCUSSION.....	81
LITERATURE CITED.....	84

PART 5: SUMMARY

1. SUMMARY AND CONCLUSIONS.....	86
VITA.....	91

LIST OF TABLES

TABLE	PAGE
PART 2: THE EFFECT OF ENVIRONMENTAL FACTORS ON METABOLIC RESPONSES OF FISH TO HYPOXIA	
1. Mahalanobis distances and significance level for group comparisons based on the discriminant model including S_D , I_D , and COC.....	34
2. Pooled within-class correlations between canonical variables and parameters included in the first discriminant model.	35
3. Mahalanobis distances and significance level for group comparisons for the second discriminant model including I_I , and S_D , and MMS.	37
4. Pooled within-class correlations between canonical variables and response parameters for the second discriminant model.	38
PART 3: FIELD TRIAL OF METABOLIC INDICATORS OF STRESS	
5. Comparison of critical oxygen concentration with three other indicators of fish health at several levels of biological organization.....	64
PART 4: EFFECT OF HOLDING PERIODS ON METABOLIC RESPONSES TO HYPOXIA	
6. Comparison of effects of holding time on RMR_I and weight loss.	80

LIST OF FIGURES

FIGURE	PAGE
PART 1: GENERAL INTRODUCTION AND OVERVIEW	
1. Metabolic rate versus dissolved oxygen for several levels of activity.	4
PART 2: THE EFFECT OF ENVIRONMENTAL FACTORS ON METABOLIC RESPONSES OF FISH TO HYPOXIA	
2. Respirometer tank with respiratory medium, respirometer chamber and equipment for measurement and control of experiments.	21
3. Derivation of metabolic response parameters from the relationship of routine metabolic rate and dissolved oxygen concentration.....	27
4. Representative RMR:DO curves for treatment groups.....	32
5. Projections of group mean responses onto the first and second canonical axes for the discriminant model that included S_D , I_D , and COC.	33
6. Projections of group mean responses onto the first and second canonical axes for the discriminant model that included I_I , and S_D , and MMS.	36
PART 3: FIELD TRIAL OF METABOLIC INDICATORS OF STRESS	
7. Representative RMR:DO curves for site groups.	63
PART 4: EFFECT OF HOLDING PERIODS ON METABOLIC RESPONSES TO HYPOXIA	
8. Representative RMR:DO curves for groups held at test conditions for differing lengths of time prior to testing.....	79

PART 1

GENERAL INTRODUCTION AND OVERVIEW

Chapter 1

LITERATURE REVIEW

Fish are adapted to maintaining homeostasis in the face of changing environmental conditions in natural aquatic systems. Stress has been defined as “the effect of any environmental alteration or force that extends homeostatic processes beyond their normal limits, at any level of biological organization” such that the result will be either an enhancement or diminishment in the probability of mortality, natality, or permanent change (Esch and Hazen 1978). This definition of stress is broad enough to include biochemical or physiological responses to stressors, forces causing stress, as well as possible consequences at the organism, population, or ecosystem level. The purposes for studying stress in fish often include a desire to gauge the effects of human activities on the condition, abundance, or probability of persistence of natural or cultured fish populations (Pickering 1981, Wedemeyer et al. 1990). Human activities include everything from release of pollutants in natural aquatic systems to management of water quality in fish culture systems.

Stress can be studied by quantifying immediate biochemical or physiological responses to stressors, or by measuring long-term changes in an animal's performance capacity (Pickering 1981). Performance capacity, the ability to perform energy intensive activities, responds more slowly than biochemical responses, but it integrates many lower-level responses and may be a better indicator of the probability of impairment of growth, reproduction, survival, or the ability to tolerate additional stressors. Performance capacity provides a better indication of the biological, and perhaps

ecological, significance of long-term exposure to sublethal stressors.

The ability of fish to tolerate additional stressor inputs is difficult to estimate when either environmental factors or stressor inputs fluctuate over time. Even if past exposure conditions could be known with certainty, their combined effects on the ability to respond to additional stressors would be difficult to estimate. Performance tests can measure how the capacity of fish to perform various physiological functions has been altered by exposure history (Schreck 1990). Challenge tests, a type of performance test, are based on the presumptions that (1) levels of stress that exceed acclimation limits reduce performance capacity; (2) reduced performance capacity can be measured as reduced tolerance of subsequent stress; and (3) survival probability of the population as a whole will be reduced to the extent that reduced performance capacity impairs growth, survival or reproduction of individual fish (Wedemeyer et al. 1990).

Hypoxic challenge tests can be used to detect altered functional capacity of respiratory or metabolic systems or altered metabolic demand. The simplest type of hypoxic challenge test is the residual oxygen bioassay. This bioassay tests the ability of a fish exposed to a toxicant to tolerate decreasing oxygen concentrations (Carter 1962, Vigers and Maynard 1977, Carmichael et al. 1988). The residual concentration of dissolved oxygen at the time of death increases as toxicity increases. A lethal endpoint may be less sensitive than necessary for detection of sublethal stress (Heath 1987). To measure sublethal differences in metabolism, hypoxic challenge tests can be conducted within a respirometer for measurement of routine metabolic rates (RMR) over the range of dissolved oxygen concentrations (DO) included in the test. The RMR measured during a hypoxic challenge test can be plotted versus DO to construct a curve that demonstrates oxygen-independence at DO concentrations above the critical oxygen concentration (COC) and oxygen-dependence below the COC (Figure 1).

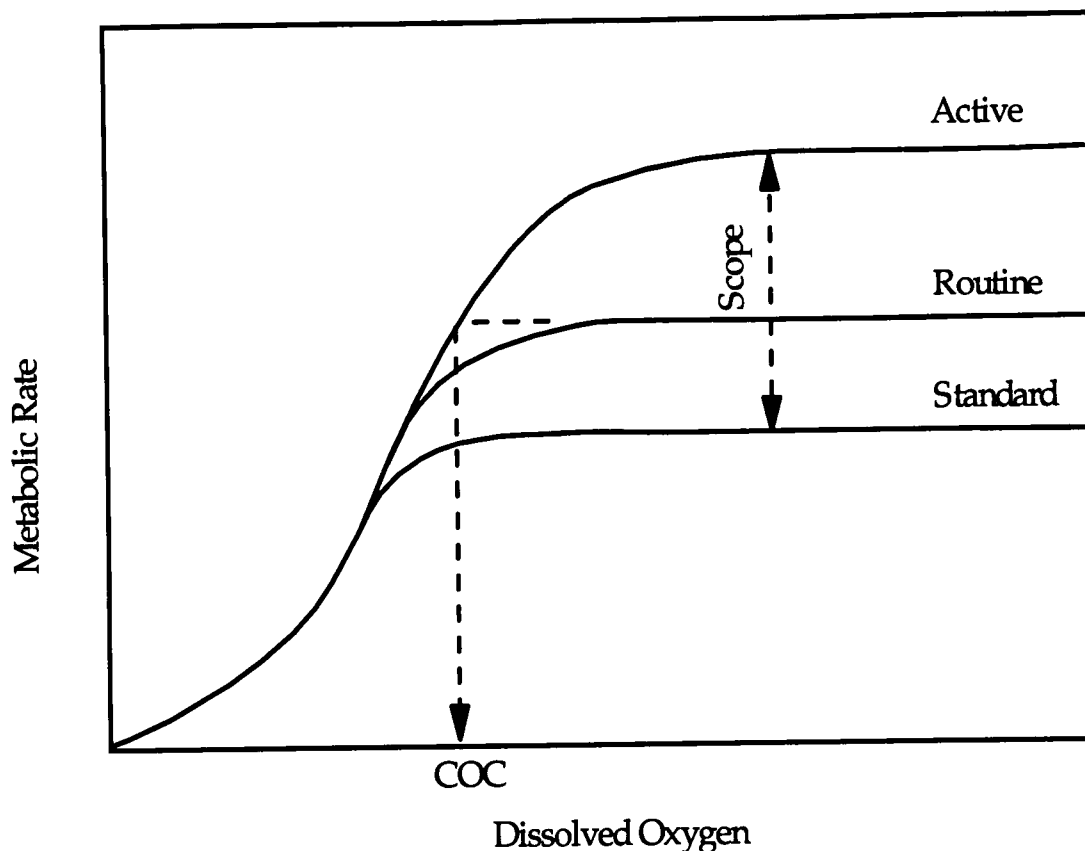


Figure 1. Metabolic rate versus dissolved oxygen for several levels of activity. Critical oxygen concentration (COC) is illustrated for routine metabolism. Scope is the difference between standard and active metabolism. This figure generalizes aspects of figures presented in Fry (1971) and Hughes (1981).

Active and standard levels of metabolism can be measured to estimate scope for activity (Fry 1947), but measurement of those levels of metabolism depends upon a high degree of control over activity. Routine metabolism is easily measured in simple respirometers because spontaneous activity is included (Cech 1990). It may be possible to derive surrogate estimates of metabolic scope from the results of routine respirometry (Neill and Bryan 1991). Because scope for activity (Fry 1947) is a measure of the limits of metabolism dictated by the interaction of energy supply and demand, it may be possible to derive an acceptable estimate of scope by examining how energy supply and

demand place limits on the homeostatic control of routine metabolism.

Most measures of environmental effects on routine metabolism include limited ranges of oxygen availability that fail to reveal how both supply and demand have been altered. Many studies have measured oxygen-independent routine metabolic rates during exposure to stressful environmental conditions or levels of toxicants to assess immediate effects of those conditions on the organism (O'Hara 1971, Kloth and Wohlschlag 1972, Almatar 1984, Felts and Heath 1984, Ye et al. 1991). A few studies have reported the effects of exposure to environmental stressors on critical oxygen concentrations (Ultsch et al. 1980, Springer and Neill 1988). The oxygen-dependent region of the curve, however, is usually measured only to allow estimation of COC, even though some investigators have demonstrated that this region of the curve can be defined rather precisely using flow-through respirometry (Ultsch et al. 1980, Berschick et al. 1987). To assess how both energy supply and demand have been altered by prior exposure conditions, this investigation measured the trend of RMR over a broad range of DO concentrations. At high DO, routine demand controls metabolism. At low DO, the ability to supply oxygen is insufficient to meet the routine demand and supply limits metabolism.

Comparison of RMR:DO curves can reveal much about how exposure conditions prior to the challenge test have altered metabolic performance, but it may not reveal which exposure factors were most influential. Metabolism is sensitive to many factors (Heath 1987), and differences revealed by hypoxic challenge must be interpreted in the context of exposure factors other than the stressors of interest. A study may focus on stressors that are related to a specific source, such as an industrial effluent, or it may focus on factors that are candidates for remediation. Other exposure factors that are not the object of study, such as temperature, may influence the measured results, and may

reduce the ability to gauge the influence of the stress factors under study.

The experiments in this study investigate the influence of environmental factors, stressors, and experimental techniques on the responses measured during hypoxic challenge. In order to use the metabolic responses to hypoxia as indicators of stress, metabolic differences must be demonstrated to be caused by the stressors of interest in the study in spite of the other influences on metabolism. These experiments should help determine whether detection of stressors is possible among field sites subject to normal environmental variation, or whether modifications of technique or interpretation will be necessary to achieve the detection of stressors.

Chapter 2

RESPONSES TO HYPOXIA AS STRESS INDICATORS

Because metabolism integrates energy use, metabolic differences could provide estimates of the probability that adverse effects will occur at higher levels of biological integration (Wedemeyer et al. 1990). Skadsen et al. (1980) suggested that metabolic responses could be related through energy budgets to more complex ecological responses. However, that study found that a single measure of effect based on swimming performance was inadequate to measure the complex metabolic responses to sublethal stress. To realize the integrative potential of metabolism as an indicator of sublethal stress, a more comprehensive assessment of metabolic changes caused by stressors was needed.

The present investigation compared response patterns measured during hypoxic challenge to reveal how prior exposure conditions altered metabolic performance. The RMR:DO curve formed in an individual hypoxic challenge test was represented as a multivariate model formed from the regression coefficients of line segments fitted to the oxygen-independent and oxygen-dependent portions of the curve and COC, the intersection point of the line segments. The COC indicates the point of failure of metabolic homeostasis and its value indicates the relative resistance to hypoxia (reviewed by Davis 1975). Additional measures of metabolic performance were compared among groups, but none were as powerful for differentiating among groups as the multivariate model that combined individual measures. The multivariate response model integrates the combined effects of energy supply and demand because it

represents the RMR:DO relationship for a broad range of oxygen concentrations.

Metabolic performance during the hypoxic challenge is a function of test conditions as well as prior exposure conditions. To investigate the effects of immediate test conditions on metabolism, laboratory studies usually attempt to minimize the effects of prior exposure conditions by careful attention to acclimation, nutrition, and health of fish prior to testing (Cech 1990). To investigate the influence of prior exposure conditions instead, a different approach was required. The effects of prior conditions can be measured by testing fish at standard conditions that are not stressful to the fish, other than the challenge factor (in this case, hypoxia). Metabolic differences may be the result of many exposure factors in addition to the stressors of interest. Differences in metabolic rate among groups being tested might be expected if the groups are not acclimated to a common temperature (Fry and Hart 1948, Kanungo and Prosser 1959, Job 1969a, Job 1969b, Jobling 1982, Moffit and Crawshaw 1983). The effects of environmental and other factors must be understood if any interpretation of metabolic differences among groups is to be made with reference to specific stressors.

The experiments conducted here investigate how environmental and experimental factors affect the ability to detect prior exposure to stressors. Metabolic response differences among groups indicate the influence of all exposure factors. In order to detect prior exposure to stressors, the effects of stressors must be differentiated, either qualitatively or quantitatively, from the effects caused by other factors. If the effects of stressors can be differentiated from the effects of other factors, it will be possible to use these responses as an indicator of prior exposure to stressors.

Chapter 3

OBJECTIVES

The principal objective of the experiments conducted in this study was to investigate whether hypoxic challenge of fish at standard conditions would reveal any alterations of metabolic state caused by prior exposure to stressful conditions. A secondary objective was to determine if effects of prior exposure to stressors could be detected among groups of fish collected from field sites, where environmental factors may also differ among sites. Many factors can affect metabolism, and an accurate interpretation of the effects of prior exposure conditions requires that responses to stressors be detectable among other potential sources of variation. The nature and relative strength of influence of other factors on metabolic responses to hypoxic challenge must be known if metabolic responses are to provide an accurate evaluation of prior exposure conditions for fish collected at field sites. This study investigated the effects of environmental and experimental factors as well as the effects of stressors on metabolic responses to hypoxia.

A first experiment was conducted to investigate the effects of temperature, food availability, and pH as environmental factors that might affect the responses to subsequent hypoxic challenge. The primary objective here was to evaluate the sensitivity of the RMR:DO curve measured during hypoxic challenge to exposure to these factors prior to testing. A secondary objective was to determine if differences in the RMR:DO curve could reveal complex differences caused by multiple exposure factor differences prior to testing. This experiment was used to evaluate the influence of

these environmental factors alone, and in combination, on fish metabolic responses during hypoxia.

A second experiment was conducted to compare metabolic differences among groups of fish from four field sites subject to different quantities and types of pollution. The primary objective here was to determine if metabolic responses to hypoxic challenge at standard conditions in the laboratory would reveal differences caused by prior exposure of some groups to pollution. Indicators of fish and aquatic system health measured in other studies were also compared among sites (Adams et al. 1994, Ryon and Schilling 1994, personal communication, S. M. Adams, Oak Ridge National Laboratory, and personal communication, C. F. Saylor, Tennessee Valley Authority). Metabolic response parameters were compared with indicators of health at a range of levels of biological organization. A secondary objective of this experiment was to determine if the influence of normal environmental variation among field sites was great enough to obscure metabolic differences caused by pollution.

A third experiment was conducted to investigate how holding periods in the laboratory prior to testing could affect the responses of fish to the hypoxic challenge test. The primary objective here was to determine if thermal acclimation, starvation, or other changes occurring during holding at test conditions would affect the metabolic responses. This experiment attempted to determine whether holding periods might change the responses to the hypoxic challenge tests and, if so, whether those changes would affect the ability to detect stressors or other important influences.

These three experiments are described in the following three parts (Parts 2-4). Each part is designed to stand alone as manuscripts for journal publication. The foregoing introductory information was intended to introduce the concepts needed to provide the reader with background information for the experiments as a group.

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PART 2

THE EFFECT OF ENVIRONMENTAL FACTORS ON METABOLIC RESPONSES OF FISH TO HYPOXIA

Chapter 1

INTRODUCTION

Stress and Metabolism

Stress has been defined by Esch and Hazen (1978) as "the effect of any environmental alteration or force that extends homeostatic processes beyond their normal limits, at any level of biological organization." A stressor is an environmental change that is severe enough to require a response on the part of the fish, population, or ecosystem (Wedemeyer and McLeay 1981). The ability to tolerate additional stressors can be altered by prior exposure to sublethal stressors (Adams 1990). Challenge tests can determine whether the ability to tolerate additional stressors has been altered by prior exposure to stressors or other environmental factors such as temperature, food availability, or pH.

Hypoxic Challenge Tests

Performance during a hypoxic challenge test can be altered by present and past exposure conditions. If test conditions are held constant, the differences measured during hypoxic challenge should reflect only the effects of prior exposure conditions. Challenge tests can help determine whether the effects of prior exposure conditions will increase the probability of adverse consequences, because reduced metabolic performance capacity increases the risk that lethal or sublethal tolerance limits will be exceeded (Priede 1985). The limitations of using metabolism to detect the effects of stressors must be realized and addressed (Heath 1987, Schreck 1990, Wedemeyer et al.

1990). The metabolic responses to hypoxic challenge may also be altered by prior exposure to different levels of environmental factors or by experimental techniques unrelated to the stressors important to the objectives of the study. In order to accurately interpret the influence of specific stressors, there must be some way of accounting for the influence of these other types of factors.

In order to use metabolic responses to hypoxia to evaluate the effects of stressors, it is necessary to establish how the RMR:DO curve is affected by background environmental factors such as temperature, food availability, and pH. These factors vary both temporally and spatially in aquatic systems and are known to affect metabolism (Fry and Hart 1948, Beamish 1964a, Beamish 1964b, Beamish and Mookherjee 1964, Beamish 1970, Alderdice 1976, Crawshaw 1977, Waiwood and Beamish 1978, Fromm 1980, Jobling 1981, Almatar 1984, Barton et al. 1985, Ye and Randall 1991, Ye et al. 1991). The responses to background environmental factors will have to be recognized as such to allow an accurate interpretation of the effects of the stressors of interest.

Testing at Standard Conditions

Standard test conditions control the influence of test conditions across all groups to reveal the influence of exposure conditions prior to testing. To detect the influence of prior exposure to stressors on metabolism, it will be necessary to recognize and account for the influence of other factors that differed among groups, but are not important to the objectives of the study. The rapid change to test conditions may exaggerate the influence of some environmental factors. Previous studies on effects of temperature, food availability, or pH on metabolism will provide some indication of how these factors may influence the metabolic responses to hypoxia measured at standard test conditions. Studies on the effects of rapid temperature change on metabolic rates of fish

revealed that cold-acclimated fish generally have a higher metabolic rate than warm-acclimated fish tested at an intermediate temperature. (Job 1969a, 1969b, Jobling 1982, Moffit and Crawshaw 1983). Metabolic rate increases following feeding (Jobling 1981), while starvation depresses routine metabolic rate (Wieser et al. 1992). Acidic water at pH 4.0 had no effect on the rate of oxygen consumption by resting rainbow trout, but swimming performance was reduced, indicating impairment at some level (Ye and Randall 1991, Ye et al. 1991).

Objectives

The primary objective of this laboratory experiment was to evaluate the sensitivity of the RMR:DO curve measured during hypoxic challenge to prior exposure to different levels of temperature, food availability, and pH in combination. A secondary objective was to determine if differences in the RMR:DO curve could reveal complex differences caused by multiple exposure factor differences prior to testing. If possible, a discriminant model would be constructed to differentiate among the unique patterns of response to these environmental factors. A sufficiently developed discriminant model could diagnose factors leading to response patterns even if prior exposure conditions were not known.

Chapter 2

MATERIALS AND METHODS

Experimental Subjects

Approximately 300 small bluegill (*Lepomis macrochirus* Rafinesque), from 50 to 90 mm TL, were obtained from the U. S. Fish and Wildlife Service, National Fish Hatchery, Mammoth Springs, Arkansas, on 29 January 1992. Prior to tests, these fish were held in indoor tanks supplied with flow-through dechlorinated tap water. A size-selected subset of these fish was transferred to a temperature-controlled holding tank at least one week before the first individual was transferred to a treatment tank. Conditions during this acclimation period were: 25°C, pH 7.0, and constant artificial light. Disturbance was minimized by use of visual screening of tanks and minimization of activity in the area. Temperature was maintained by immersion heaters connected to temperature controllers (YSI 71A) rated to within $\pm 0.5^\circ\text{C}$. The pH in the holding tank was maintained by the addition of potassium phosphate compounds to the water to raise or lower the pH. During the holding period, fish were fed flake food at approximately 2% of body weight per day on a dry food per wet weight basis (approximate routine caloric requirements).

Treatment Exposures

A three-way factorial design was employed with two treatment levels each for temperature, food availability, and pH before challenge with hypoxia. Temperature was either 15 or 30°C, pH was either 5 or 7, and food availability was either excess or none

(starvation). Two levels for each of three factors results in eight treatment combinations. Four fish were exposed to each treatment combination in 32 individual tests. Treatment combination replicates were randomly assigned to a challenge test date. Two weeks prior to the test date, an individual fish was chosen randomly from the holding tank, weighed and measured, then transferred to a treatment tank. Fish were exposed individually for two weeks in 11.6-liter tanks containing approximately 9 liters of water buffered to the appropriate pH and held at the assigned treatment temperature. On the test date, the individual fish was removed from the treatment tank, weighed and measured a second time, then placed in a respirometer chamber and tested. Experimental treatments began on 9 March 1992, and the final respirometer test was conducted on 8 May 1992.

Treatment tanks were prepared by scrubbing, sterilization with a solution of bleach, and a final rinse. Water of the appropriate pH was mixed and introduced to the tank and allowed to come to temperature in a bath of either 15 or 30°C. Fish ranged from 2.96 to 6.01 grams and from 63 to 76 mm TL before exposure to treatment conditions. Water in the exposure tank was changed twice per week, and fish in treatment groups receiving food were fed three times per week. Temperature and pH of each treatment tank were checked daily Monday through Friday and adjusted if necessary.

Respirometry

Apparatus

A static respirometer was used to measure RMR during progressive hypoxia. The respirometer was automated with data-acquisition and process-control capabilities to allow for unattended operation after initiation of a test. A microcomputer monitored

and controlled the respirometer during habituation and testing periods. The basic respirometry system used was patterned after the system of Springer and Neill (1988), but the final system incorporated many design and operational modifications. Swimming of fish in the test chambers was neither forced nor restricted, so the oxygen consumption rates recorded by this system approximated routine metabolism. Because sunfish are not active swimmers and habituate well to confinement, the metabolic rates recorded in this study may approach resting routine or even standard metabolism, but most properly may be referred to as routine (Cech 1990).

Fish were tested individually in one of two independently controlled respirometer chambers (Figure 2). Each chamber and its associated equipment were housed within a separate 37-L respirometry tank that contained the respiratory medium. The respiratory medium was made by buffering distilled water to pH 7 with potassium phosphate and calcium phosphate buffers, that also increased calcium hardness to approximately 25 mg/L (as CaCO_3). Each of these respirometry tanks was set in a constant-temperature bath maintained at 25°C by a quartz immersion heater attached to a YSI model 71A temperature controller rated to $\pm 0.5^\circ\text{C}$. The constant-temperature bath was electrically grounded to reduce inductive interference to the oxygen measurement system from the heater and temperature controller. The constant-temperature bath was aerated to prevent thermal stratification that otherwise might disrupt temperature control.

Each respirometer chamber consisted of a 335-ml transparent polystyrene jar attached to an air-driven magnetic stirrer. An oxygen probe was mounted through the rubber closure of each jar and positioned so that the membrane was near the stir-bar. A disk shaped stir-bar with top vanes spun within a circular compartment on the bottom of the chamber. This compartment prevented bumping of the stir bar by the fish. Two

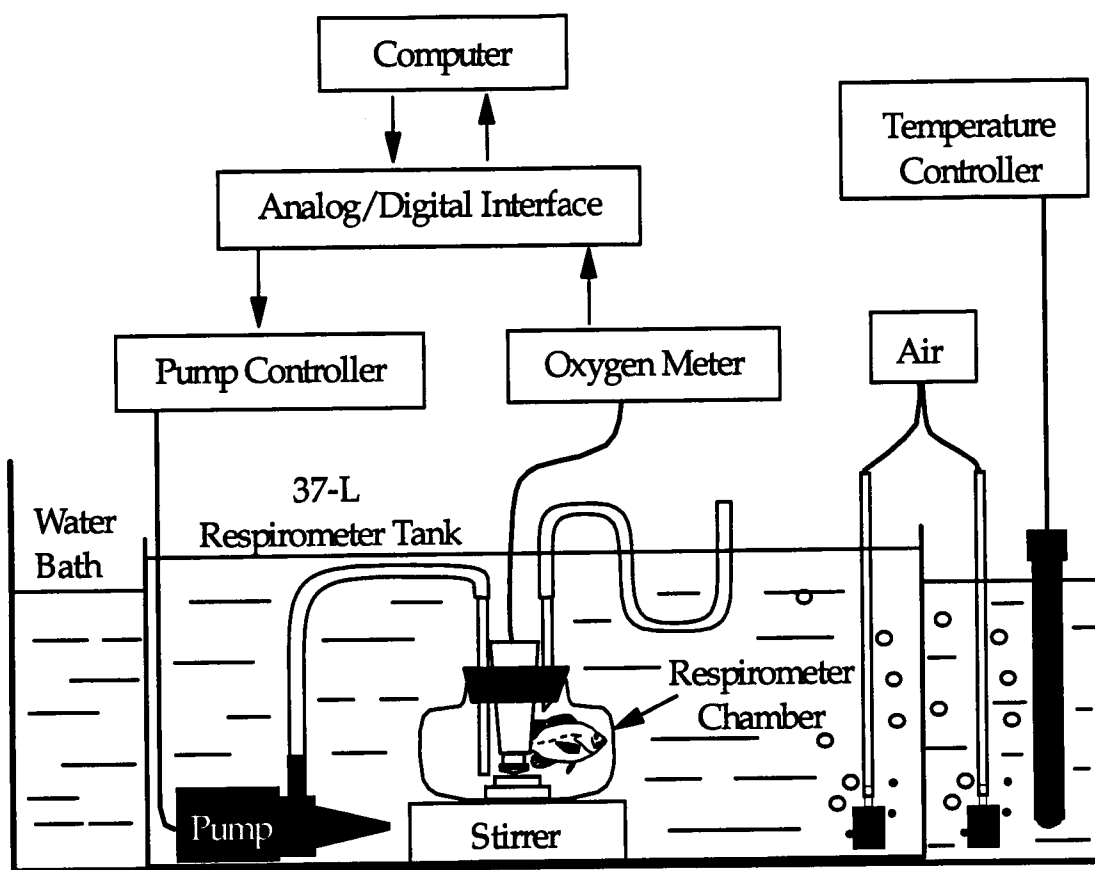


Figure 2. Respirometer tank with respiratory medium, respirometer chamber and equipment for measurement and control of experiments.

tubes mounted through the closure allowed circulation of the respiratory medium through the chamber before and after hypoxia testing periods. A small submersible pump in each tank circulated the respiratory medium through the chamber during a habituation period. The outlet tube was adjusted so that bubbles were forced out of the chamber during flow periods. A loop in this tube remained partially submerged to prevent flow of water during the testing period.

Dissolved oxygen was measured in each respirometer chamber and recorded on a computer. Polarographic oxygen probes (YSI 5750) were connected to either dissolved oxygen meters (YSI Model 57) or dissolved oxygen transmitters (YSI 1255)

which were functionally equivalent to the meters. An analog output signal proportional to DO concentration was fed from these meters or transmitters to a data-acquisition and control system (Omega WB-40) which converted these signals to digital equivalents. The digital values were then sent to an IBM PC-compatible computer. The respirometer control program converted the digital signals back to concentrations of DO. Each measurement was an average of 100 analog-to-digital conversions, and the reading for each 5-minute interval was based on 16 measurements. The DO of each chamber was sampled 1600 times for each 5-minute reading. This rate of DO monitoring was the highest that could be achieved with the assembled components, the limit being the rate of analog-to-digital conversion. The rapid rate of analog-to-digital conversion helped overcome electrical interference, and averaging readings over 5 minutes helped dampen transient probe readings that did not reflect actual changes in DO within the chamber.

The volume of the chamber, the weight of the fish, the change in DO, and the times between readings (based on the computer's internal clock) were used to calculate RMR for on-screen display. Fish weight, time, and DO for 5-minute intervals were recorded to disk for subsequent analysis.

Respirometry Procedures

Before each test, the respirometer tank and equipment contained within were bleached, drained, and refilled with the respiratory medium. All tests were conducted at 25°C. For each test, temperature was allowed to equilibrate to 25°C. The electrolyte and membrane of each oxygen probe were replaced before each test, and oxygen meters were zeroed according to manufacturer's recommendations, but using the computer readout rather than the meter dial. Oxygen probes were then calibrated after installation into the chamber, with stir bars spinning, by adjusting the oxygen meter or transmitter

until the air-saturation value consistent with test temperature and the altitude where the tests were conducted was shown on the computer readout. The probes are sensitive to differences in stirring, and this calibration method provided more consistent readings than did calibration of probes in moist air. Other investigators have used similar calibration schemes with good results (Forstner 1983). The respirometer tanks were well aerated throughout the habituation and test periods to maintain oxygen saturation.

Once the computer's respirometer control program was started, the pumps were automatically turned on to flush the chambers during a habituation period before testing. Fish were habituated to the chambers for approximately 36 h with oxygen concentration maintained at air saturation by pumping the respiratory medium through the chambers. The habituation period was intended to reduce the excitement level following handling and transfer to the respirometer chamber. O'Hara (1971a, 1971b) found that respiration rates of bluegill transferred to a respirometer returned to base levels during a 24-h adjustment period at 25°C. The hypoxic challenge test was initiated at 20:00h on the second night after fish were introduced into the chambers.

At the start of a respirometric test, flow to the chambers was stopped and DO monitoring and recording were initiated. Oxygen consumption by the fish during the test caused DO in the chamber to decline. The test for each chamber continued until its DO declined to 0.75 mg/L; the flow to that chamber was then restarted. Moss and Scott (1961) reported that bluegill acclimated to normal oxygen conditions would not survive below 0.75 mg/L dissolved oxygen at 25°C, so the test ended if that level was reached. After the test was complete for both chambers, a data file was saved containing the time record of DO during the test period. Fish that failed to complete the entire treatment and test procedures were replaced by scheduling a new fish for treatment and testing when the respirometer was next available.

Many studies account for the effects of spontaneous activity during testing, but this was not possible with the challenge test method used here. Fish activity can be accounted for by taking multiple measures of metabolic rate over a period of time at each oxygen concentration, but the static respirometry system used here measured metabolic rate only once at each oxygen concentration. No comparison of high and low rates could be made that might indicate activity differences over time. Marvin and Burton (1973), however, found that, unlike trout, bluegill exhibit little excitement and remain almost motionless as DO concentrations decreased to near lethal levels. This is consistent with visual observations during preliminary tests with the present system. Considering observations and Marvin and Burton's report, the effect of spontaneous activity on the measured metabolic rates was assumed to be minimal.

Analysis of Metabolic Responses

Representative RMR:DO curves for treatment groups were constructed from mean values for the regression coefficients and COC and compared among sites. The fifth parameter (in this case, S_I) was redundant and was therefore computed from the other parameters to assure consistency.

The metabolic responses to the hypoxic challenge test were evaluated by examining the changes in RMR as DO declined. Each respirometry test produced a computer file that contained the DO and time for each measurement. RMR was computed for each 5-minute period. Oxygen readings were corrected for changes (usually reductions) of probe response during the habituation period. This correction was made by assuming air saturation at the beginning of the test period, and is appropriate for water with ionic content that affects oxygen activity (Hitchman 1983). This method of calibrating to water at air saturation has other practical advantages for

automated respirometry, especially when multiple chambers are involved (Forstner 1983, Gehrke and Fielder 1988). The rate of downward drift in probe response was low enough so that no correction was required for drift during the relatively brief testing period.

Microbial respiration was estimated with blank respirometer tests, which involved measuring oxygen consumption in clean chambers and in chambers immediately after the fish had been removed following a complete test. In either case, microbial consumption of oxygen in the chambers was so low that it was insignificant during the time it took to complete a respirometry test. Therefore, no correction was made for microbial consumption of oxygen during a test.

Measured metabolic rates were standardized for size differences by expressing them relative to body mass raised to the 0.67 (2/3) power, which produces "mass-independent" rates. The size range of individuals used in the present experiments was too narrow to reliably compute a unique mass exponent for each group. The mass exponent of 0.67 is based on the theoretical arguments of Heusner (1984). A previous study conducted with redbreast sunfish from area streams found a mass exponent of 0.665 (Shepard 1988). Other studies have found a range of mass exponents for sunfish ranging from 0.72 to 1.06 (Wohlschlag and Juliano 1959, Evans 1984).

The regions of oxygen-independent and oxygen-dependent respiration were examined graphically by plotting RMR versus DO. To set the boundaries between the two regions, a uniform procedure to characterize the curve was defined. The procedure was performed only for DO concentrations less than 5 mg/L because RMR at higher DO concentrations were more variable in preliminary tests. The causal factors for this variability were not established in those tests, though activity or excitement may have been important during this first part of the test. In preliminary tests for the present study,

a point was often reached, as DO dropped to very low levels, where RMR deviated rapidly from the downward trend in the oxygen-dependent portion of the curve, presumably because the fish was no longer able to control RMR. Because this discontinuity in consumption rate was perceived to signal the failure of normal metabolic regulation, and because the onset of this deviation from the downward trend was difficult to measure for individual fish, a minimum RMR of 50 mg O₂/kg/h (not adjusted for body mass) was set beyond which data points were discarded, even if the minimum test DO had not been reached. Above the minimum RMR established in preliminary testing, it appeared reasonably certain that RMR was responding to the changes in DO in a consistent way. The data falling within the boundaries set by preliminary tests was used to evaluate changes in RMR for individual fish for the range of DO experienced during the test.

A segmented regression line was fitted to the RMR:DO data points by joint least-squares minimization using the NLIN procedure (SAS 1988). This segmented function will be referred to as the RMR:DO curve. Two line segments were fitted to the RMR:DO curve for each individual (Figure 3). One line segment was fitted to the oxygen-independent portion of the curve, the other was fitted to the oxygen-dependent portion. The breakpoint of the line segments was set by NLIN procedure to minimize joint least-squares error of the two segments. The intersection of these two segments indicates the onset of oxygen dependence and this breakpoint defines the critical oxygen concentration for routine metabolism. The regression coefficients (slopes and intercepts) of the two line segments and the critical oxygen concentration were computed for each individual. These metabolic response parameters reflect the dynamics of metabolism represented in the RMR:DO curve for an individual. Differences in the shape of the curve among treatment groups reflect how prior exposure to treatment conditions altered

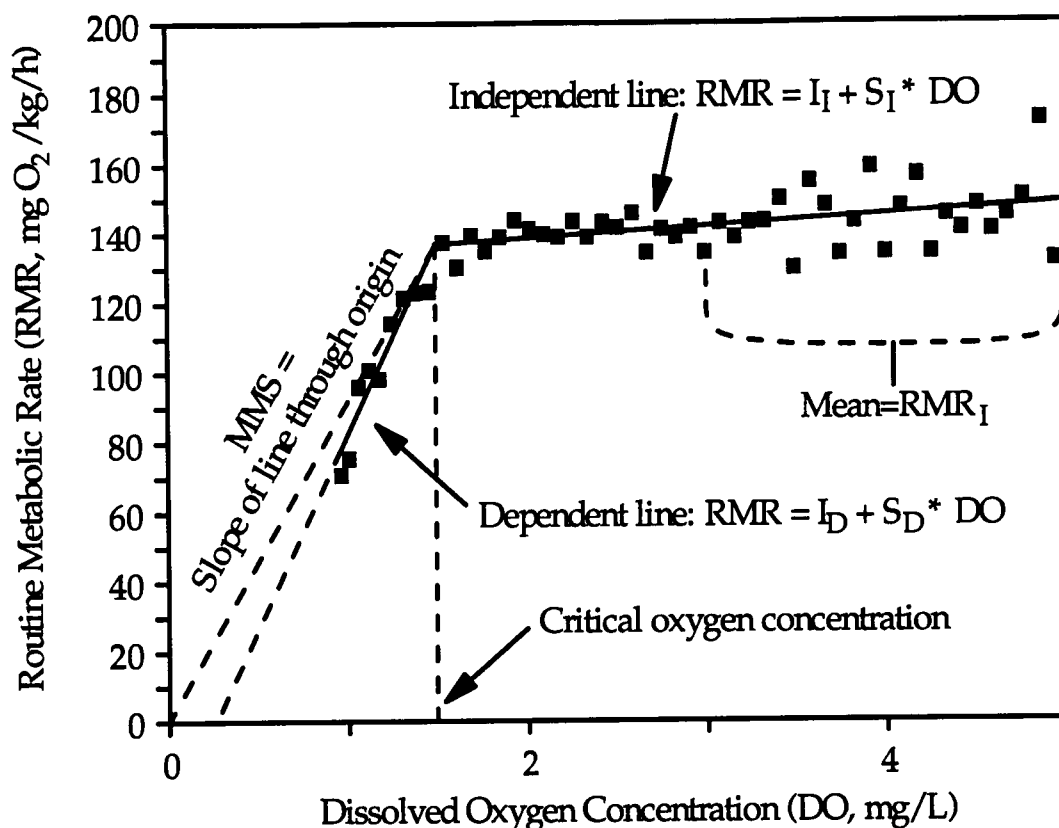


Figure 3. Derivation of metabolic response parameters from the relationship of routine metabolic rate and dissolved oxygen concentration. Solid line segments were fitted by joint minimization of least squares. Squares are 5-min average RMR measured during the test of an individual fish from the 30°C, starved, pH 5 treatment group.

metabolic state.

The regression coefficients of each line segment were a slope (S) and an intercept (I). The four response parameters of the fitted line segments were S_I and I_I for the oxygen-independent portion, and S_D and I_D for the oxygen-dependent portion of the curve. The response parameter COC was not independent of the other parameters (Figure 3). Because the COC marks the failure of metabolic homeostasis, this response parameter might be a more useful measure of metabolic performance than the regression coefficients of the line segments that have less obvious physiological interpretation.

Additional measures of fish metabolic functional capacity were compared to the regression coefficients of the fitted line segments and COC. The first additional measure was marginal metabolic scope (MMS), which is a surrogate for scope for activity or metabolic capacity that can be computed from the results of routine respirometry (Neill and Bryan 1991). In the present study, MMS was calculated as the slope of a line passing from the COC, at the RMR for that oxygen concentration, through the origin of the metabolic rate and oxygen concentration axes (Figure 3). The other measure was the mean routine metabolic rate for the oxygen-independent region (RMR_I), measured at DO concentrations great enough to avoid oxygen-dependence. For this study, RMR_I was computed as the mean rate for a portion of the oxygen-independent region (Figure 3). The MMS estimates metabolic capacity, and RMR_I estimates routine oxygen demand when oxygen is not limiting.

Statistical Procedures

The treatment groups were compared first using the regression coefficients of the fitted line segments and COC, but excluding MMS and RMR_I . Treatment groups were then compared using the coefficients of the fitted line segments, COC, MMS, and RMR_I , to determine if the additional response parameters improved the ability of the multivariate response model to differentiate among groups. This approach also was used to determine if MMS and RMR_I were individually more powerful for detecting differences than were the individual regression coefficients of the line segments or COC. When necessary, response parameters were transformed by box-cox transformation, which involves estimating the best power transformation (Sokal and Rohlf 1981) to satisfy the assumption of homogeneity of variance, homogeneity of covariance, and normality for the response parameters.

A canonical discriminant analysis was used to increase the ability to visualize and evaluate complex response differences among groups relative to treatment factors (CANDISC procedure, SAS 1988). To reduce the dimension of the response model for ease of visualization and interpretation, a stepwise variable selection procedure (STEPPDISC procedure, SAS 1988) was used to select a subset of the response parameters to maximize discriminatory power among exposure groups. This procedure used both forward and backward selection and removed redundant parameters and retained only the most powerful combination of parameters in the final discriminant model.

The canonical variables are linear combinations of the response parameters that summarize group differences in fewer dimensions for ease of visualization and interpretation. Group mean response vectors were projected onto the canonical axes with 95% confidence ellipses computed by the method of Schott (1990). Bartlett's test of the contribution of eigenvalues was used to determine if the third, and higher, eigenvalues did not differ significantly from zero (Seal 1964). If only the first two eigenvalues differed significantly from zero, a two-dimensional plot is sufficient to illustrate group differentiation. The sufficiency of the two-dimensional representation at representing group differences in the response parameters was tested by comparing the graphical representation with the significance of Mahalanobis distances, or squared statistical distances (D^2 , page 552, Johnson and Wichern 1992).

Univariate analysis of variance, ANOVA, (GLM procedure, SAS 1988, $p \leq 0.05$), was used to partition the variation among observations into portions associated with treatment factors for each response parameter included in the discriminant model. This technique revealed which treatment factors were most likely to be influencing the response parameters. The ANOVA also allowed additional supposedly random factors

such as fish size to be tested for influence on the included parameters. The structure of the canonical model indicates which response parameters were correlated with each canonical variable. The ANOVA and the canonical structure together indicate which treatment or random factors were associated with the canonical axes and are therefore correlated with the most powerful group differences.

To investigate the energetic costs of the treatment exposures, the net gain or loss of energy was determined by comparing the percentage change in weight during exposure to treatment conditions prior to testing. Treatment factors were tested by ANOVA to determine which factors explained significant variation in the difference in weight changes during exposure to treatment conditions. The ANOVA results were used to identify the factors that had the greatest effect on the energy budget of the fish during exposure.

Chapter 3

RESULTS

Comparison of Group Responses

RMR:DO Curves

The pH treatments did not result in significant differences in the shape of the RMR:DO curve ($p > 0.05$) between pH 5 and 7. To increase statistical power for comparisons among temperature and food availability treatments, groups differing only in pH were pooled to form four groups of eight individuals each. This approach assumes that variability in the groups due to exposure pH differences was negligible. The four new groups were: 15°C-starved (15S); 15°C-fed (15F); 30°C-starved (30S); and 30°C-fed (30F).

The RMR:DO curves measured during hypoxic challenge differed widely among temperature and food availability treatment groups (Figure 4). Treatment temperature differences resulted in the greatest differences among groups. Fish exposed to the higher treatment temperature, 30°C, demonstrated lower metabolic rates throughout the tested range of oxygen concentrations than fish exposed from 15°C treatments. Starved fish demonstrated higher metabolic rates than fed fish if treatment temperature of both groups was high (30°C), but demonstrated lower metabolic rates than fed fish if treatment temperature was low (15°C) for both groups. Treatment factor differences influenced other aspects of the curves, as is evident in the oxygen-dependent region of these curves.

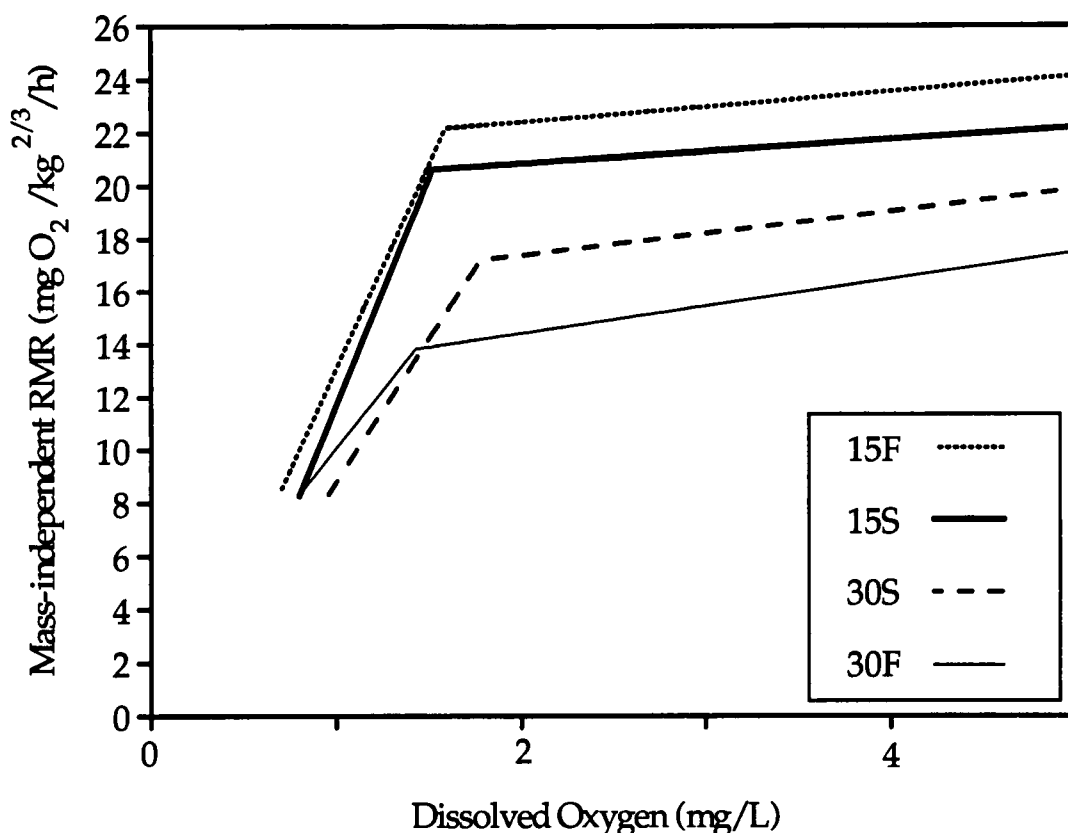


Figure 4. Representative RMR:DO curves for treatment groups. 30 = 30°C; 15 = 15°C; F = fed; and S = starved.

Regression Coefficients and COC

Four response parameters (of the regression coefficients and COC) differed among temperature and food availability treatment groups. Only S_I did not differ among groups. The stepwise variable selection procedure retained only three parameters in the response model: S_D , I_D , and COC. Individually, I_I was the most powerful for group discrimination, but it was removed from the final model by the variable selection procedure because it was redundant when the three other parameters were included. The multivariate response model was much more powerful for differentiating among groups than any single response parameter.

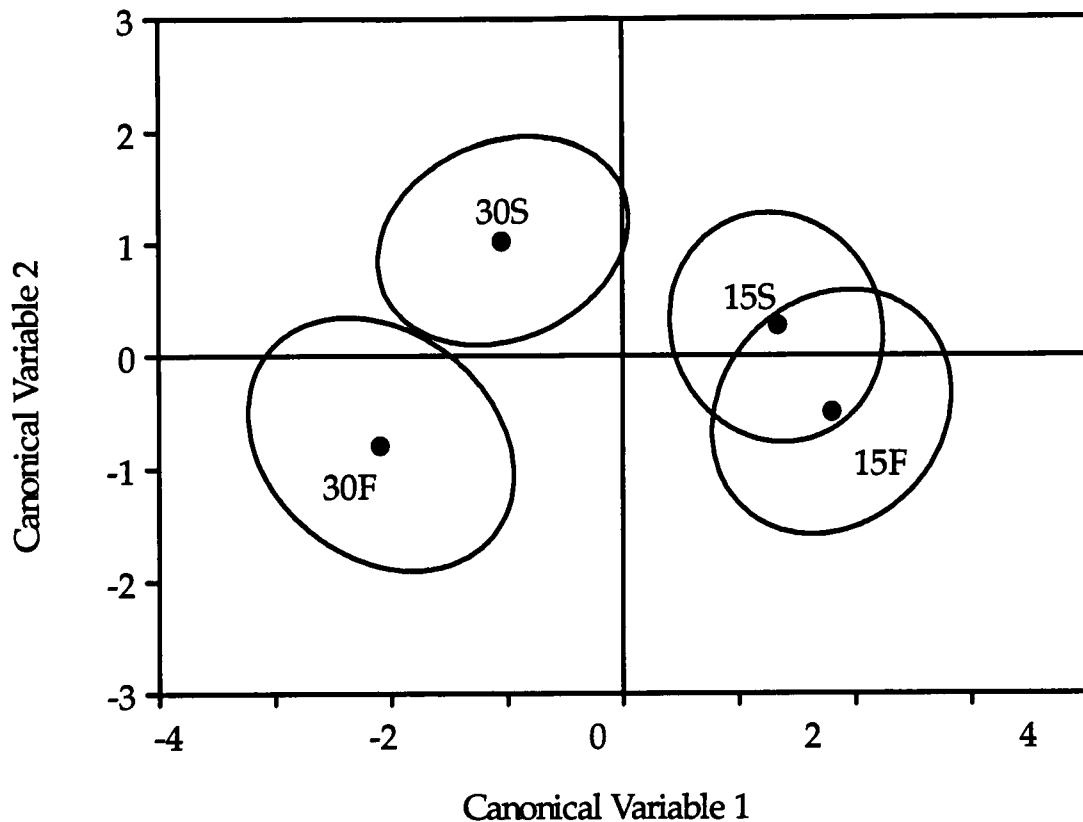


Figure 5. Projections of group mean responses onto the first and second canonical axes for the discriminant model that included S_D , I_D , and COC. Ellipses are 95% confidence regions for the means. 30 = 30°C; 15 = 15°C; F = fed; and S = starved.

The graphical representation of the canonical discriminant model revealed differences among temperature and food availability treatments (Figure 5). Because Bartlett's test of the contribution of eigenvalues determined that the third eigenvalue did not differ significantly from zero (Seal 1964), the relationships among groups can be visualized by plotting only the first two canonical variables. In this canonical model, 97% of the variation explained by the model was attributed to the first two eigenvalues. The projections differed significantly among groups as evidenced by the separation of confidence regions, except for 15F and 15S. The mean projections for these two groups did not differ significantly, as evidenced by the overlap of the means by the confidence

Table 1. Mahalanobis distances and significance level for group comparisons based on the discriminant model including S_D , I_D , and COC.

Comparison	Mahalanobis Distance	p-value
15S vs 15F	1.45740	0.1711
15S vs 30S	6.59980	0.0005
15S vs 30F	12.86226	0.0001
15F vs 30S	10.36868	0.0001
15F vs 30F	15.39258	0.0001
30S vs 30F	4.50471	0.0043

region of the other group.

The placement of treatment group means along the canonical axes revealed that the first canonical variable, which summarizes the greatest axis of differentiation among groups, differentiated between temperature treatments in this experiment. It was also evident that the second canonical axis differentiated between food availability treatments, but this differentiation was not as great. The overlap between the confidence ellipses of the 15°C groups, but not the 30°C groups (Figure 5), suggests that feeding did not significantly influence group response at 15°C, but did have a significant effect on the responses of groups exposed to 30°C. Thus, there was an interaction effect between temperature and food availability treatments.

The significance of Mahalanobis distances corresponded well with the separation of groups along the canonical axes (Table 1). The graphical representation of the canonical model and the Mahalanobis distances both indicated that the mean responses of all but the two 15°C treatment groups (15S and 15F) differed significantly. The agreement between these two measures of separation suggests that the plot of the first two canonical variables provided a valid representation of the differentiation among

Table 2. Pooled within-class correlations between canonical variables and parameters included in the first discriminant model.

Parameter	Canonical Variable 1	Canonical Variable 2
S_D	0.56	0.12
I_D	-0.31	-0.45
COC	0.09	0.68

groups.

The structure of the canonical model indicated that S_D was most highly correlated with canonical variable 1 (Table 2). The COC was most highly correlated with canonical variable 2. The graphical representation (Figure 5) revealed that canonical variable 1 was associated with treatment temperature differences and canonical variable 2 was associated with food availability treatment differences.

A further investigation of the influence of treatment factors by ANOVA indicated the influence of additional factors not revealed by the canonical analysis. Temperature explained a highly significant portion of the variability in S_D , and initial weight also explained a significant amount. Temperature, food availability, and initial weight explained a significant amount of the variability in I_D . Food availability and a temperature-food availability interaction explained significant variability in COC.

Additional Measures of Metabolism

Five response parameters (of the regression coefficients, COC, MMS, and RMR_I) differed among temperature and food availability groups. The stepwise variable selection again retained only three parameters in the response model: the marginal metabolic scope, MMS; the intercept of the independent line segment, I_I ; and the slope of the dependent line segment, S_D .

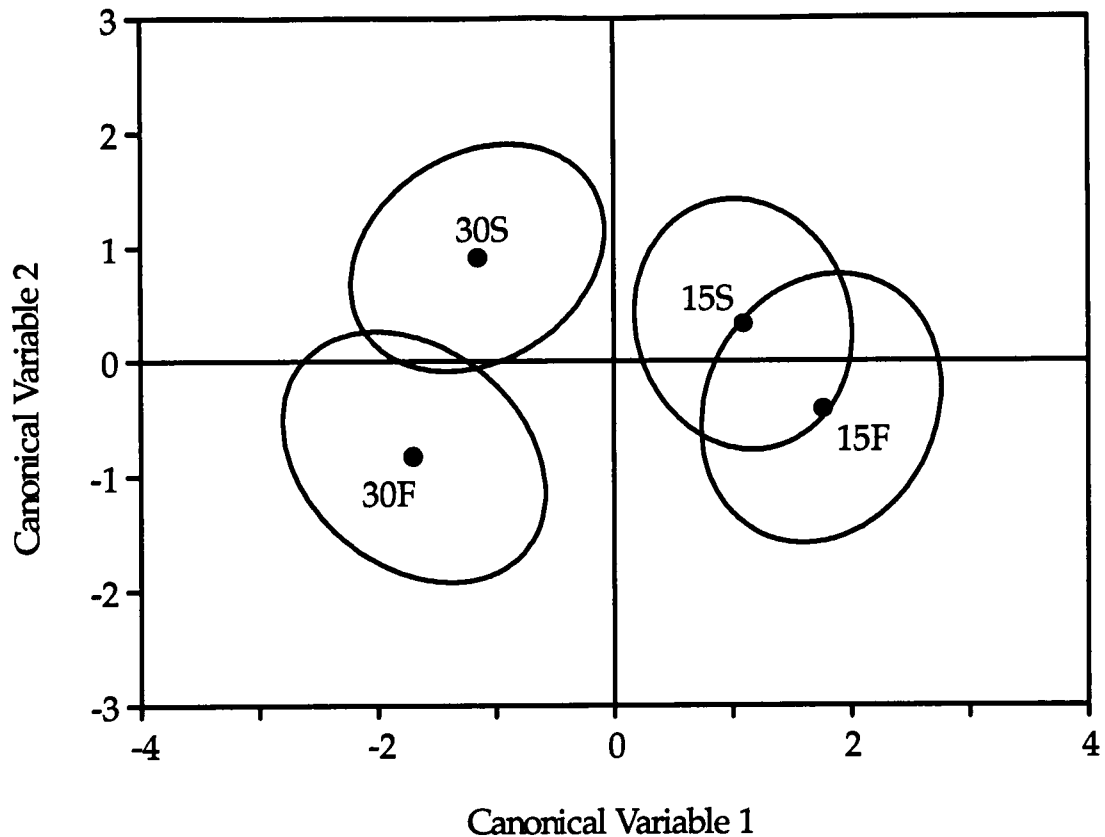


Figure 6. Projections of group mean responses onto the first and second canonical axes for the discriminant model that included I_1 , and S_D , and MMS. Ellipses are 95% confidence regions for the means. 30 = 30°C; 15 = 15°C; F = fed; and S = starved.

The graphical representation of the second canonical discriminant model revealed differences among temperature and food availability treatments (Figure 6). Bartlett's test of the contribution of eigenvalues determined that the third eigenvalue did not differ significantly from zero, and therefore the relationships among groups can be visualized by plotting only the first two axes. In this canonical model, 95% of the variation explained by the model was attributed to the first two eigenvalues.

The relative position and separation of group mean responses in the graphical representation based on the second canonical model (Figure 6) were similar to the relationships among groups in graphical representation of the first canonical model

Table 3. Mahalanobis distances and significance level for group comparisons for the second discriminant model including I_L and S_D , and MMS.

Comparison	Mahalanobis Distance	p-value
15S vs 15F	1.81352	0.1068
15S vs 30S	6.01056	0.0009
15S vs 30F	9.28008	0.0001
15F vs 30S	10.18486	0.0001
15F vs 30F	12.29708	0.0001
30S vs 30F	3.47010	0.0137

(Figure 5). The means of the two 15°C exposure groups again were statistically similar, but all other pairs differed. The placement of group means along the canonical axes is only slightly different from the previous model. The increased set of response parameters from which to choose had little effect on the overall ability of the second canonical discriminant model to differentiate among group mean responses.

The Mahalanobis distances for the second discriminant model agreed well with the graphical differentiation of group mean projections and associated confidence regions. The distances and their level of significance are listed in Table 3. The levels of significance differed slightly from those computed for the same comparisons using the parameters included in the first canonical model. The changes in significance were small, however, and did not affect the interpretation of group differences.

The additional parameters in the second canonical analysis did improve separation of means among treatment groups, but different parameters were included in the second model. The response parameter I_L had the greatest individual discriminant power of all the parameters tested, with MMS the second greatest. However, the inclusion of both of these parameters in the canonical model did not increase the

Table 4. Pooled within-class correlations between canonical variables and response parameters for the second discriminant model.

Parameter	Canonical Variable 1	Canonical Variable 2
I _I	0.88	-0.40
S _D	0.61	-0.29
MMS	0.84	0.12

significance of the overall model much relative to the first model. Instead, it seems that it is possible to account for metabolic differences among groups with more than one possible combination of parameters from the group of seven. The redundancy and correlation among response parameters could explain why the discriminant power of one combination was improved only slightly by selecting among more response parameters.

As with the first canonical model, the canonical variables in the second canonical model were graphically associated with the temperature and food availability differences (Figure 6). The canonical structure of the second canonical comparison revealed that, though I_I was the most highly correlated with canonical variable 1, all parameters contributed to group differentiation along the first canonical axis (Table 4). I_I also was highly (negatively) correlated with the second canonical variable.

MMS and I_I were tested by ANOVA to determine which treatment combinations had the greatest influence on these parameters. Temperature was the only factor that accounted for a highly significant amount of the variation in MMS. The ANOVA for I_I revealed that both water temperature and initial weight of the fish accounted for highly significant amounts of variation; the temperature-food availability interaction term also was significant. The relationships between treatment conditions and S_D remain the same as reported above: temperature and initial weight each accounted for a highly significant

amount of the variation.

Treatment Factor Effects on Weight

Change in weight during exposure periods differed among treatment factor groups. Percentage weight change during the exposure periods were usually negative, indicating that energy supply was insufficient to meet demand even in treatments that received food. An ANOVA test of the association between percentage weight loss and the temperature and food availability treatments revealed that both temperature and food availability explained highly significant amounts of the variability ($p = 0.0004$ and $p = 0.0001$, respectively) in weight loss. A temperature-feeding interaction also explained significant variability in the weight loss ($p = 0.022$). The treatment factors significantly influenced the energy budgets of these groups of fish as indicated by the significance of the overall ANOVA model ($p < 0.0001$).

Chapter 4

DISCUSSION

The metabolic responses of bluegill to hypoxia indicated that prior exposure to treatment conditions affected metabolic status. The hypoxic challenge test, and the method that I used to compare responses, revealed complex metabolic differences among treatment groups. Temperature, food availability, and an interaction between these two factors all resulted in significant response differences among groups. Treatment levels of pH did not lead to measurable differences in response, but this is believed to have been because the levels used were insufficient to tax the homeostatic mechanisms of the fish, and because the number of individuals in each original treatment group was too low to establish statistical differences. Nevertheless, the multivariate comparison of metabolic response parameters is capable of detecting even complex metabolic differences caused by prior exposure conditions.

The response parameters based on the fitted line segments include measures of routine oxygen demand independent of oxygen concentration (at higher DO levels) and measures of the ability to maintain supply to metabolic processes under oxygen-dependent conditions (at lower DO levels). Although the response parameters are artificial constructs, their meaning may be discovered by examining how they fit into the representation of the RMR:DO relationship. If S_I is nearly zero, or at least consistent across groups, which was the case here, I_I indicates routine metabolic demand. The value of S_I indicates whether oxygen independence is somewhat less than perfect in the oxygen-independent region. COC marks the failure of metabolic homeostasis due to

oxygen limitation. S_D indicates how strongly decreasing oxygen concentration decreases aerobic metabolism. I_D indicates the combined effects of oxygen supply and demand, but is best interpreted relative to the other parameters because of redundancy (only four of five parameters needed to completely define the two line segments).

Comparisons of group responses based on individual response parameters may fail to account for complex differences in the RMR:DO relationship. A multivariate response model including the most powerful combination of response parameters can better evaluate complex response differences among groups. The canonical analysis simplified the comparison of groups further by compressing the important aspects of groups response onto two canonical axes. This dimensional reduction had the effect of condensing important differences among groups into a simple visual comparison of differences. This graphical representation reveals how metabolic responses differ among groups. The clear graphical associations between the first canonical axis and treatment temperature differences and between the second canonical axis and food availability demonstrate how this procedure simplifies the interpretation of response relationships.

The two additional measures of metabolic functional status, MMS and RMR_I , had little effect on the ability to differentiate among groups. The canonical model included MMS, S_D and I_I . The relationships among group responses based on this model differed little from the first model which excluded MMS and RMR_I . These multivariate models produced a similar picture of group response differences relative to the treatment factors. The response parameters MMS, RMR_I and COC may individually have the clearest physiological interpretations, but they are not necessarily better able to capture the complex metabolic responses to hypoxia. The multivariate models can account for physiologically complex responses without requiring that the individual response parameters in the model closely match the mechanisms of response.

Although the oxygen-dependent region of metabolism is rarely studied because anaerobic energy use is not reflected in short-term oxygen consumption, present results suggested that oxygen-dependent rates of oxygen consumption were a sensitive indicator of metabolic differences among groups. S_D was included in both canonical models, and I_D was included in the first model. MMS and S_D differ only because the line segment for which S_D is the slope is not forced to pass through the origin of the RMR and DO axes. If the placement of the dependent line segment, like MMS, was a function only of where COC falls on the independent line segment, then the value of S_D would equal or be proportional to MMS. The inclusion of both MMS and S_D in the second canonical model suggests that S_D contains additional information about group metabolic differences. Thus, the results suggest that S_D is not equal to or highly correlated with MMS.

Temperature is an important factor in the metabolism of fishes, and it is not surprising that the most significant group response differences were associated with the temperature treatments. Treatment temperature had a significant effect on S_D , I_D , I_I and MMS. By design, the experimental protocol did not allow complete acclimation to the test temperature prior to the start of the hypoxic challenge test. When testing unacclimated fish, measured metabolic rates represent uncompensated metabolism (Prosser and Brown 1961). Uncompensated metabolic rates are generally lower for warm-acclimated fish than for cold-acclimated fish at an intermediate temperature. Evans (1990) also points out that routine rates may be altered due to changes in activity metabolism following temperature change.

If temperature changes cannot be eliminated, it may be possible, for a limited number of well-studied species, to draw upon the results of previous studies to accurately predict the effects of a given temperature change on metabolic rate. Some

correction for temperature could then be applied prior to the interpretation of the influence of stressors or other factors. Such a correction would be difficult to apply when interaction effects occur involving temperature, as they did here. The exposure treatment temperatures used in the present investigation were selected to represent an extreme case of temperature differences among groups in order to determine how prior temperature exposure experience might alter the measured responses to the hypoxic challenge test.

What are the implications of the large response differences caused by temperature treatments for the use of the responses to hypoxic challenge to evaluate the effects of stressors? In a study of stressors unrelated to temperature, it would be unreasonable to compare fish from sites that exhibited such large differences in temperature. Even smaller differences in temperature experience could alter the metabolic responses to hypoxia in ways that might hide, exaggerate, or confound the responses used to detect the presence of the stressors under study. Present results suggest that temperature differences among groups should be minimized for comparison of the effects of other factor differences among groups.

Food availability treatments also resulted in significant differences in metabolic responses of the fish to hypoxia. The responses of bluegill differed more among food availability treatment groups that had been held previously at 30°C than among food availability groups at 15°C. Fish would be expected to require more energy at higher temperatures due to increased metabolic rate (Pierce and Wissing 1974, Jobling 1981, Jobling 1982, Hogendoorn 1983). Thus, a ration level acceptable for maintaining homeostasis at 15°C would not be so at 30°C. Food availability treatments affected many of the parameters which were included in the discriminant models (i.e., I_D , I_I , and COC), with the notable exceptions of MMS and S_D .

What are the implications of the significant effects of food availability for the use of metabolic responses to hypoxia to evaluate the effects of stressors? Food availability may differ among sites independent of their respective levels of stressor inputs. If food availability differences are related to the stressor inputs under study, metabolic responses related to food could be an inherent part of the stress response. Otherwise, investigations should compare metabolic responses at stressed sites with reference (unstressed) sites that are similar with respect to food availability, if possible.

Although the pH levels tested in this study did not result in significant differences among treatment groups, more extreme levels of this factor would be expected to significantly influence metabolic status. Ultsch et al. (1980) found no difference in standard metabolic rate or scope for spontaneous activity between pH 7.4 and 4.0 for both carp and trout, although further acidification resulted in significant differences in both measures of metabolic status in both species. In that study, differences were found in critical oxygen tension (equivalent to COC but expressed in terms of oxygen partial pressure instead of concentration) between groups exposed to pH 4 and pH 8 for both species. In well-buffered aquatic systems, however, fish will rarely be exposed to a larger range of pH than was tested in this study. Thus, natural variability in pH in most aquatic systems should not greatly affect the metabolic responses measured during a hypoxic challenge test at standard conditions.

The influence of fish size, expressed as the weight of fish before exposure to treatment conditions, on the response parameters of individual fish was unexpected because the size range of fish used in the test was small. Although metabolism of sunfish is known to be influenced by body size (Wohlschlag and Juliano 1959, O'Hara 1968, Evans 1984), metabolic rates were adjusted based on the theoretical arguments of Heusner (1982) to account for this influence. The method applied here for minimizing

the size-dependent differences in oxygen consumption rates has been used successfully by others studying similar species when sufficient individuals were available to compute a unique mass exponent for the tested population (Wohlschlag and Juliano 1959, Evans 1984, Shepard 1988). In the present study, the use of an approximate mass exponent apparently did not eliminate individual differences related to fish size. The failure to eliminate mass-dependence of individual responses probably had little effect on the comparisons of mean response among groups because groups were composed of randomly assigned individuals and mean sizes of the treatment groups were similar. Although a mass exponent more appropriate than 0.67 might be found by testing a larger size range of fish, the present results suggest that mass-dependent metabolic differences in small sunfish will be difficult to eliminate. To minimize mass-dependent metabolic differences, fish size differences should be minimized or mass relationships should be more precisely defined using large numbers of fish.

The response parameters retained in the response models were each affected differently by the treatment factors. The S_D was influenced by temperature and by the initial weight of the fish, and I_D was influenced by temperature, food availability, and initial weight of the fish. In contrast, COC was influenced by food availability and a temperature-food availability interaction. The I_I was influenced most strongly by temperature, initial weight of the fish, and a temperature-food availability interaction, whereas MMS was influenced almost solely by temperature. Each parameter represents a separate aspect of the relationship between RMR and DO during the hypoxic challenge test. The alteration of metabolic processes by treatment factors will affect only those parameters involving the aspects of the RMR:DO curve that change in response to that treatment factor.

If treatment factors alter metabolism in unique ways, differences in the patterns

of response to hypoxic challenge among groups may have diagnostic power. Group responses might then be compared to determine which factors differed in their exposure experience prior to the test. Discriminant models, such as the canonical discriminant models used above, could allow the composite of responses of individuals or groups with unknown exposure histories to be plotted along temperature or food availability axes, for example, to evaluate whether fish had been previously exposed to high or low levels of those factors. Because only a limited number of factors were tested here, this technique could only be applied to the elimination of these as possible causal factors, and even then it must be assumed that other factors, alone or in combination, would not result in a similar response pattern. For example, if the responses of two groups of fish did not differ significantly for a parameter known to be sensitive to temperature, such as MMS, it can be assumed that temperature differences are not the principal factor affecting group response differences. More background studies are needed to establish how factors and combinations of factors influence the responses of fish to hypoxia to assure these responses are interpreted correctly relative to major exposure factors.

Treatment conditions were shown to affect the energy budgets of fish during exposure. I expected that temperature and food availability would affect the energetic status of fish, and this expectation was met. The statistical significance of the differences in mean percentage weight loss of groups of fish during exposure to treatment factors prior to testing supports the other evidence that temperature and food availability treatment levels were sufficiently different to cause meaningful differences in fish metabolic status after only a two week exposure. The differences in integrated metabolic responses to hypoxia among treatment groups agreed with the group differences in energetic status based on the measurement of weight differences before and after the exposure period. This agreement, in turn, suggests that the metabolic

indicators are correlated with differences in energetic status that could affect fish growth, reproduction, or survival.

An ideal stress indicator should reflect the holistic, or integrated, response of an organism to environmental stressors. An integrative indicator should allow detection and quantification of the relative impact of multiple stressors on the organism. The individual response parameters tested in this study cannot be considered integrative indicators because each one responds differently to the various treatment factors. The multivariate response model formed by combining all individual response parameters does provide an integrative assessment of the effects of stressors on metabolic performance. Each individual parameter may not differ in every group comparison, but the complete response model is a starting point which assures that important differences will not be missed. The complete response model can be pared down to include only the response parameters important to a particular comparison of groups. This multivariate approach can reveal the importance of complex interactions between animal and environment that might go unnoticed if response parameters were analyzed individually.

Multivariate response models selected from the complete group of response parameters, (the regression coefficients, COC, MMS, and RMR_I), were clearly able to discriminate among groups of fish after exposure to different levels of background environmental factors, but specific stressors were not tested here. Additional experiments will be necessary to determine whether specific stressors, such as anthropogenic pollutant inputs, will have detectable effects on the metabolic responses to hypoxia. Pollution-related changes in the metabolic responses to hypoxic challenge must be quantitatively greater or qualitatively different from the responses to variation in background environmental factors among sites if the metabolic responses to hypoxic challenge are to be used as indicators of pollution stress. The next experiment in this

study attempted to detect metabolic differences among groups of fish collected from field sites subject to various levels of pollution, in spite of the influence of normal environmental variation among field sites.

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PART 3

FIELD TRIAL OF METABOLIC INDICATORS OF STRESS

Chapter 1

INTRODUCTION

This experiment investigated whether metabolic responses to hypoxia could be used to detect differences among groups of fish collected from field sites subject to different levels of pollution. Because metabolism is sensitive to many environmental factors the effects of pollution or other stressors of interest may be difficult to detect by comparison of metabolic differences of fish from various field sites (Brett and Groves 1979, Heath 1987, Wedemeyer et al. 1990). The previous laboratory experiment demonstrated that two important environmental factors, temperature and food availability, caused significant differences in the metabolic responses of fish to hypoxic challenge in the absence of pollution effects.

In order to use the relationship of RMR:DO measured during hypoxic challenge as an indicator of pollution stress, environmental factor differences prior to testing must be taken into account. Most physiological processes are influenced to some extent by these background environmental factors, but physiological parameters used as indicators of stress must be shown to indicate this stress as distinct from some other physiological reaction (Schreck 1990). The influence of background environmental factors can be evaluated by comparing the ability of metabolic indicators to detect differences among groups of fish from field sites known to be subject to different levels of stressors, but also subject to normal environmental variation.

I conducted an experiment to test the ability of metabolic responses of fish to hypoxia to reveal the influence of pollution inputs despite background environmental

differences among sites. The metabolic responses to hypoxic challenge were compared among groups of redbreast sunfish [*Lepomis auritus* (Linnaeus)] collected from three polluted sites and one reference site. Previous fish health assessment studies of this species in these aquatic systems demonstrated that fish health was reduced at the polluted sites (Adams et al. 1992a, 1992b). The metabolic responses to hypoxia of groups from these sites should reflect the influence of pollution that has been shown to alter other indicators of fish health in those previous studies.

The relative ability of metabolic indicators to reveal the effects of known differences in pollution inputs will help determine whether these indicators have the potential to detect prior exposure to stressors among groups of fish collected from sites where the presence or absence of stressors has not been established previously. To be useful as an indicator of stress, the metabolic responses to hypoxia must first be altered by stressors. Environmental factors and other influences not related to the stressors of interest must not obscure the metabolic alteration caused by exposure to stressors. If these conditions are met, it may be possible to apply the technique among field sites to detect whether fish are affected by sublethal stressors. Even if metabolic differences can reveal the influence of pollution, this experiment may indicate a need to alter some aspects of the experimental technique or the method used to evaluate response differences to increase the reliability or sensitivity of the metabolic indicators for detecting the stressors.

Chapter 2

MATERIALS AND METHODS

Sampling Sites

Fish were collected from three polluted sites, including Upper East Fork (UE) and Jackson Farm (JF) on East Fork Poplar Creek, Anderson County TN, and the Pigeon River (PR) above the town of Newport in Cocke County, TN. The Middle Prong (MP) of the Little Pigeon River (Sevier County, TN) served as a reference site. Confirmation of biologically relevant differences in water quality among sites was provided by published integrated health assessment studies on redbreast sunfish, *Lepomis auritus* (Adams et al. 1992a, 1992b) and from unpublished data (personal communication, S. M. Adams, Oak Ridge National Laboratory).

The UE and JF sites contain PCBs, mercury, other metals, suspended solids and nitrogenous compounds from point source discharges entering the stream near its headwaters just above the upper site, UE. The lower site, JF, is approximately 9 km downstream of UE, and receives some urban and agricultural runoff. A more exhaustive list of contaminants measured in water or sediment is available in Kornegay (1992). The Pigeon River receives effluents containing dioxins and chlorophenolic compounds (EPA 1988) from a bleached pulp mill approximately 80 km upstream from the sampling site. Even at this distance downstream, foaming and water discoloration associated with pulp mill effluents were evident in the river at the time of this study. Some small municipal waste treatment plants (tertiary treatment) release effluents to the

river at two or more locations between the mill outfall and the sampling site, but they constitute a relatively small proportion of stream flow. The watershed of the Middle Prong of the Little Pigeon River above the study site does not contain known sources of industrial pollution.

Experimental Subjects

Bluegill sunfish, the species used in the previous laboratory test, were not readily available at all experimental sites, so redbreast sunfish [*Lepomis auritus* (Linnaeus)] were used for the field experiment. Fish used in the present experiment weighed from 5 to 20 grams; the length of those fish ranged from 73-106 mm TL. These sizes represented the maximum range considered acceptable for testing in the respirometer chambers. Collection of sufficient numbers of similarly sized fish from all sites was not possible due to scarcity and difficulty of capturing fish at some sites. I was able to collect and test 15 fish from UE, 6 from PR, 5 from JF, and 7 from MP.

Collection and Holding

Fish were collected from the streams by electrofishing between the dates of 01 June 1992 and 20 November 1992. After being collected, fish were brought to the laboratory in stream water. They were then either immediately introduced into the respirometer for habituation and testing, or held at test conditions until the first tests were completed. On the day of collection two fish were introduced into the respirometer chambers immediately upon return to the laboratory to begin habituation. Holding of additional fish collected during a trip was necessary because of the limited number of respirometer chambers, but may have allowed fish to acclimate more fully to the test conditions. Fish were not fed during holding. The effects of holding on the metabolic responses to the hypoxic challenge test are investigated in a subsequent experiment.

Visual screening of the holding tanks minimized disturbance.

Respirometry

The methods for the respirometric tests are described in detail in Part 2. Briefly, fish were weighed and measured, placed in the respirometer chambers and allowed to habituate to the chamber for approximately 36 h. Respiration rates of the fish then were measured by discontinuing flow of the respiratory medium and measuring the decline of dissolved oxygen concentration within the chamber until a preset minimum of 0.75 mg/L oxygen was reached. Water temperature in the tests was 25°C, and the pH was 7. The respiratory medium was prepared as in Part 2, above.

Analysis of Metabolic Responses

Representative RMR:DO curves for treatment groups were constructed from mean values for the regression coefficients and COC and compared among sites. The fifth parameter (in this case, S_I) was redundant and was therefore computed from the other parameters to assure consistency.

Data analysis was conducted by the method described in Part 2. A segmented regression was fit to the RMR:DO curve. Each line segment was characterized by a slope and an intercept. The four parameters of the equations of the fitted line segments were S_I and I_I for the segment fitted to the oxygen-independent portion, S_D and I_D for the segment fitted to the oxygen-dependent portion of the curve. The COC was defined as the oxygen concentration where the segments intersected (See Figure 3, Part 2). The computation of COC was not independent of the other parameters. The additional parameters of metabolic function, MMS, and RMR_I , were also included (Figure 3, Part 2). None of the variables required transformation because distributions were not found to differ from normal, and because variance and covariance were not found to differ

among sites. The variables were tested by stepwise variable selection for inclusion in a canonical discriminant model (STEPDISC procedure, SAS 1988). The selected variables were compared among site groups.

The precision of parameter estimates was dependent on the variability of oxygen consumption rates of fish during each test. In this experiment, some individuals exhibited more variable oxygen consumption rates than other individuals. To reduce the influence of the tests where parameters were estimated with less precision, I used a weighting variable based on the variance of oxygen consumption rates near the beginning of the test. Weighted means were compared among groups using ANOVA (Tukey option, $\alpha = 0.05$, GLM procedure, SAS 1988).

Indicators of Aquatic System Health

The differences in the metabolic responses to hypoxia among sites were compared to the differences in other indicators of fish and aquatic system health measured prior to the present study. The indicators were the index of biotic integrity (IBI), 7-ethoxyresorufin *O*-deethylase (EROD) activity, and instantaneous true growth rate. These health indicators were compared among groups of fish collected from these sites in 1990 (Ryon and Schilling 1994, Adams et al. 1994, personal communication, S. M. Adams, Oak Ridge National Laboratory, and personal communication, C. F. Saylor, Tennessee Valley Authority). Growth rates of fish were compared among sites to illustrate bioenergetic differences among sites. Mean instantaneous true growth rates (Ricker 1975) computed from scales of age 2 fish, were compared among sites at or near the present sampling sites. Age 2 fish were most similar in size to the fish collected from these sites for hypoxic challenge tests in the present experiment. Means were compared with a Tukey test with experiment-wise level of significance controlled to $\alpha =$

0.05 (GLM procedure, SAS 1988).

A biochemical indicator of contaminant exposure (7-ethoxyresorufin *O*-deethylase (EROD) activity) was compared among groups of adult redbreast sunfish collected from these sites. To make these measurements, hepatic microsomes containing mixed function oxidase enzymes were prepared by differential centrifugation (McKee et al. 1983). The EROD activity in the hepatic microsomal fraction was measured fluorimetrically using a luminescence spectrometer (Perkin-Elmer LS50) at 30°C (Burke and Mayer 1974) with an excitation wavelength of 530 nm (15-nm slit) and an emission wavelength of 582 nm (20-nm slit). Mean EROD activities of from 15 to 20 males at each site were compared with a Tukey test ($\alpha = 0.05$, GLM procedure, SAS 1988).

The Index of Biotic Integrity (IBI) was used to provide a general assessment of environmental quality by measuring certain aspects of the aquatic community at each site, including species composition and richness, trophic structure, abundance, and condition. A composite score was computed for each site by a modification of the method developed by Karr et al. (1986), as described by Adams et al. (1992b). These composite scores were classified into categories representing different levels of environmental quality from very poor to excellent by the classification system developed by Karr et al. (1986). No statistical comparison of the differences among composite scores was possible, but scores not classified into the same environmental quality categories were considered to differ for the purposes of comparing these sites.

Chapter 3

RESULTS

Comparison of Group Responses

RMR:DO Curves

The representative RMR:DO curves for fish from all sites were similar (Figure 7). Response lines generally intertwined within a narrow band. Only MP appeared to stand out to any degree, having a slightly higher RMR_I and COC. The RMR:DO relationships of the two sites in the same aquatic system, JF and UE, were the most similar. These curves differed much less than those for treatment groups of bluegill exposed to temperature and food availability treatment differences in the previous experiment (Figure 4, Part 2).

Response Parameters

Of the seven response parameters, the regression coefficients, COC, MMS, and RMR_I , the variable selection procedure retained only one, COC, in the final response model. None of the other response parameters, alone or in combination, were retained in the model, indicating that they did not differ significantly among sites. With only one variable retained in the final response model, the canonical discriminant analysis was not conducted.

The mean critical oxygen concentrations of groups of fish collected at each polluted site were significantly lower (Tukey test, $\alpha = 0.05$) than the mean for the group

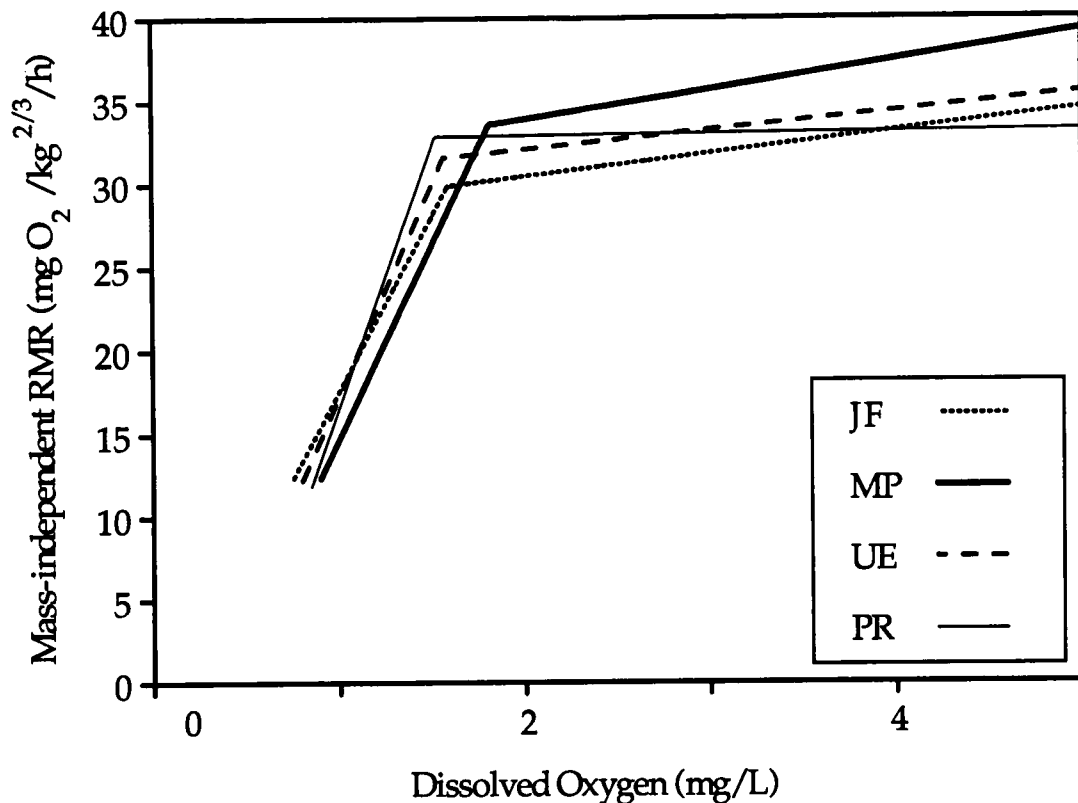


Figure 7. Representative RMR:DO curves for site groups. UE = Upper East Fork; JF = Jackson Farm; PR = Pigeon River; MP = Middle Prong.

collected at the reference site, MP (Table 5). This finding suggests that resistance of redbreast sunfish to hypoxia is greater (low COC = high resistance to hypoxia) at polluted sites. Mean COCs were more similar among the group of polluted sites, though significant differences existed for fish from different polluted sites in different aquatic systems.

Indicators of Fish and Aquatic System Health

Indicators of fish and aquatic system health measured for fish collected in other studies during 1990 differed significantly among sites but did not consistently rank polluted sites relative to each other (Table 5). These indicator parameters responded

Table 5. Comparison of critical oxygen concentration with three other indicators of fish health at several levels of biological organization. In order of increasing level of biological organization: EROD = 7-ethoxyresorufin *o*-deethylase activity; COC = critical oxygen concentration; Growth = instantaneous true growth rate; IBI = index of biotic integrity.

Parameter	Reference value	Ranking relative to reference		
		Most similar		Least similar
EROD	MP	UE	PR	JF
	<u>24.6</u>	<u>72.0</u>	<u>88.5</u>	<u>124.8</u>
COC	MP	JF	UE	PR
	<u>1.81</u>	<u>1.59</u>	<u>1.57</u>	<u>1.52</u>
Growth	MP	PR	JF	UE
	<u>1.33</u>	<u>1.67</u>	<u>1.78</u>	<u>1.91</u>
IBI	MP	JF	UE	PR
	<u>48</u>	<u>30</u>	<u>26</u>	<u>24</u>

EROD activity expressed as #pmol/ min/ #nmol-P450. COC expressed in mg/ L O₂. Growth is expressed as instantaneous true growth rate of age 2 fish, a measure of the proportional weight increase during the last annular increment (Ricker 1975). IBI expressed as composite score based on a modification of Karr et al. 1986. Means underlined by the same line segment do not differ significantly (Tukey test, $\alpha = 0.05$). IBI scores underlined by the same line segment are in the same environmental quality grouping in the classification scheme of Karr et al. (1986).

strongly to the presence of pollution. The mean values of EROD, growth rate, and the composite score for IBI measured at the reference site differed from the means or scores measured at each polluted site. The ranking of differences among polluted sites by these indicators, however, did not indicate a consistent trend of the severity of pollution among polluted sites.

Comparison of COC with Other Parameters

All parameters demonstrated significant differences between the reference and each polluted site, but the rank order of sites by COC was consistent only with that of IBI. The rank order of polluted sites relative to the reference by COC was not consistent with the rank order of polluted sites by either EROD or Growth. The significance of differences among polluted sites was not consistent for COC and any other parameter. Considering this comparison, the metabolic parameter, COC, was as effective as the other tested indicators of fish and aquatic system health at discriminating among reference and polluted sites, but it was not possible to evaluate its ability to assess of the relative severity of contaminant stress because the other indicators did not provide a uniform assessment for comparison.

Although each parameter indicates that fish from the reference differ from fish from each polluted site, parameters provide conflicting information about the effects of pollutants. The parameters EROD and IBI indicate lower contaminant exposure and better environmental quality at the reference site, but growth was slower, and the ability to resist hypoxia was lower (i.e., COC was higher) for small fish collected from the reference site.

Chapter 4

DISCUSSION

Because previous studies had demonstrated the effects of pollutant stressors at a wide range of levels of biological organization, it was surprising that only one of the metabolic response variables tested in the present experiment differed significantly among sites. Other sources of variation, such as environmental factor differences among sites, could have reduced the ability to detect differences in other metabolic response parameters among sites. The small number of fish collected and tested from some sites could also have reduced the ability to demonstrate statistical significance for apparent differences in the RMR:DO curves.

The objective of this experiment was to determine if metabolic responses to stressors could be detected in spite of background environmental variation among field sites. The differences in the metabolic response parameter, COC, among sites did reflect the presence or absence of industrial pollution. Background environmental factors did not obscure the effects of prior exposure to pollution on this metabolic response parameter. The ability of COC to differentiate polluted sites from the reference was comparable to that of other indicators of fish and aquatic system health measured in previous studies. Each indicator parameter proved capable of differentiating polluted sites from the reference, but failed, as a group, to provide a uniform assessment of the relative severity of varying degrees of pollution.

Individual parameters provided conflicting information about the effects of pollution inputs relative to the reference site. Response parameters indicated that

pollutants increased contaminant exposure and lowered environmental quality but also increased growth rates and increased resistance to hypoxia. Both measures, growth rate and COC, that indicated improved energetic status or metabolic performance of fish collected from polluted sites are based on small (age 2) fish. Growth rates of other age classes did not exhibit the same trend among sites (Ryon and Schilling 1994, personal communication, S. M. Adams, Oak Ridge National Laboratory). The apparent contradiction among these parameters suggests that the effects of pollution are not uniform for all age classes and all levels of biological organization.

Growth rates generally reflect energetic status of the organism. Pollution can increase available energy by nutrient enrichment effects through the food chain, reduced competition for a limited food resource, or both. In the Pigeon River, increased growth rates have been attributed to reductions in intra- and inter-specific competition as a result of reproductive impairment (Adams et al. 1992b). In East Fork Poplar Creek, growth rates are only higher than reference sites for age 2 fish, so any increased food availability or reduced competition should only apply to that age class. Sprague (1971) reviewed the use of sublethal measures of toxicity and found instances where toxicant exposures decreased growth, increased growth, or had no effect. A more extensive investigation of factors contributing to differences in energy budgets is needed to interpret the biological significance of altered growth rates relative to pollution exposure.

Fish chronically exposed to stressors have been shown to compensate for acute reductions in performance measures such as swimming stamina, even when other evidence of chronic stress was apparent (Webb and Brett 1973, Waiwood and Beamish 1978, McLeay and Brown 1979). Wedemeyer and McLeay (1981) suggest that lack of impairment in swimming stamina tests for fish chronically exposed to stressors may be

the result of the development of improved respiratory efficiency, relative to unstressed fish. Increased respiratory efficiency could result in increased performance after removal from stressful water conditions, as in the hypoxic challenge tests used here. Fish at polluted sites might increase their respiratory or metabolic efficiency if they are exposed to low concentrations of environmental oxygen (Shepard 1955) or other conditions that might result in tissue hypoxia, such as high temperatures, extremes of pH, high concentrations of free CO₂, and certain other toxic substances (Doudoroff 1957). The existence of such compensatory changes makes interpretation of metabolic performance relative to pollution inputs difficult.

Energetic status and COC may be interrelated. In the laboratory experiment described in Part 2, COCs of bluegill were found to be affected by food availability. Higher mean weight loss during two-week laboratory exposures was associated with higher mean COCs. In the present comparison among sites, higher growth rates were associated with lower mean COCs. Better net energetic status appears to be generally associated with lower mean COCs. This relationship has not been tested here, but is noted as a prospect for future study to investigate how energetic status influences COC.

The metabolic response parameter, COC, proved its ability as an indicator of prior exposure to stressors by differentiating each polluted site from the reference. Lower mean COCs for small redbreast sunfish at polluted sites indicate increased resistance to hypoxia, which suggests pollution exposure increased, rather than decreased, metabolic homeostatic capacity. Altered resistance to hypoxic challenge reflects the effects of prior exposure conditions, but stressors may not necessarily reduce resistance to hypoxia. It is difficult to judge by this indicator whether a group of fish was previously exposed to stressors. The differences that are measured may be judged to indicate stress only in comparison to a reference group or to additional indicators.

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PART 4

EFFECT OF HOLDING PERIODS ON METABOLIC RESPONSES TO HYPOXIA

Chapter 1

INTRODUCTION

Measurement of metabolic rates characteristic of a given set of conditions is difficult because metabolic rate responds rapidly to extraneous factors (Brett and Groves 1979, Heath 1987). In the previous experiment, fish collected at field sites were tested in a laboratory respirometer after rapid transfer from ambient field conditions. Standardization of test conditions helped minimize disturbance and other factors that may affect metabolic rates, but the rapid transfer to test conditions did not allow complete acclimation to temperature and other factors prior to the hypoxic challenge test. To allow more fish to be tested from a sampling trip, some fish were held at test conditions for a 2-d period in the laboratory prior to the beginning of habituation and testing procedures. The use of a holding period for some of the fish raised questions about the effects of the holding period on the metabolic responses measured during the hypoxic challenge test. Further study was needed to determine how the alteration of metabolic responses during holding would affect the ability to detect differences among sites indicative of differing exposure conditions or stressors.

Changes in thermal acclimation state and starvation during holding periods may alter the metabolic responses to hypoxia measured in tests following the holding period. The previous laboratory experiment (Part 2) demonstrated that temperature and food availability treatment conditions prior to testing had a strong effect on the metabolic responses to hypoxia. Fry (1957) noted that the thermal history of fish had a major effect on metabolic rate. Starvation can also have a major effect on metabolic rate

(Jobling 1981). If the influence of these factors during holding is established, the impact of holding times on the comparison of responses among groups can be evaluated.

An experiment was conducted to establish the effects of holding periods on the metabolic responses to hypoxic challenge. A large difference between prior acclimation temperature and holding temperature was used to maximize the effects of temperature acclimation differences on the metabolic responses. 15°C-acclimated fish were either tested immediately or held at 25°C without food for 4 or 9 d prior to testing. Food was withheld during holding periods to mimic the inability, in my experience, to rapidly coax field-collected fish to feed upon return to the laboratory. The effects of holding periods on the metabolic responses to hypoxia will be evaluated to determine the implications for the use of metabolic responses to detect differences related to prior exposure conditions or stressors.

Chapter 2

MATERIALS AND METHODS

Experimental Subjects

The bluegill sunfish (*Lepomis macrochirus*) used in this experiment were from the same lot as used in the laboratory experiment in Part 2 of this study. Initial weight of the fish at the beginning of acclimation treatments ranged from 4.87-9.64 g, and initial length ranged from 71-85 mm TL. All fish were held at 15°C in carbon-filtered dechlorinated tap water for at least two weeks prior to the beginning of treatment exposures to assure complete acclimation to 15°C before transfer to holding or test temperature, 25°C. The source water in the holding, treatment, and testing tanks had a pH of 7.9, a hardness of 134 mg/L (as CaCO₃), and 105 mg/L of alkalinity (as CaCO₃). The fish were fed frozen brine shrimp and frozen *Daphnia* while in the 15°C holding tank. Fish were not fed during acclimation treatments or following transfer to the respirometer for habituation and testing.

Experimental Design

Three groups of eight fish each, all originally acclimated to 15°C, were either introduced into the respirometer immediately or held for 4 or 9 days at the test temperature (25°C), yielding three acclimation states ranging from 15 to 25°C. After completion of the holding period, each fish was tested by respirometry at 25°C. Available times for testing in the respirometer were randomly assigned to one of the three treatments. Fish were then either tested immediately or transferred to 25°C for an

acclimation period of either 4 days or 9 days prior to initiation of a 36-h habituation period in the respirometer chamber prior to hypoxic challenge. The water for the 25°C exposure period was renewed slowly in a large flow-through bath and fish were held individually in plastic minnow traps to allow constant water flow but prevent aggressive social interactions.

The rule for assuring complete temperature acclimation is to allow one day for each degree Celsius difference in temperature following transfer (Fry 1971). It is unlikely that acclimation occurs in such a simple linear fashion and many environmental factors can modify the acclimating responses to temperature (Prosser and Brown 1961). Therefore, the acclimation states that are reached during the present acclimation treatments can not be interpolated to a specific temperature acclimation state. However, the 9-day acclimation period, when combined with the habituation period in the chamber, was expected to be sufficient for complete acclimation to test conditions, especially at the relatively warm final temperature of 25°C.

Respirometry

The methods for the respirometric tests are described in detail in Part 2. Briefly, fish were weighed and measured, placed in the respirometer chambers and allowed to habituate to the chamber for approximately 36 h. Respiration rates were measured by stopping the flow through the respirometer chamber and measuring the decline of DO concentration within the chamber. Test temperature was 25°C. The respiratory medium for this experiment was carbon-filtered, dechlorinated tap water from the same source as the holding and prior exposure tank water. Unlike the other experiments in this study, the pH of the respiratory medium was not adjusted, and was identical to the holding and treatment tank water.

Analysis of Metabolic Responses

Representative RMR:DO curves for treatment groups were constructed from mean values for the regression coefficients and COC and compared among sites. The fifth parameter (in this case, S_I) was redundant and was therefore computed from the other parameters to assure consistency.

Data were analyzed using the method described in Part 2. A segmented regression was fit to the RMR:DO curve for each individual test. Each line segment was characterized by a slope and an intercept. The coefficients of the equations of the fitted line segments, COC, MMS, and RMR_I were used as response parameters (See Figure 3, Part 2). None of the variables required transformation to meet the assumptions of normality, homogeneity of covariance, or homogeneity of variance. The variables were tested by stepwise variable selection for inclusion in a canonical discriminant model (STEPDISC procedure, SAS 1988). The selected variables were also compared among groups using ANOVA (Tukey option, $\alpha = 0.05$, GLM procedure, SAS 1988).

To examine the correlation of response variables with the energetic status of the acclimation groups, the weight change during the acclimation was compared among groups. The 0-day group of fish had no acclimation period and, therefore, no weight loss. The reductions in weight of fish during 4-day and 9-day acclimation periods were tested to determine whether they differed from zero, and whether they differed from each other. Fish were not fed during the acclimation period and, therefore, the weight of the fish was expected to decrease.

Chapter 3

RESULTS

Comparison of Group Responses

RMR:DO Curves

The representative RMR:DO curves for fish in all groups were similar, except in the oxygen-independent region (Figure 8). Curves differed little in the oxygen-dependent region, but increased holding time decreased metabolic rate in the oxygen-independent region. The 9-d group appeared to differ in the oxygen-independent region from both the 0-d and 4-day groups. The RMR:DO curves for the 0- and 4-d groups did not appear to differ from each other.

Response Parameters

Holding periods reduced mean RMR_I . Of the seven response parameters, the regression coefficients, COC, MMS, and RMR_I , the variable selection procedure retained only one, RMR_I , in the final response model. This response parameter reflects the metabolic differences evident among groups in the representative RMR:DO curves. With only one variable retained in the final response model, the canonical discriminant analysis was not conducted. Short holding periods had no significant effect on RMR_I . The mean RMR_I did not differ between 0-d and 4-d holding period groups. Longer holding periods resulted in significant differences in RMR_I among groups. The mean for the 9-d holding period group differed significantly (Tukey test, $\alpha = 0.05$) from the

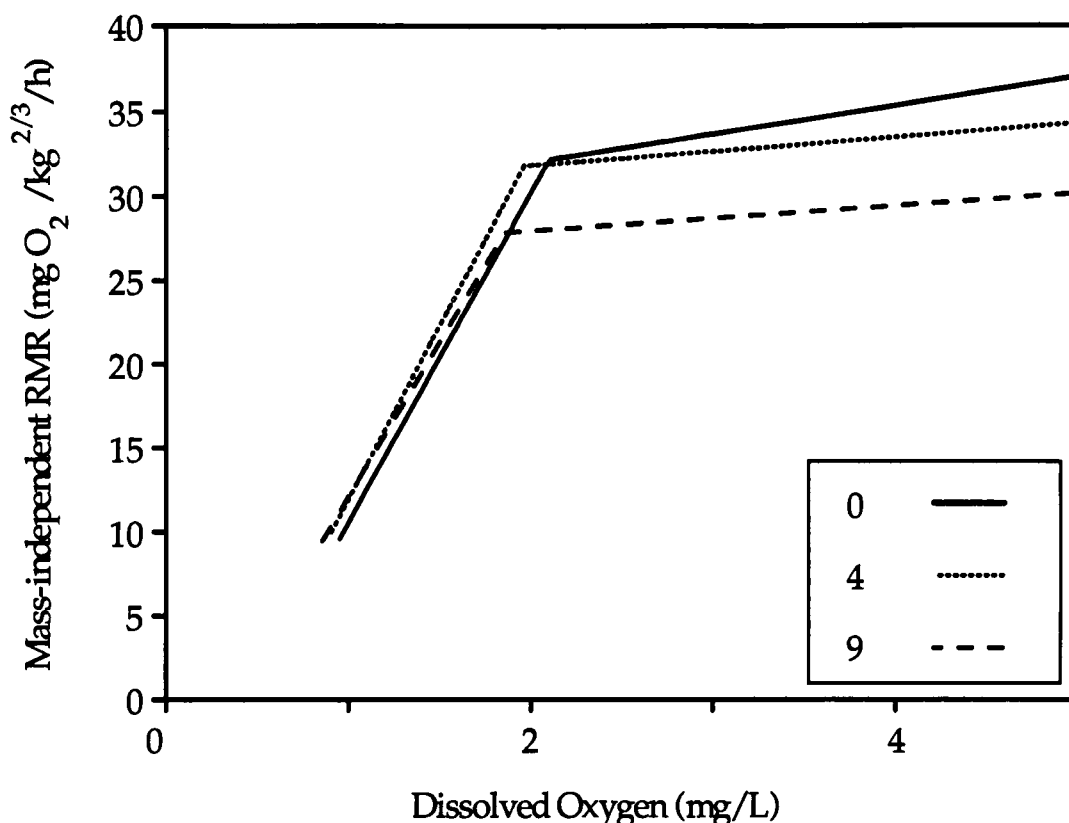


Figure 8. Representative RMR:DO curves for groups held at test conditions for differing lengths of time prior to testing. 0 = held 0 d at 25°C; 4 = held 4 d at 25°C; 9 = held 9 d at 25°C.

mean of each of the shorter holding periods (Table 6).

Weight Loss

Starvation during the holding periods resulted in weight loss. Longer holding periods produced greater percentage weight loss. No weight loss was measured for the 0-d group because there was no holding period during which weight loss could occur. The mean percentage weight loss was significantly greater than zero for 4-d and 9-d acclimation groups (Table 6). Mean percentage weight loss did not differ between 4-d and 9-d groups. Thus, the detectable influence of starvation appeared to have been complete by the fourth day.

Table 6. Comparison of effects of holding time on RMR_I and weight loss.

Parameter	Holding time (d)		
	0	4	9
RMR _I	34.6	33.4	27.8
Weight Loss	0	-4.6	-6.0

RMR_I expressed as mg O₂/kg^{2/3}/h. Weight loss expressed as percentage reduction of original weight. Means underlined by the same line segment do not differ significantly (Tukey test, $\alpha = 0.05$). The dashed line indicates that weight loss could not occur because there was no holding period.

Chapter 4

DISCUSSION

The effects of even the longest holding periods tested here on the RMR:DO curves were surprisingly limited. The oxygen-dependent line segments for the three holding period groups are nearly coincident, and COC did not change much with holding time. A single metabolic response parameter differed significantly among holding period groups. The mean oxygen-independent routine metabolic rate, RMR_I , of the 9-d holding period group differed from that of each of the shorter holding periods. No other metabolic response parameter differed significantly among holding period groups.

Even those response parameters found to respond strongly to temperature or food availability in the previous laboratory experiment (Part 2) did not differ significantly among holding periods. The metabolic differences among holding period groups due to temperature acclimation and starvation should be similar to the types of metabolic differences realized among temperature and food availability groups in the previous experiment, but of a lesser magnitude. Response parameters that responded strongly to the large treatment temperature differences (15 vs 30°C) in the experiment described in Part 2, such as MMS, did not differ significantly among holding period groups even though temperature acclimation states probably differed greatly for 0- and 9-d holding period groups. COC, which responded to food availability differences in the previous laboratory experiment did not differ among holding period groups even though starvation caused significant weight loss for 4- and 9-d groups.

Some aspect of the holding periods did alter RMR_I after sufficient time elapsed. The first possibility is temperature acclimation. Temperature transfer results in a progression of metabolic responses prior to the completion of acclimation to the final temperature. Prosser and Brown (1961) have classified metabolic responses following temperature transfer into three types of reaction. Immediately after transfer, an initial overshoot reaction results in temporarily extreme metabolic rates. Rates soon become stabilized at a non-compensated level that is slowly changed by acclimation until rates characteristic of full acclimation are reached. The downward trend in RMR_I with increasing acclimation is consistent with the changes expected due to progression from stabilized non-compensated rates to rates characteristic of full acclimation as thermal acclimation temperature rose toward 25°C as holding time prior to testing increased.

Many physiological and biochemical changes occur during acclimation (Hoar 1983). These changes are energy intensive and could increase metabolic rate during the acclimation period. As acclimation nears completion, the energy demands of this process should decrease, eventually reducing RMR_I to a lower value characteristic of full acclimation. The magnitude of energy requirements is likely to be small relative to the changes in metabolic rate due to the effect of acclimation state, however, or temperature transfer would rarely result in lower uncompensated rates for warm-acclimated fish relative to fully acclimated fish. Thus, changing acclimation state is a more likely cause of changes in RMR_I than are the changing energy demands of the acclimation process.

Starvation during acclimation periods also may have affected metabolic rate. Starvation reduces metabolic rate over time (Jobling 1981). The influence of food availability treatments on RMR_I in the previous laboratory experiment (Part 2) was not highly significant, especially in comparison to the influence of treatment temperature

differences. In that experiment, COC was the only measured metabolic parameter that was influenced more by food availability than by temperature, but COC did not differ among groups in the present experiment. Considering these results, temperature acclimation differences seem more likely than starvation or changing energy requirements to have caused the differences in RMR_I among groups in the present experiment.

Whether temperature acclimation, energy requirements, starvation, or some combination of the three are acting upon metabolism during holding periods prior to testing, the combined effects were not significant unless holding exceeded 4 days. Because the most extreme thermal acclimation differences that were tested affected only RMR_I , holding periods during the field experiment (≤ 2 d) probably had no effect on the comparisons of metabolic differences among field site groups. No change in the present method of testing is needed based on these results, because the field experiment (Part 3) employed holding times ≤ 2 d.

The present study did not establish whether holding fish for a sufficiently long period to allow acclimation to test temperature would decrease, or possibly, increase, the ability to detect other metabolic differences related to stress. Present results suggest that most response variables change little during holding periods. Further experiments are needed to determine if stress-related metabolic differences, such as those measured for fish among field site groups in the previous experiment, will diminish with time during acclimation to the testing temperature. Until such experiments are completed, the results of the present experiments do not indicate the need for changing the present holding and testing procedures. However, to err on the side of caution, fish should be tested soon after capture, if possible, or held for fewer than 4 days, when necessary.

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PART 5

SUMMARY

Chapter 1

SUMMARY AND CONCLUSIONS

The metabolic state of fish can be altered by environmental or anthropogenic factors. At least some of these alterations can be measured by challenging fish with an additional stressor, such as hypoxia. The performance of the individual during the challenge test reveals the influence of exposure history and test conditions. Challenge tests conducted under a standard set of non-stressful conditions, other than the challenge factor itself, reveal the effects of exposure history alone, because test conditions do not differ among exposure groups. The metabolic responses to hypoxic challenge indicate how metabolic performance has been altered by previous exposure conditions.

Changes in metabolic performance can be used as an indicator of previous exposure to stressors. Metabolism responds to many factors, however, and response differences must be demonstrated to indicate the effects of stressors, rather than some other factors. This study investigated the ability of metabolic responses to hypoxic challenge to detect stressors of interest, such as pollutants, in spite of the influence of environmental factors. The three experiments in this study investigated factors believed critical to the application of metabolic responses as indicators of exposure to stressors at field sites. One experiment investigated the influence of background environmental factor treatments (pH, temperature, and food availability), on metabolic responses to hypoxic challenge among groups of bluegill sunfish in the laboratory. Another experiment investigated the influence of pollution on metabolic responses of redbreast sunfish collected from field sites. The third experiment investigated how holding fish at

test conditions for differing lengths of time prior to testing could alter the metabolic responses of bluegill sunfish to hypoxia.

Results of the first experiment (Part 2) demonstrated that prior exposure of fish to environmental factors can be detected as changes in their metabolic responses to hypoxic challenge. Temperature treatments, in particular, caused highly significant differences in the measured responses. Food availability treatments also caused significant response differences. An interaction effect between temperature and food availability treatment factors also caused significant response differences among treatment groups. The multivariate comparisons of group mean responses, and the graphical representations of these comparisons, indicated how two treatment factors, temperature and food availability, jointly altered metabolic responses. Treatment differences in pH did not cause significant differences in the measured responses of fish to hypoxic challenge. The pH levels tested probably did not tax the homeostatic mechanisms sufficiently to result in measurable metabolic response differences. Multiple environmental factors can jointly influence metabolic responses to hypoxic challenge, and may limit the ability to detect other factors among sites with different environmental conditions.

The second experiment (Part 3) demonstrated that a single response parameter, COC, could be used to detect differences in resistance to hypoxia among groups of fish collected from field sites known to have different levels of pollutant inputs. Despite the possible influence of environmental differences among sites, COC differentiated polluted sites from the reference site. None of the other metabolic response parameters proved capable of differentiating among these sites. The ability of COC to differentiate polluted sites from a reference matched that of three indicators of fish and aquatic system health measured in other studies. The indicator parameters EROD, growth, and

IBI and COC each demonstrated significant differences between the reference site and each polluted site but did not, as a group, to provide a consistent ranking of relative magnitude of pollution stress among polluted sites.

The differences in COC among sites did not indicate reduced metabolic performance for fish collected at polluted sites. Lower mean COCs measured at polluted sites indicated that resistance to hypoxia had been increased by exposure to pollution. Exposure to stressors might be expected to decrease metabolic performance, and reduce the ability to resist hypoxia because of the energetic demands of responding to stressors. Testing under standard conditions may reveal the effects of compensation to stressors prior to collection and testing. The challenge test would reveal the improvement in performance resulting from compensatory adjustments, but this improved performance could only be interpreted as the result of an exposure to stressors by comparing it to a reference value. Thus, detecting prior exposure to stressors with this metabolic response parameter is not possible unless a reference group is available for comparison.

The third experiment (Part 4) demonstrated that routine metabolic rate for the oxygen-independent region of the RMR:DO relationship could be altered by long holding periods in the laboratory. These results indicate that the 2-d holding periods used for some fish in the field experiment probably had little effect on the outcome of the comparisons among groups. Short holding periods in the laboratory would not be expected to have much effect on the ability to detect differences among groups caused by exposure to stressors prior to collection. Although thermal acclimation and starvation during holding periods had little impact on metabolic responses to hypoxia, temperature and food availability differences prior to collection may have a greater influence on metabolic responses. The first laboratory experiment demonstrated that thermal history and food availability treatments strongly affected metabolic responses to hypoxia. The

third experiment focused only on the changes during holding, not on the influence of exposure history differences prior to the holding period. Short holding periods had little effect on metabolic responses to hypoxia, but extended holding periods did result in significant differences, and should be avoided.

The results of these three experiments indicate that the present technique was capable of detecting metabolic differences among groups of fish resulting from a range of exposure factors. Environmental factors, temperature and food availability, resulted in large differences after laboratory exposure to extremes of these factors. Environmental factor differences among field sites did not obscure the influence of prior exposure to pollution on groups of fish collected from field sites. Thermal acclimation and starvation during short holding periods had little effect on metabolic responses. These results suggest that metabolic responses to hypoxia, and COC in particular, may be useful in comparing exposure history differences among sites, even under the influence of normal variations in other environmental factors.

The results of these experiments have also provided insights on how the comparisons of metabolic responses among groups of fish might be improved. A flow-through respirometer system would allow better control of activity level during testing because repeated measures of oxygen consumption could be made at an oxygen concentration, eliminating the possibility that elevated activity would go unnoticed. It could also eliminate the influence of anaerobic metabolism in the oxygen dependent region of the RMR:DO curve. These improvements would also help generate data that could more easily be integrated into an energy budget. An energetic approach could help predict the effects of a metabolic alteration.

As a challenge test, progressive hypoxia can reveal how prior exposure conditions have altered the ability of organisms to tolerate additional stressors. The

metabolic response parameters can indicate other differences among exposure groups, but the implications of those differences have not been investigated fully here. Further study may increase the ability to predict the consequences of exposure history from the information contained in the metabolic responses to hypoxia. An increased understanding of the consequences of exposure history would improve the ability to protect populations of fish in dynamic aquatic systems.

VITA

Kenneth D. Ham was born in Dalton, Georgia on December 12, 1963. He attended elementary schools in Georgia, California, and South Carolina. Following completion of the 11th grade at Orangeburg-Wilkinson High School, Orangeburg, SC in 1981, he entered the College of Charleston, Charleston, SC. In September 1982 he transferred to the University of Tennessee, Knoxville, and in March 1986 graduated with high honors and as top graduate in the College of Agriculture with a Bachelor of Science in Wildlife and Fisheries Science. He entered Texas A&M University, College Station, in September 1986 and in December 1989 received a Master of Science in Wildlife and Fisheries Sciences. In August 1989 he entered the University of Tennessee, Knoxville, and in May 1994 received a Doctorate in Ecology.

The author is a member of the American Fisheries Society and the Society of Environmental Toxicology and Chemistry. He is also a member of the honor society, Phi Kappa Phi. After graduation, Mr. Ham will enter a post-doctoral position at the Environmental Sciences Division of Oak Ridge National Laboratory, Oak Ridge, Tennessee.