ONTOGENETIC CHANGES AND ENVIRONMENTAL HYPOXIA: RESPONSES OF TWO
FISH SPECIES TO LOW OXYGEN CONCENTRATIONS AT EARLY LIFE STAGES

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(ABSTRACT)

Hypoxia refers to any condition in which the water is less than fully saturated with oxygen. Although it is generally accepted that adults are more tolerant of hypoxic conditions than larval stages, there is little information to support this assumption. To determine whether reduced concentrations of dissolved oxygen (DO) affect fishes differently during various early life stages, I examined the responses of two species of fish (fathead minnows (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*)) exposed to low dissolved oxygen concentrations at different ages during the first 100 days post-hatch.

The changes in oxygen requirements and respiratory patterns that occur during ontogeny and exposure to hypoxia were observed. The results of this study suggest that the early larval stages appear to be at least as tolerant of short-term exposure to low dissolved oxygen concentrations as the older, more developed stages. Fathead minnows underwent a gradual transition from being metabolic conformers to regulators during development. Hemoglobin appeared to be playing a larger role in oxygen supply in the early post-hatch trout than in the minnows. Fathead minnow larvae produced relatively low concentrations of lactate upon exposure to hypoxia. Conversely, rainbow trout larvae exhibited significant increases in lactate concentration under similar conditions. This implies that there is a threshold oxygen concentration below which trout larvae utilize anaerobic metabolism to provide additional energy. Lactate dehydrogenase activity increased as the rainbow trout larvae aged, suggesting that they develop an anaerobic capacity which could be used to provide additional energy during hypoxia. The minnows did not exhibit this increase in activity.

The ability of larval fishes to detect and avoid hypoxic conditions was also examined. The overall trends suggest that throughout this period of development, both fish species gradually leave an area as the dissolved oxygen concentration decline. Both species appeared to leave the hypoxic areas with deliberate motions, indicating that a directed sensor system allowed them to detect oxygen gradients. The results suggest that a combination of physiological, biochemical, and behavioral mechanisms may allow fishes to cope with hypoxia.

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Background

Low concentrations of dissolved oxygen (DO) may cause physiological stress and even death in many fish species. As a result, the species richness and assemblages may change as species that are unable to tolerate the reduced DO concentrations are extirpated (Tonn and Magnuson 1982, Doudoroff and Shumway 1970). Hypoxia refers to any condition in which the water is less than fully saturated with oxygen. Because of the large number of parameters that may affect DO, hypoxia is a potentially widespread problem in aquatic habitats (Boutilier 1990). Lentic and lotic systems undergo dynamic seasonal and even daily changes in temperature, flow, depth, and light levels. These parameters are intricately linked to DO; consequently, DO concentrations may change quickly as these parameters fluctuate (Boutilier 1990).

Besides providing a useful ecological parameter of water quality for researchers, aquaculturists monitor DO concentrations in order to maximize survivorship in holding ponds (Morrissy et al. 1984). Smale and Rabeni (1995) reported that hypoxia tolerance does not differ with size in adult fishes. However, there is little information concerning the effect of low DO on the early life stages of fishes. A review of the literature by Rombough (1988) suggested that the effects of the lowered DO concentrations vary with the developmental stage and species, with younger fishes in general showing sensitivity to hypoxia. Breitburg (1992) observed that severe hypoxia caused high mortality of new naked goby (*Gobiosoma bosc*) recruits. Krammer and Smith (1962) reported that heightened mortality during the first two weeks of life makes this period vitally important in determining year class strength for largemouth bass (*Micropterus salmoides*). Dudley and Eipper (1975) proposed that exposure to low DO concentrations while fish are young may determine the strength of year classes. Rapid changes in body structure and physiology during early development, however, make it difficult to quantify the effects of hypoxia for specific life stages in fish. The differences in testing methodologies between experiments also complicates comparisons between experiments (Rombough 1988).

Although the ontogenetic period of greatest sensitivity to hypoxia varies with different species (Siefert and Spoor 1974, Spoor 1977, Spoor 1984, Barton and Taylor 1996), it is generally believed that the larval stage is most vulnerable (Doudoroff and Shumway 1970, Davis 1975). However, the most sensitive time for a fish exposed to hypoxia may be the period within the larval stage when the fish "switches over" from cutaneous respiration to gill-dominant respiration (Rombough 1988). Rombough (1988) implied that the timing of this switchover could be a primary factor in determining the timing of a species' maximum tolerance to hypoxia.

There is however, some concern about whether the developmental process in fish larvae is continuous, with changes occurring slowly, or discontinuous, with rapid changes occurring at set time points. Balon (1979) argued that discontinuous, or saltatorial, changes occur in the ontogeny of most fishes, and that distinct and "abrupt functional changes," occur at the start of each life stage. It is presently unknown whether changes in the respiratory mechanisms/abilities in developing fishes are saltatorial or continuous.

In addition to the development of gill respiration, there is the appearance of the oxygen-carrier hemoglobin in the bloodstream of the fish. Many fish species hatch without hemoglobin and depend on simple diffusion to supply oxygen needs until body size/activity becomes too great for oxygen demands to be met by diffusion alone (De Silva 1974). While the timing of hemoglobin appearance has been noted for the larvae of several fish species, such as walleye, *Stizostedion vitreum*, the relative importance of this oxygen-carrier in larval fishes has not been

investigated (McElman and Balon 1979). Although Cech and Moyle (1988) state that the importance of hemoglobin to most fishes is "difficult to overstate," Antarctic icefish can live without hemoglobin (Holeton 1970 1972) and goldfish can survive for days when their hemoglobin is bound to carbon monoxide (Anthony 1961). Holeton (1971 *a, b*) observed that adult rainbow trout (*Oncorhynchus mykiss*) were unable to survive for more than a few hours when their hemoglobin was bound by carbon monoxide, suggesting that these fish may require hemoglobin. The conflicting conclusions drawn from studies of adults and the small number of studies involving larvae indicate that more research on the value of this carrier molecule to developing fishes is warranted.

Rates of oxygen consumption have been found to increase dramatically in the period between fertilization and hatching (Rombough 1988). Peterka and Kent (1976) reported that the tolerance to low dissolved oxygen concentrations decreases from the embryonic to larval stages for northern pike (*Esox lucius*), smallmouth bass (*Micropterus dolomieu*), and bluegill (*Lepomis macrochirus*). While oxygen consumption rates for some species of fish larvae have been determined (e.g. Holeton, 1971a, Rombough 1988, Chulakasem et al. 1989, Walsh et al. 1989 1991), they were usually related to other values collected for the larvae under physical stress (e.g. exercise, temperature). Few studies have attempted to directly determine whether changes occur in oxygen requirements during ontogeny.

It has been debated whether fishes have the ability to detect and avoid hypoxic waters. Jones (1952) reported that random swimming and struggling caused by hypoxia-induced stress allows rainbow trout to exit a hypoxic area. Doudoroff and Shumway (1970) concluded in their review that many fishes do not leave hypoxic areas. However, Whitmore et al. (1960) reported that chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*O. kisutch*), largemouth bass (*Micropterus salmoides*), and bluegill (*Lepomis macrochirus*) avoid areas of low DO. Adults and large juvenile naked gobies (*Gobiosoma bosc*) migrate inshore during occasional episodes of offshore hypoxia according to Breitburg (1992), and rainbow trout (*O. mykiss*) and acclimated Atlantic cod (*Gadus morhua*) seek areas with cooler temperatures as DO concentrations fall (Schurmann et al. 1991, Schurmann and Steffensen 1992). These movements indicate that mobile fishes may move from one area to another in response to the DO content of the water. While many species are mobile, fishes at early life stages may not have the same capability of directed movement as the adults and may instead rely on water flow as their primary mode of movement. Furthermore, if fishes have varying tolerances to hypoxia at different life stages, movement away from areas with low DO concentrations may account for the physical separation of life stages exhibited by some species.

Upon entering hypoxic waters, an adult fish may deal with the low DO in one of two ways: "burst activity," by which the fish attempts to rapidly move away from the hypoxic area, or by remaining in the low DO area and decreasing physical activity and hence metabolic rate. As indicated by Schurmann and Steffensen (1994), each of these two mechanisms has advantages and disadvantages. Burst activity allows a fish to move away from an area quickly, but may raise the metabolic rate above a level that can be supported by the ambient oxygen supply, requiring the use of anaerobic metabolism. If the area of hypoxic water is large, the fish may be exhausted before reaching an area of higher DO. Decreasing the rate of activity may also cause problems for a fish in hypoxic waters because it could lead to a reduction in swimming speed, requiring the fish to spend a longer period of time in the hypoxic water.

Regardless of whether a fish swims away from a hypoxic area or reduces its activity, there is a metabolic cost associated with prolonged exposure to low DO. Hughes et al. (1983)

reported that carp exposed to low DO concentrations accumulate lactate in their blood and Burton and Heath (1980) observed that the threshold for the switch to anaerobic metabolism varies greatly among species. These and other studies suggest that a fish may become increasingly dependent on anaerobic mechanisms of energy production as it attempts to cope with severe hypoxia.

Hinterleitner et al. (1987) reported that changes in enzyme activities may be correlated with certain activities in larval fishes. They found that for several species, the activity associated with the aerobic enzyme citrate synthase decreased as the larvae grew during the first 100 days post-hatch, while the activity associated with the glycolytic enzyme lactate dehydrogenase increased. The rainbow trout (*O. mykiss*) used in their study however, proved to be a notable exception as the trout exhibited an increase in citrate synthase activity as they aged. With the exception of Hinterleitner et al. (1987) and Wieser et al. (1985), there have been few studies that have focused on the changes in the aerobic and anaerobic enzyme capacities in developing larval fishes.

Fish Species

Fathead minnows (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*) were tested for several reasons. The larval stages of development in these species are somewhat different, providing ecological and ontogenetic contrasts. According to Cech and Moyle (1988), trout larvae are "vestigial" because of they hatch with appearances and behaviors already similar to older juvenile fish. Conversely, fathead minnows emerge as relatively undeveloped individuals. Since many researchers (e.g. Knight 1963, Davis 1975, Tompkins and Gee 1983, also see review by Barton and Taylor 1996) have worked with fathead minnows and rainbow trout, a large amount of basic information exists concerning the life history characteristics and physiology of the adults. Although fathead minnows are widely used in toxicity tests, little information exists on larval tolerance to low DO concentrations. Rainbow trout are stocked in many areas as game fish and so information on larval DO concentrations could be valuable to hatchery programs seeking to increase yield.

The natural habitats of these two fishes are different in that fathead minnows are commonly found in shallow, slow moving waters (e.g. pools of small streams and marshes), and so may encounter unfavorable conditions such as hypoxia (Tompkins and Gee 1983). Because spawning occurs from May to August, increased water temperatures and drought conditions may expose the eggs and larvae to low dissolved oxygen concentrations for brief periods (Markus 1934).

In contrast, rainbow trout are found in small rivers and lakes that are usually cold and well oxygenated (Moyle and Cech 1988). The salmonids, in general, are considered to be quite sensitive to low DO (Barton and Taylor 1996). However, trout larvae remain in nests located below the sediment surface after hatching, and are dependent on the flow of water through the substrate for the supply of oxygen (Wickett 1954). Because the flow rates may be slower than flow rates in the water column, trout larvae may encounter hypoxic conditions before leaving the nest. Although fathead minnows and rainbow trout hatch in different environments, it is likely that fishes of both species face brief periods of hypoxia as larvae – a life stage that has received little attention in this regard. Additionally, the different times of the year that the larvae of these species were available from hatcheries allowed for fathead minnows to be tested in the spring and rainbow trout in the late summer. This permitted the experiments to be conveniently completed without overlap of time and lab space.

General Overview

Given the scarcity of information that exists concerning the effects of hypoxia on juvenile fishes, I examined the responses of two species of fish exposed to low DO concentrations at different ages during their first 100 days post-hatch. The results are described in the following chapters. Chapter 1 discusses the changes in oxygen requirements and respiratory patterns that were observed. The results of biochemical assays measuring the whole body lactate concentrations are also discussed in the context of how developing fishes utilize anaerobic metabolism under conditions of severe hypoxia. Chapter 1 also includes a discussion of the changes in the activities of the oxidative enzyme citrate synthase and the glycolytic enzyme lactate dehydrogenase during early development, and how these changes relate to the anatomy and physiology of the developing fishes. The focus of chapter 2 is larval fish behavior with the discussion centering on questions of whether young fishes can detect and avoid areas of low DO. Chapter 3 includes a summary of the results and a brief exploration of possible future work involving hypoxia and fish.

CHAPTER 1:

ONTOGENETIC CHANGES AND ENVIRONMENTAL HYPOXIA: RESPONSES OF TWO
FISH SPECIES TO LOW OXYGEN CONCENTRATIONS AT EARLY LIFE STAGES

(ABSTRACT)

Larval fishes may use a suite of physiological mechanisms to tolerate short-term exposure to hypoxia. Hemoglobin, biochemical mechanisms, alterations in ventilation patterns, and changes in behavior may all act in concert to allow the fish to survive brief periods of hypoxia. Fathead minnows (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*) were exposed to low dissolved oxygen concentrations at different ages over the first 100 days post-hatch and their responses to hypoxia were monitored. The fathead minnows appeared to undergo a gradual transition from being oxyconformers towards becoming oxyregulators as they developed. Hemoglobin appeared to play a more vital role at an earlier age in the rainbow trout than in the fathead minnow. Fathead minnows less than 17 days of age likely utilized cutaneous respiration for the majority of their oxygen uptake, while older minnows increased breathing rates as dissolved oxygen concentrations declined. In contrast, rainbow trout larvae coped with hypoxia by increasing breathing amplitude.

Increased lactate concentrations in the rainbow trout larvae indicate that there was a threshold oxygen concentration below which larvae utilized anaerobic metabolism. Increased lactate dehydrogenase activity suggests that trout develop an anaerobic capacity which may be used to provide energy under conditions of low dissolved oxygen. Fathead minnows did not appear to develop this anaerobic capacity during the first 100 days post-hatch. The observation of larval hypoxia tolerance challenges the assumption that larval fishes are more sensitive to low oxygen than their adult counterparts.

Introduction

Adult fish may employ a variety of physiological mechanisms to tolerate water that is less than fully saturated with oxygen, a condition known as hypoxia. To meet oxygen requirements, the adults of some fish species increase ventilation volume during hypoxia. This is achieved by a combination of increases in breathing frequency and amplitude (reviewed by Shelton et al. 1986). Hemoglobin is a critical oxygen carrier in mammalian systems and facilitates survival of hypoxia (Schmidt-Nielson 1990). Hemoglobin is also present in many fishes, however its role in oxygen supply is unclear. The adults of some fish species (e.g. icefish) can survive without hemoglobin, while adults of other species (e.g. rainbow trout) die when hemoglobin is inactivated as an oxygen carrier (Holeton 1970, 1971*a*). When oxygen becomes limiting, increased anaerobic metabolism may provide the fish with an alternative form of energy production. In this situation, lactate accumulates and can cause fatigue and disrupt the pH of body fluids (Fievet et al. 1988, Hill and Wyse 1988). Some species may lower metabolic rates as dissolved oxygen (DO) concentrations decline in an attempt to reduce overall energy demand. Diverse species of fish utilize a combination of these mechanisms to deal with environmental hypoxia (Kramer et al. 1978). The degree to which fish can survive hypoxia is dependent on the capacity to invoke these mechanisms under periods of oxygen stress.

Organisms that reduce oxygen consumption rates in response to declining environmental DO concentrations are termed metabolic conformers. In contrast, metabolic regulators maintain oxygen consumption rates despite variations in DO concentrations (Heath 1995). Metabolic conformity during hypoxia has been observed in the adults of many species of fish and may assist the fish in surviving low oxygen levels (Marvin and Heath 1968, Gerald and Cech 1970, Hughes et al. 1983). The metabolic conformers catfish (*Ictalurus punctatus*) and carp (*Cyprinus carpio*) commonly encounter hypoxic areas within their aquatic environments. In apparent attempts to continue to function in low DO concentrations, these fishes reduce their oxygen consumption rates (Marvin and Heath 1968, Hughes et al. 1983). DeSilva and Tytler (1973) reported that plaice and herring larvae appeared to change from metabolic conformers to metabolic regulators after metamorphosis. This indicates that developmental milestones may alter the manner by which a fish responds to hypoxia.

Changes in ventilation patterns in response to hypoxia have been investigated in several fish species including trout (Holeton 1971 *a, b,* Boutilier et al. 1988), catfish (Gerald and Cech 1970), and carp (Lomholt and Johansen 1979). According to Holeton and Randall (1967 *a*) and Stevens and Randall (1967), adult trout increase breathing amplitude more than breathing rate to compensate for hypoxic conditions. According to Johansen et al. (1967), increasing ventilation amplitude may be more energy efficient for the fish than increasing breathing rates because of the high density of water and the large amount of energy required to move it over surfaces. It has not been determined however, whether the ability to alter breathing rate and amplitude emerges as muscle structure develops or if it is innately present from hatch.

Fox (1954) first suggested that most fish use hemoglobin as a means of oxygen supply only in "oxygen emergencies." Anthony (1961) reported that goldfish could survive for several days in the presence of carbon monoxide, a substance known to block the oxygen carrying ability of hemoglobin. These findings sparked a debate over the importance of hemoglobin in supplying oxygen to fishes. A decade later, it was found that icefish could live without hemoglobin (Holeton 1970, 1972). Pelster and Burggren (1996) reported that the binding of

hemoglobin by carbon monoxide in developing embryos of zebra fish did not impact survival. However, Holeton (1971 *a, b*) found that blocking a high percentage of the adult trout's hemoglobin with carbon monoxide resulted in death after several hours. This implies that the function of hemoglobin was important to the survival of adult rainbow trout. Following Holeton's (1971 *a, b*) discussion of trout hemoglobin, it was assumed prior to the initiation of the current study that if oxygen consumption is depressed by carbon monoxide (CO), then hemoglobin is playing a role in carrying oxygen.

Lactate, a by-product of anaerobic metabolism, increases in adult fishes in response to various stressors including hypoxia (Burton and Heath 1980, Dunn and Hochachka 1986, Wieser et al. 1986). Klinger et al. (1982) observed that fathead minnows were able to survive over the winter in an ice-covered lake with seasonally low DO concentrations. They hypothesized that the minnows survived through increased use of anaerobic pathways for the production of energy. The long-term use of anaerobic metabolism is not efficient for the organism since this form of metabolism produces less ATP per molecule of glucose than aerobic metabolism. Additionally, anaerobic metabolism results in the buildup of potentially toxic by-products, such as lactate. Burton and Heath (1980) reported that brown bullhead adults did not increase use of anaerobic metabolism except at extremely low DO concentrations. This metabolic alteration was coupled with a decrease in energy demand to increase survival. In the same study, however, rainbow trout adults switched to a more anaerobically driven metabolism at less hypoxic DO concentrations than the bullhead. This implies the trouts' limited mechanisms for oxygen acquisition had been saturated. However, anaerobic metabolism supplied the energy necessary for the trout to survive for a limited period of time past this point. The short-term use of anaerobic metabolism may allow fishes to tolerate brief periods of hypoxia while maintaining metabolic rates necessary for survival (Hochachka and Somero 1971, Heath 1995).

Brief use of anaerobic metabolism may confer an advantage to fishes exposed to short episodes of hypoxia. Therefore, key glycolytic enzymes must be present during normal conditions to provide anaerobic capacity. It is possible that differential activity of glycolytic and oxidative enzymes may be exhibited during different life stages since larval fish experience changes in the relative amounts of aerobic (red) and anaerobic (white) muscle (Nag and Nursall 1972, Forstner et al. 1983). Elevated activity of the glycolytic enzyme lactate dehydrogenase (LDH) is indicative of high anaerobic capacity (Torres and Somero 1988). Compared to fishes with less LDH activity, fishes with high LDH activity are more likely to have the capability to invoke anaerobic metabolism. Increased anaerobic capacity may confer increased tolerance of short-term hypoxic events by providing an alternate pathway for ATP production, thus generating the energy necessary to sustain metabolism (Hochachka et al 1977).

Considerable research has been performed to elucidate the oxygen requirements of adult fishes of diverse species (e.g. Beamish 1964, Boutilier et al. 1988, Cech et al. 1979) and several major reviews of this literature have been published (Doudoroff and Shumway 1970, Fry 1971, Hughes 1973, Davis 1975, Rombough 1988 *b*, Heath 1995). In general, it has been assumed that the young of most species are less tolerant of low DO concentrations than their older counterparts (Doudoroff and Shumway 1970, Davis 1975). However, Shepard (1955) noted that the hypoxia tolerance of brook trout (*Salvelinus fontinalis*) did not change during the first year of life. Respiration patterns have not been extensively studied throughout fish development, but are generally speculated to change in a way that contributes to increased hypoxia tolerance with increasing age.

With the exception of the work by Holeton (1971 *b*), Brungs (1971), Rombough (1988 *b*), and Marty et al. (1995), most studies focused on adult or juvenile fishes and did not include the early stages of larval development. In the cases in which oxygen consumption was measured for the larvae (e.g. Rombough 1988 *b*, Chulakasem et al. 1989, Walsh et al. 1989, 1991), rates were usually collected during conditions of physical stress (e.g. exercise, high/low temperatures). It is therefore difficult to make ontogenetic comparisons based on these limited studies.

My overall objective was to investigate the development of respiratory responses in two diverse species of fish exposed to periodic hypoxia. My specific objectives were:

1) to monitor respiration changes with exposure to declining DO concentration by measuring oxygen consumption and breathing rate.

2) to determine the relative importance of hemoglobin as an oxygen carrier during ontogeny by comparing oxygen consumption rates of untreated fish with oxygen consumption rates of fish that were treated with carbon monoxide to competitively block the binding of hemoglobin.

3) to determine the utilization of anaerobic metabolism during exposure to different DO concentrations by quantifying lactate accumulation in the fishes.

4) to determine the anaerobic and aerobic capacities during ontogeny by measuring lactate dehydrogenase and citrate synthase activity in the fishes.

Study species

Fathead minnows (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*) were used as test species because adult fathead minnows are generally considered to be relatively tolerant to hypoxia, while rainbow trout adults are relatively sensitive to low oxygen conditions (Davis 1975). These two species also differ greatly in their developmental patterns and in their hatching environments.

Fathead minnows lay their eggs on the underside of logs, branches, and rocks in the shallows (<1 meter deep) of ponds and pools in slow moving riverine systems (Nelson 1992). Wynne-Edwards (1932) noted that the male fathead minnow, which guards the egg mass, agitates the surrounding water in a likely attempt to keep them well oxygenated. The eggs hatch into free-swimming larvae after an incubation period of approximately 5 days. Because spawning occurs from May-August, rising water temperatures and drought conditions may cause the eggs and larvae to encounter periodic hypoxic events (Markus 1934).

Adult rainbow trout use their tails to create shallow depressions in loose gravel areas in which they lay eggs during the spawning period from February to early May. The eggs are covered with the substrate and the nest, termed a redd, is left unguarded. The incubation time for the eggs ranges from 44-101 days depending on water temperature (Jenkins and Burkhead 1994). After hatching, the larval trout remain in the redd for a period of 2-4 weeks, consuming only their yolk sacs to meet nutritional needs. After the yolk sacs are consumed, the trout larvae perform swimup, and emerge from the redd to rest near the bottom in hollows between stones. In these areas, the larvae can feed exogenously and be protected from being washed downstream (Sedgwick 1982). While in the redd, the trout larvae are dependent on the interstitial flow of water between the substrate particles for the delivery of oxygen (Wickett 1954). Because interstitial flow rates may be slower than the flow rates of the water column, DO concentrations may be relatively low in the redd. As a result, trout larvae may encounter hypoxic conditions, especially before swimup (Sowden and Power 1985).

Although fathead minnows and rainbow trout eggs hatch in different environments, it is likely that the larvae of both organisms face periodic hypoxic events during early development. While it is probable that some of the mechanisms these fishes use to deal with hypoxia are similar, the differences in environments and developmental patterns may cause the fishes to respond with different physiological tools. This paper focuses on the development of the mechanisms that these fishes use to cope with hypoxia.

Materials and Methods

Organisms and Holding Facilities

Rainbow trout larvae < 24 hours post hatch were obtained from the state fish hatchery in Paint Bank, Virginia in July 1997 and 1998. Fathead minnow larvae < 24 hours post hatch were obtained from Sachs Systems Aquaculture in Florida in February, 1997 and March, 1998. Both species of fish were reared, and all experiments were conducted, in dechlorinated Blacksburg, Virginia municipal water (average pH~8.0 and hardness ~50mg/L $CaCO_3$). Upon arrival at Virginia Tech, fish were divided into roughly equal numbers and placed in modified 1-L Nalgene^{TM} beakers that served as holding tanks. A large hole was cut in the sides of each beaker and was covered with Nytex^{TM} mesh that allowed the circulation of water without permitting the fish to escape. The beakers were suspended in a 500-L Min-o-cool tank^{TM}, and a constant flow of water was pumped through the beakers. Airstones were placed in each beaker and the dissolved oxygen concentration within the beakers was maintained near saturation. At the start of the study, each beaker contained approximately 150 larvae, but the numbers dropped daily as fish were used in experiments and died at an expected attrition rate. Fathead minnows were held at a water temperature of 22 ^{o}C $\pm$ 2 ^{o}C, and rainbow trout were held at a water temperature of 14 ^{o}C $\pm$ 1 ^{o}C. The fathead minnows were fed a mixture of *Artemia* and ground TetraMin^{TM} flake food twice a day for the first three weeks, and once a day thereafter. Because the yolk sac supplied their nutritional needs, the rainbow trout were not fed for the first 10 days post-hatch. The trout were then fed a mixture of *Artemia* and ground trout chow twice a day for the next two weeks, and then once a day thereafter.

Oxygen Consumption

To measure oxygen consumption, fish were sealed in glass containers and the dissolved oxygen concentration was measured every 10-30 minutes. The sizes of the test chambers were selected based on the size of the age class under study. The chambers ranged in size from a shot glass (22 ml) to a baby-food jar (96ml). Chambers were fitted with a rubber stopper with a hole through the center of the rubber stopper to permit the placement of a YSI^{TM} DO probe (model 5750) in the chamber. The probe was connected to a YSI^{TM} DO meter (Models 54A, or 57) which was used to monitor the DO concentrations within the chambers. Because the probe required the water to be moving for accurate sensing, a micro-stir bar (12.5 mm in length) was used to circulate the water. A small plastic jacket was constructed and placed over the DO probe, allowing water to circulate through the chamber and probe while preventing the fish from having contact with the micro-stir bar. The jacket also prevented the formation of a current that the fish would have to swim against. Water was added to each of the three replicate chambers and the fish (all of similar size) were then added using a dropper or net, depending on the size of

the fish. The number of fish included in each chamber ranged from 2-30 for the fathead minnows and from 1-9 for the rainbow trout.

To reduce the DO in the chamber over a reasonable time period, the number of fish used in each experiment during the course of the tests was varied. Care was taken to remove all air bubbles from beneath the rubber stopper/DO probe/jacket combination when it was inserted in the chamber. The tops of the chambers were wrapped tightly in parafilm. The sealed chambers were placed in a Plexiglas^{TM} tank that served as a circulating water bath to keep the temperature constant during the experiment (22 ^{o}C $\pm$ 2 ^{o}C for fathead minnows, 14 ^{o}C $\pm$ 1 ^{o}C for rainbow trout). Three stir plates were placed below the water bath to drive the micro stir bars in the chambers. Dissolved oxygen concentrations were measured every 10-30 minutes. The probe was disconnected from the meter between readings to prevent oxygen consumption by the probe. Preliminary control studies indicated that changes in oxygen concentration in the chambers without fish was negligible within the time frame of the experiments. The amount of oxygen consumed was therefore calculated to be the difference in measured DO concentrations between readings. After the last fish died, the DO concentration was measured and the fish were removed from the chamber, placed in a 60 ^{o}C drying oven for at least 24 hours, and weighed. The amount of oxygen consumed (mg/l) per unit time was calculated for the total dry weight of the fish in each replicate.

To determine the relative importance of hemoglobin during development, fish were exposed to carbon monoxide and oxygen consumption rates were measured. Carbon monoxide was used to bind hemoglobin because the hemoglobins of teleosts have a greater affinity for this gas than oxygen (Anthony 1961, Holeton 1971 *a*, *b*). In addition, Holeton (1971 *a*, *b*) reported no interference by 5% CO on tissue metabolism in larval rainbow trout. Therefore, this concentration was used throughout the study. Fish were placed in a 1-L beaker of water and exposed to a mixture of 5% carbon monoxide (CO) and 95% air which was generated by a gas-mixing flow meter (Cameron Instrument Company model GF3mp). The fish were exposed for approximately 90 minutes. It was noted in the current study that longer periods of exposure of the older fish could cause death, which agreed with the findings of Holeton (1971 *b*). Therefore, older rainbow trout were exposed to the mixture for a shorter period of time. The fish were transferred in the CO-treated water to the chambers, and the oxygen consumption tests were performed as described above.

Breathing Rate

To measure breathing rate, the fish were sealed in plastic chambers and the number of opercular movements in a 10 second period were observed through a dissecting scope. Two plastic 4.2-L tanks with tightly fitting lids served as the water sources for the breathing rate experiments. Water was added to the two tanks that were then placed in a water bath for temperature control. Below the water bath were two stir plates that drove stir bars (length = 2.5 cm) in the tanks to keep the water constantly circulating. A YSI^{TM} probe, (model 5750) connected to a YSI^{TM} DO meter (model 54A and 57), was inserted in a hole in the lid of the tanks to keep a continuous record of the DO concentrations within the source tanks. Water from these tanks was gravity-fed through Tygon^{TM} tubing (size 14) to the inlets on the test chambers.

The round test chambers were constructed from clear plastic 32-ml containers. Each chamber was divided by a piece of Plexiglas^{TM} which served as a wall to separate two equal halves. In each half, an 18-gauge syringe needle was inserted through the side to allow for an equal inflow of water (flow rate = 250 ml/hour). A screw-on lid was loosely attached to allow a

small amount of water to overflow from the chamber. Care was taken to ensure that no air bubbles remained trapped beneath the lid. After setup, the fish were allowed to acclimate for approximately five minutes before measuring breathing rate. Preliminary studies indicated that the breathing rates for the fish stabilized within this time period since readings taken more than 30 minutes later were similar.

Each week, eight fish of similar size were placed in the chambers (one fish in each half, four chambers total). The chambers were placed in a shallow Plexiglas^{TM} tray that was located on the stage of a dissecting microscope. The number of opercular movements made by each of the fish was monitored for 10 seconds and recorded. By slowly moving the tray around the stage, it was possible to observe all of the fish sequentially with minimal disturbance of the fish. Observations of each fish were made three times over a 30-minute period (at 0, 15, and 30 minutes) before lowering the DO to the next concentration. After an initial 30-minute period during which all containers received oxygen saturated water, the DO concentration in the tanks was lowered using nitrogen gas regulated through a gas-mixing flow meter. This DO concentration was maintained for 30 minutes. Every 30 minutes, the DO concentration in the tanks was lowered in a stepwise fashion. To keep the flow rates equal throughout the experiment, the source tanks were topped off with a small amount of oxygenated water at the end of each 30-minute interval. The DO concentrations used for the experiments involving the fathead minnow were 8, 6, 4, 2, 1, and 0.5 ppm, while those used for the rainbow trout were 10, 8, 6, 4, 2, 1, and 0.5 ppm.

Lactate Concentration

To determine the effects of low DO concentrations on anaerobic metabolism, whole-body lactate concentrations were measured in fish exposed to different DO concentrations. The same chamber system described for breathing rate measurement was used for this test. Each week, 60-180 fish (the number used inversely related to size) of similar size were sealed in 100-ml modified plastic beakers (10-30 fish in each container, six containers total) and allowed to acclimate for approximately five minutes. An 18-gauge syringe needle in the bottom sidewall of the container supplied the chamber with water at a flow rate of 1500 ml/hour. A Plexiglas^{TM} top provided a barrier to oxygen penetration. A hole was cut in the center of the top and was plugged with a small rubber stopper. An 18-gauge syringe needle penetrated the midpoint of the stopper. This needle allowed excess water to flow out from the top of the chamber, while preventing the fish from contact with the air.

The chambers were placed in a shallow Plexiglas^{TM} tray located 1.1 m below the source water tanks. After an initial 20-minute period during which all chambers received water saturated with oxygen at the test temperature, the DO concentration in the source tanks was lowered using nitrogen gas, which was regulated by a gas-mixing flow meter. This DO concentration was then maintained for 20 minutes. After each 20 minute period, the DO concentration in the tanks was lowered in this stepwise fashion. To keep the flow rates equal throughout the experiment, the source tanks were topped off with a small amount of oxygenated water at the end of each 20-minute interval. Every 20 minutes, the fish from one of the containers were quickly poured from their chamber and snap frozen in liquid nitrogen. The fish were stored in tubes at –20 ^{o}C. The DO concentrations in the experiments were the same as those used in the breathing rate experiments.

Fishes were assayed for whole body lactic acid using a modified enzymatic method of Noll (1984). All chemicals were obtained from Sigma Chemical Company. The fishes were

removed from the freezer, weighed, and placed on ice until assayed. The fishes were homogenized by hand in 30 ul of 0.6-mol/L perchloric acid. The solution was neutralized with 50 ul of 2M potassium bicarbonate (KHCO$_3$). As the size of the fishes increased, proportionately larger volumes of homogenation buffer neutralization solution were used. The neutralized samples were centrifuged at 3000 g for 15 minutes at 4 ^{o}C to remove insoluble material. Reagents were added to the cuvette in the following order: 134 ul of glutamate buffer (0.52 mM, pH 8.9), 20 ul of 24 mM nicotinamide-adenine dinucleotide (B-NAD), 20 ul of cleared homogenate, 10 ul of 80kU/L alanine aminotransferase (ALT), and 392 ul of deionized water. The contents were mixed thoroughly and incubated at 25 ^{o}C for 10 minutes. The initial absorbance was measured at 365nm, then 10 ul of 550 kU/L of lactate dehydrogenase (LDH) was added to the cuvette to facilitate the reaction. Absorbance was measured periodically until a constant final absorbance was reached. The difference between the final and initial absorbance was determined to calculate lactate concentration. A Kruskal-Wallis analysis of variance was used to determine whether lactate concentration varied significantly ($p < 0.05$) upon exposure to declining DO concentrations in fish of the same age. In the cases in which significant differences were found, a Dunnett's multiple comparison test was used to determine whether the lactate concentrations of fish exposed to declining DO concentrations differed significantly from lactate concentrations of the same aged fish in the normoxic "control" group. The DO concentrations in the control groups were 8 and 10 ppm for the fathead minnows and rainbow trout, respectively.

Anaerobic and Aerobic Metabolism

To determine whether developmental changes occurred in the anaerobic and aerobic capacities of the organisms, whole-body assays for lactate dehydrogenase and citrate synthase were performed. Each week during the first 100 days post-hatch, up to 120 fish (the number used dependent on size) were removed from their source tanks and snap frozen in liquid nitrogen. From 1 to 5 fish (depending on size and species) were pooled, frozen, and stored in tubes at –20 ^{o}C until analyzed for both lactate dehydrogenase and citrate synthase. Chemicals and enzymes used for determination of both lactate dehydrogenase and citrate synthase were obtained from Sigma Chemical Company. The fish were removed from the freezer, weighed, and placed on ice until assayed. The fish were homogenized by hand in 500 ul of 50 mM phosphate buffer and the volume brought to 1000 ul with buffer. The samples were vortexed for 1 second and centrifuged for 15 minutes at 3000 rpm. Bio-Rad^{TM} protein assay reagent was used to determine protein concentration of each sample by a modification of the method of Bradford (1976). Standard curves were generated using bovine serum albumin. After 5 minutes, the absorbance was measured at 595 nm and the amount of protein in each sample was determined from the standard curve.

Determination of Lactate Dehydrogenase

Fish were assayed for lactate dehydrogenase following a modified method of Vassault (1984). A Tris/NaCl/reduced nicotinamide-adenine dinucleotide (NADH) stock solution (500 ul, 80 mM Tris pH~7.2, 200 mM NaCl, and 0.2 mM NADH, pH~7.2) was placed in a cuvette. The supernatant from the centrifuged sample (1 ul) and 100 ul of a Tris/NaCl/pyruvate stock solution (80 mM Tris, pH ~7.2, 200 mM NaCl, 1.6 mM pyruvate) was added to the cuvette. The contents of the cuvette (601 ul total volume) were mixed, and the linear decrease in absorbance at 339nm due to the oxidation of NADH was immediately determined. The linear decreases in absorbance

were recorded every 30 seconds for 4 minutes. The difference between the initial and final reading was used to calculate the LDH activity (U/mg protein) for each sample.

Determination of Citrate Synthase

Fish were assayed for citrate synthase using a modified method of Srere (1969). Reagents were added to the cuvette in the following quantities and order: 530 ul of deionized water, 300 ul of Tris/Ethylenediaminetetraacetic acid (EDTA)/5,5[1]-dithio-bis (2-nitrobenzoic acid) (DTNB or Ellman's reagent) solution (200mM Tris/5mM EDTA/0.333mM DTNB), 60 ul of S-acetyl Coenzyme A (2.09mg/ml in Tris/EDTA pH~7.5) and 10 ul of the supernatant from the cleared sample. The contents (1000 ul total volume) were mixed and the absorbance read every 30 seconds for 4 minutes at 412 nm. 100 ul of oxalacetic acid (OAA 0.66 mg/ml in Tris/EDTA, pH ~ 7.5) were added to the cuvette to start the reaction. The contents were mixed, and the increase in absorbance due to the interaction between reduced coenzyme A and DTNB was measured every 30 seconds for 4 minutes. The changes in absorbance that occurred between the initial (time 0) and the final reading (time = 4 minutes) were determined for the readings before and after the addition of OAA. The difference between these two values was used to calculate the CS activity (U/mg protein).

Results

Physical observations

Over the first 100 days post-hatch, the wet weights of the fathead minnow ranged from approximately 0.0010 g – 0.4300 g while the wet weights of the rainbow trout larvae ranged from approximately 0.0100-1.500g. Representative age-weight relationships are shown for fathead minnows and rainbow trout larvae from the current study in Figures 1A) and 1B), respectively. High mortality did not occur in either species over the course of the study, indicating that the holding facilities and care were adequate.

Oxygen Consumption Patterns

During development, both fathead minnows and rainbow trout appeared to be primarily metabolic conformers (Figures 2-5). The decreases in oxygen consumption rates concomitant with the decline in DO concentrations indicated that the larvae of both species lowered their metabolic rates in response to hypoxia. The patterns of oxygen consumption indicated that the minnows may have been undergoing a transition toward becoming metabolic regulators (Figures 2-3). However, minnows less than 100 days post-hatch were not yet sufficiently developed to maintain a constant oxygen consumption rate during hypoxia. At the stages tested, minnows had not yet fully become metabolic regulators.

Breathing Rate

Fathead minnows older than 17 days greatly increased their breathing rates as DO concentrations declined. Depending on the age of the minnow, mean breathing rates at a DO concentration of 1-2 ppm were 1.5-2 times the rate of the fish in normoxic water at 8 ppm (Figure 6). Minnows < 17 days old, which demonstrated slight increases in breathing rate, proved to be a notable exception. As DO was lowered, breathing rates increased until a DO concentration of 1-2 ppm was reached, after which they quickly declined as the fish expired. In

comparison to the older minnows, the rainbow trout of all ages tested did not dramatically increase their breathing rates in response to declining DO concentrations. In fact, 87 day old rainbow trout reduced their breathing rates as DO concentrations declined (Figure 7).

Fathead minnow larvae were observed to move to the surface of the chamber as the DO concentration fell below 4 ppm (Figure 8). The surfacing response was strongest at 1-2 ppm, with the majority of the minnows present at the surface. The rainbow trout of all ages tested did not appear to surface in response to hypoxia.

Although breathing amplitude was not quantified in this experiment, obvious differences in this parameter were observed in the two fishes. As the rainbow trout aged, they exhibited marked increases in breathing amplitude in response to decreased DO concentrations. In contrast, a dramatic increase in breathing amplitude was not observed with the developing fathead minnows.

Importance of Hemoglobin

Fishes were exposed to the carbon monoxide regime to determine the role of hemoglobin in hypoxic conditions. Exposed minnows nine days of age were unable to survive at oxygen concentrations below approximately 3 ppm (Figure 2). By 65 days of age, the minimum oxygen concentration for survival of exposed minnows increased to approximately 5 ppm (Figure 3). This concentration (5 ppm) continued to be the value for the lower limit for survival throughout the ages tested. For minnows 9-11 days of age, there was little difference in the minimum oxygen concentration (~3 ppm, ~2 ppm, respectively) for fish exposed to CO compared to fish that were not exposed to CO. As the fathead minnows developed, the minimum oxygen concentration for survival of fishes exposed to CO increased (e.g. 4 ppm for 23 day old minnows; Figure 2). However, the minimum DO concentration did not appear to change with development for unexposed fishes, but remained between 1 and 2 ppm (Figures 2-3).

Rainbow trout of all stages of larval development that were exposed to carbon monoxide died at higher oxygen concentrations, in contrast to trout that were not exposed to carbon monoxide. As the trout developed, the minimum required oxygen concentration for those fish exposed to carbon monoxide increased from approximately 4 to 6 ppm (Figures 4-5). The 2-day-old rainbow trout exposed to carbon monoxide died at a markedly higher oxygen concentration (~6 ppm) than did fish that were only a few days older (~4 ppm) (Figure 4). This implies that hemoglobin plays a large role in the early larval stages of trout.

Lactate Concentrations

The Kruskal-Wallis test indicated, with all ages tested, no significant difference between the lactate concentrations of minnows exposed to normoxic water (8 ppm) and those exposed to hypoxia. With all ages tested, lactate concentrations in the minnows did not rise significantly as DO declined. This indicates that there was no threshold DO concentration at which anaerobic metabolism started to be highly utilized (Figure 9).

In contrast, lactate concentrations in the rainbow trout held under normoxic conditions (DO=10) were significantly lower than the lactate concentration in trout exposed to the low DO conditions. Lactate concentrations generally increased as the DO concentration dropped below 4 ppm, indicating that around this DO concentration, anaerobic metabolism started to play a role in meeting some of the energy needs of the trout (Figure 10).

Anaerobic and Aerobic Metabolism

The series of lactate dehydrogenase assays at different ages showed that the activity of LDH increases in both species. LDH increased approximately 10-fold in the fathead minnow during the course of development (Figure 11A). In rainbow trout, the activity of LDH increased approximately 20-fold from hatch to day 60 (Figure 11B). The increased LDH activity in the trout may contribute to the greater tendency of this species to produce lactate under hypoxic conditions. Although LDH activity increased as the fathead minnow aged, it does not appear that the fish utilized anaerobic metabolism under hypoxic conditions because there is no corresponding increase in lactate.

The citrate synthase assays demonstrated that the activity of this aerobic enzyme increases during development in both species. Activity remained approximately the same in fathead minnows over the course of the study (Figure 11 A). In rainbow trout, citrate synthase activity increased 10-fold in rainbow trout 85 days post-hatch (Figure 11B). For both species, the increase in activity of this enzyme is probably a result of the differentiation of an increased number of aerobic red muscle cells.
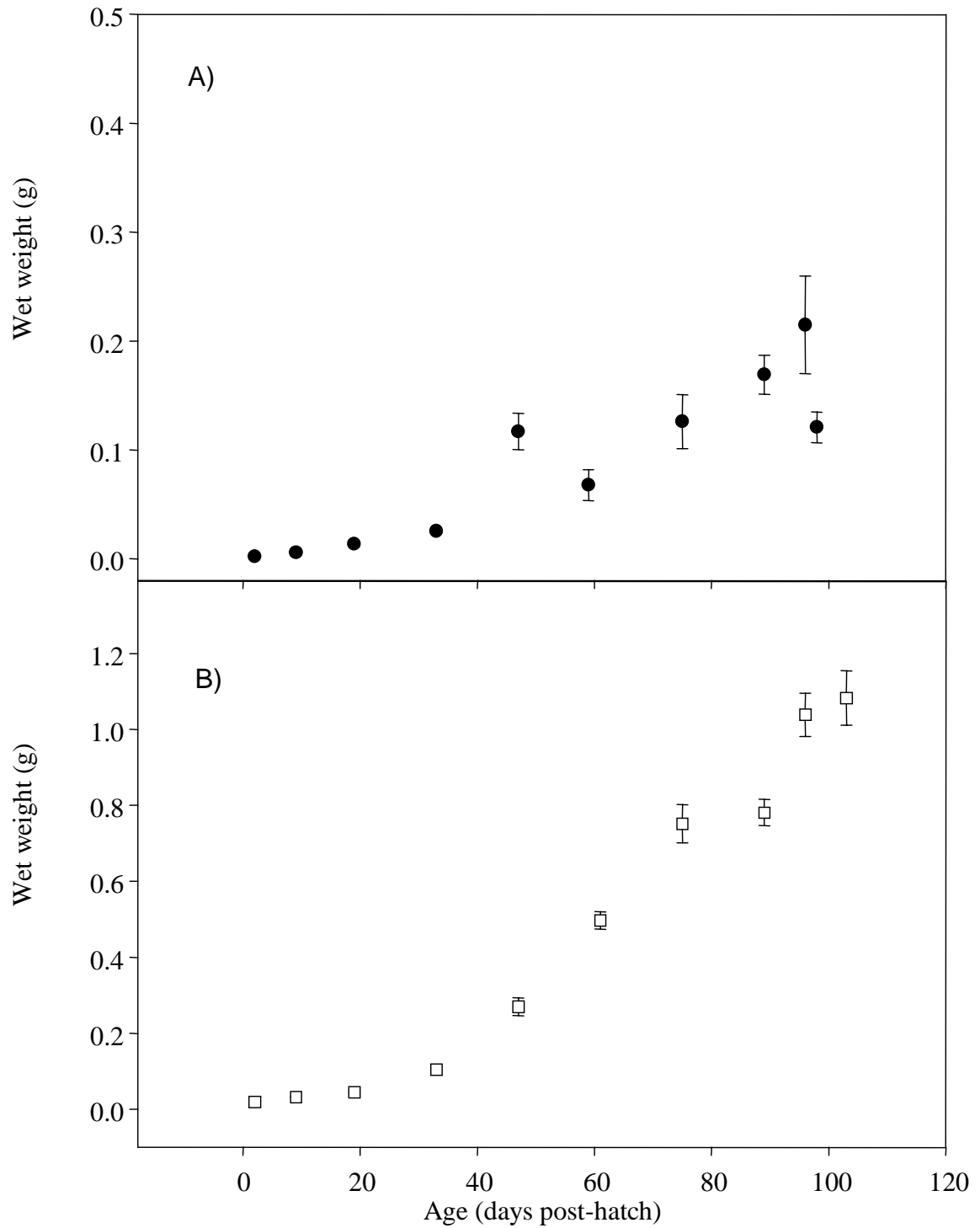
Figure 1. Representative mean weights for fathead minnows and rainbow trout at selected ages over the first 100 days post-hatch. Graph A) contains the mean weights for fathead minnows used in the lactate dehydrogenase and citrate synthase assays, while B) contains the mean weights for rainbow trout used in the same assays.

Figure 2. Oxygen consumption rates at declining dissolved oxygen concentrations for fathead minnows of different ages. ● represents fish that were exposed to 5% carbon monoxide (CO) to bind hemoglobin, while □ represents fish in untreated water. Error bars represent the standard error of both the DO concentration (horizontal), and oxygen consumption rate (vertical). Sample size = 3 for each symbol except where the symbol does not have error bars and then n • 2.
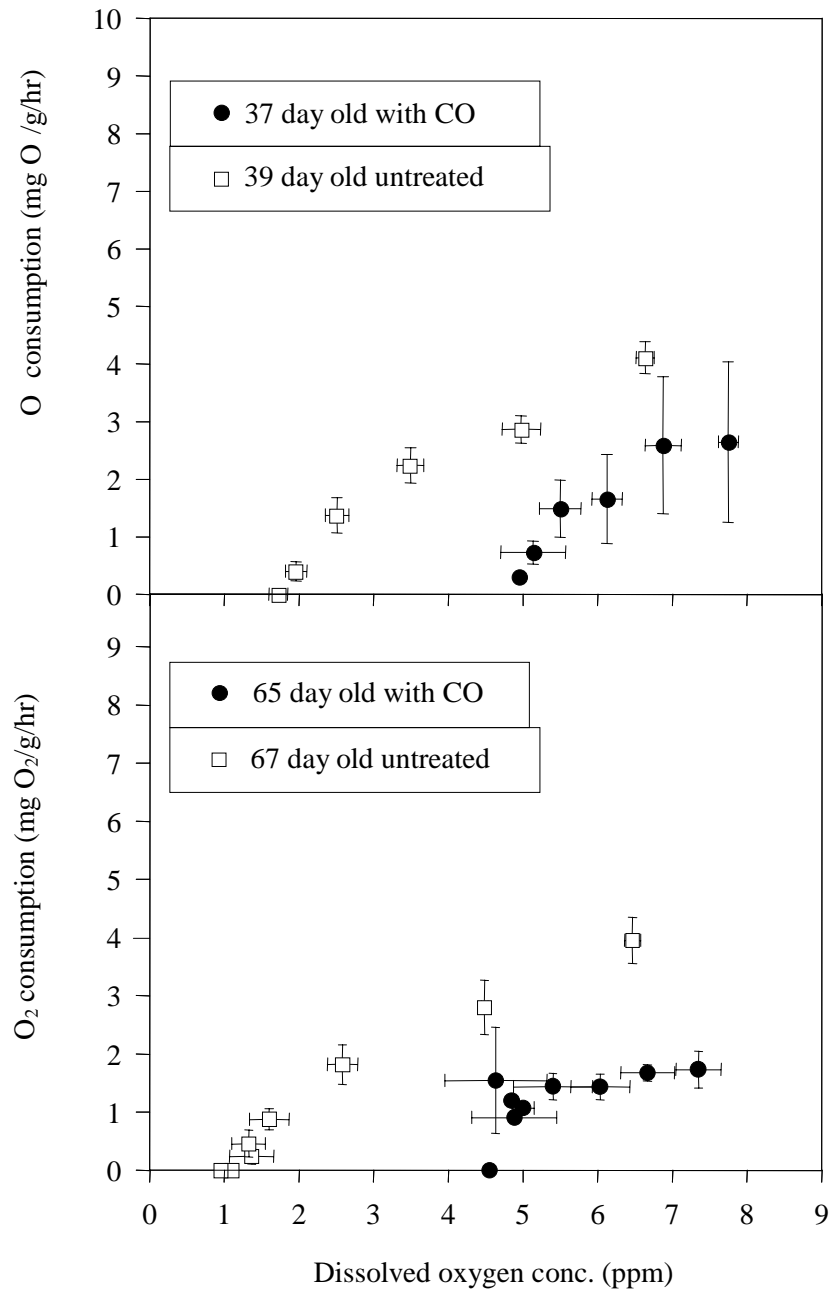
Figure 3. Oxygen consumption rates at declining dissolved oxygen concentrations for fathead minnows of different ages. ● represents fish that were exposed to 5% carbon monoxide (CO) to bind hemoglobin, while □ represents fish in untreated water. Error bars represent the standard error of both the DO concentration (horizontal), and oxygen consumption rate (vertical). Sample size = 3 for each symbol except where the symbol does not have error bars and then n • 2.
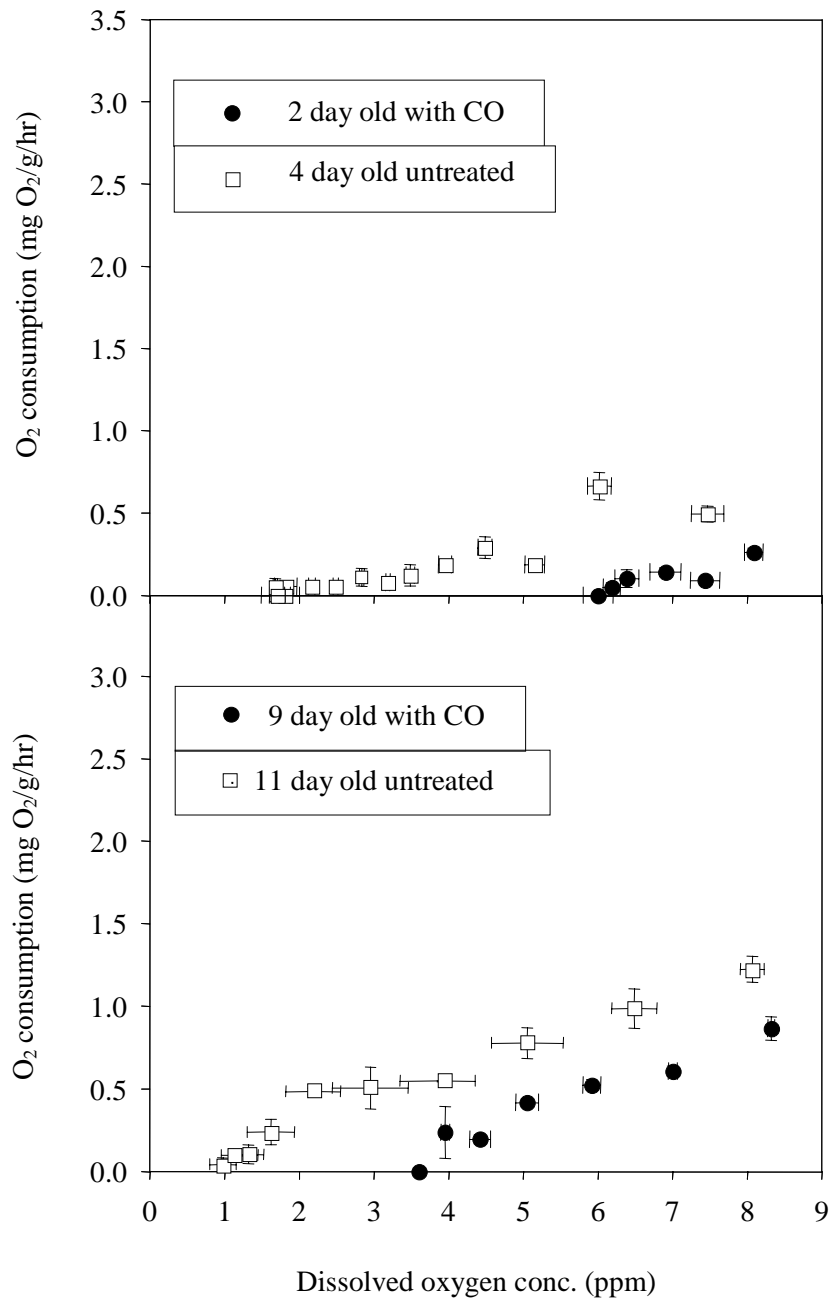
Figure 4. Oxygen consumption rates at declining dissolved oxygen concentrations for rainbow trout of different ages. ● represents fish that were exposed to 5% carbon monoxide (CO) to bind hemoglobin, while □ represents fish in untreated water. Error bars represent the standard error of both the DO concentration (horizontal), and oxygen consumption rate (vertical). Sample size = 3 for each symbol except where the symbol does not have error bars and then n ● 2.
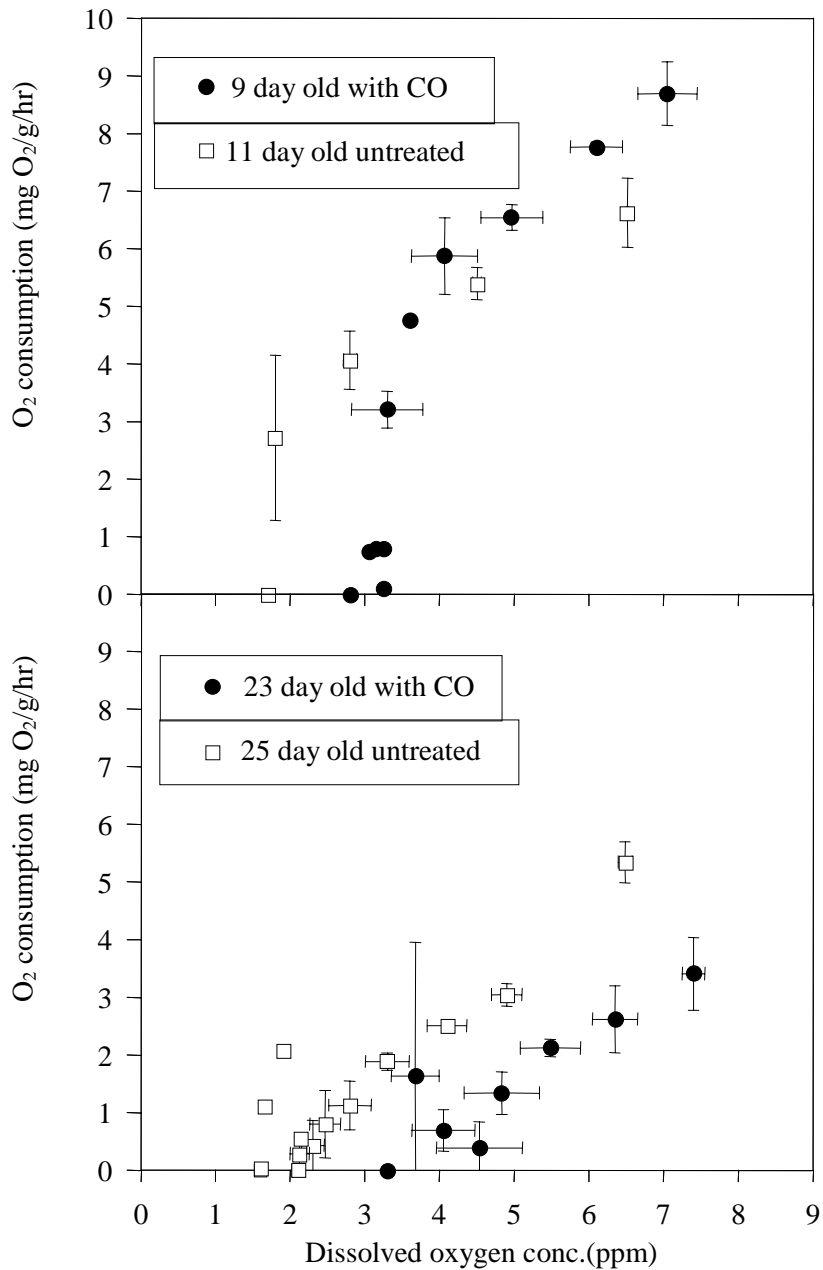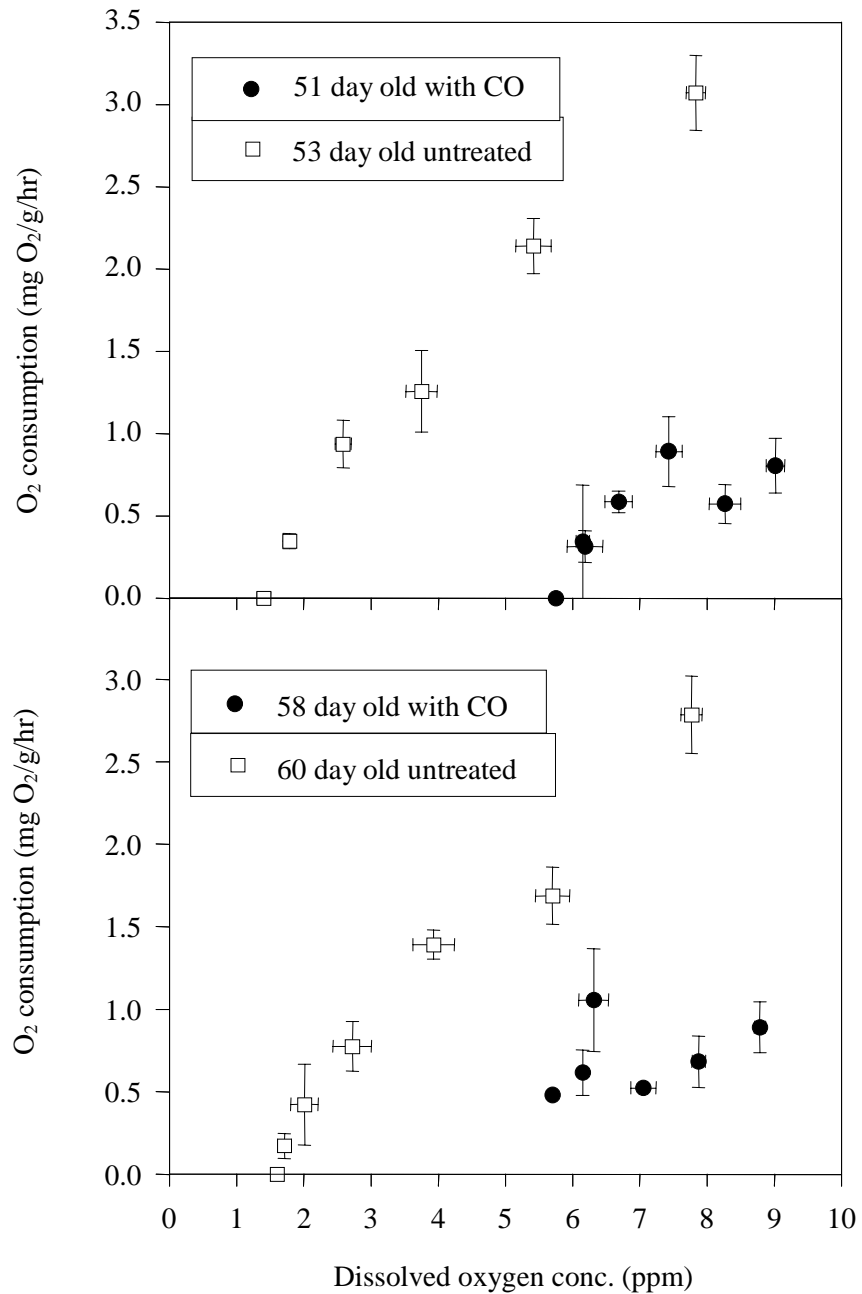
Figure 5. Oxygen consumption rates at declining dissolved oxygen concentrations for rainbow trout of different ages. ● represents fish that were exposed to 5% carbon monoxide (CO) to bind hemoglobin, while □ represents fish in untreated water. Error bars represent the standard error of both the DO concentration (horizontal), and oxygen consumption rate (vertical). Sample size = 3 for each symbol except where the symbol does not have error bars and then n ∙ 2.
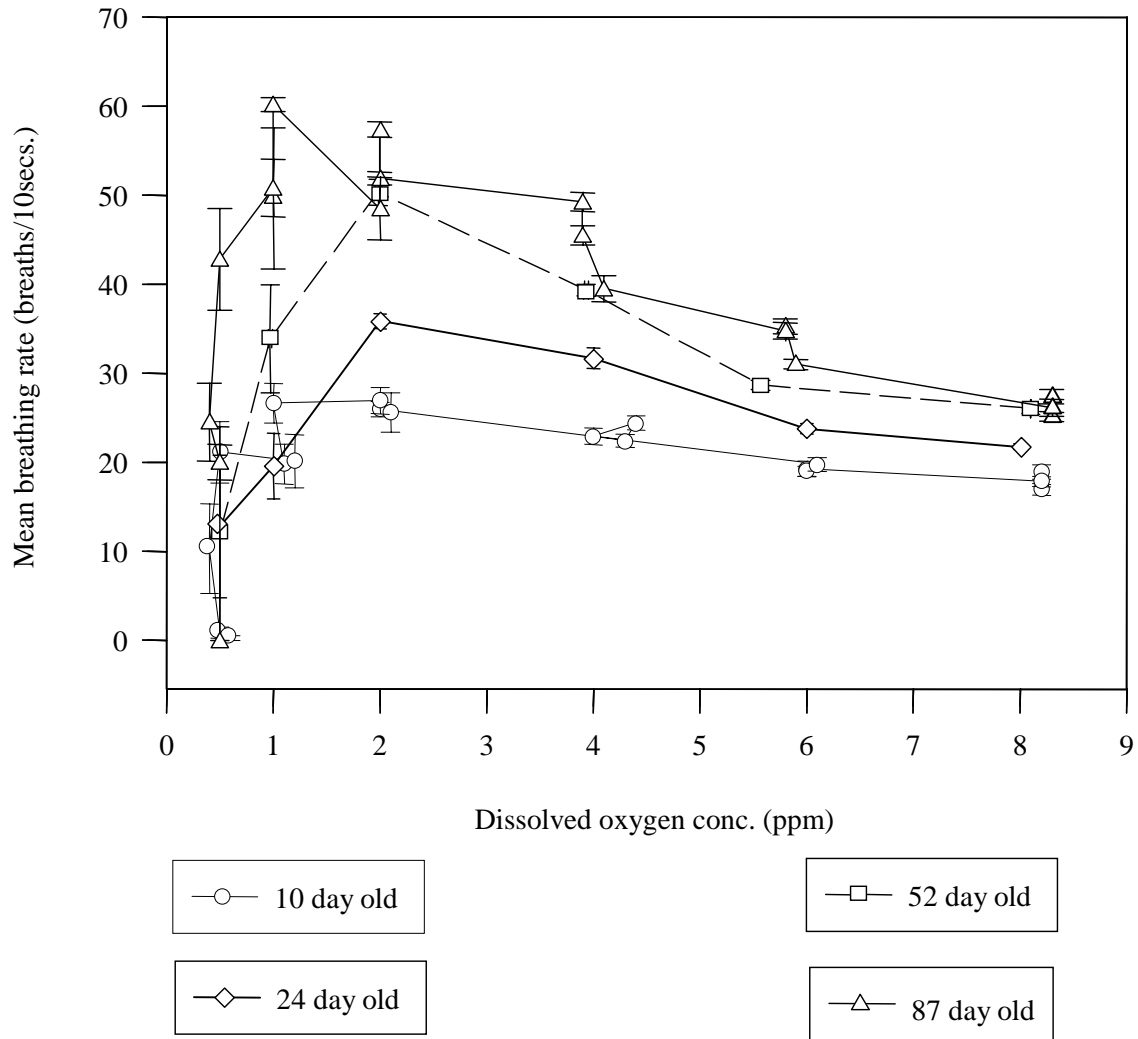
Figure 6. Representative mean breathing rates for fathead minnow larvae of different ages subjected to declining dissolved oxygen concentrations. Data points are the mean breathing rates determined by counting the number of breaths in a 10 second period. The DO concentration was held constant over a 30-minute period and three readings were taken (time 0, 15 and 30 minutes). The DO concentration was then reduced. Error bars represent the standard error. Sample sizes for the 10, 24, 52, and 87 day old fish are 12, 11, 12 and 16 respectively.
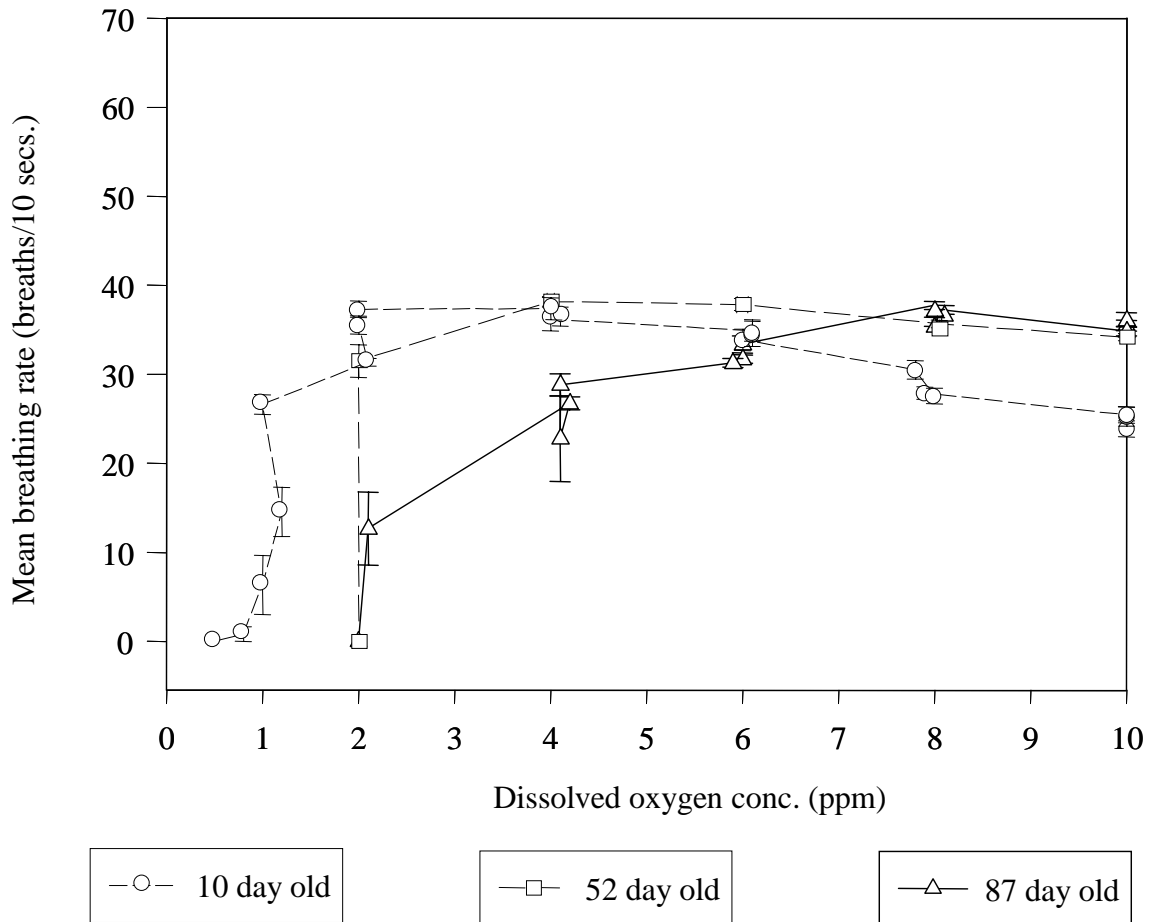
Figure 7. Representative mean breathing rates for rainbow trout larvae of different ages subjected to declining dissolved oxygen concentrations. Data points are the mean breathing rates determined by counting the number of breaths in a 10 second period. The DO concentration was held constant over a 30-minute period and three readings were taken (time 0, 15 and 30 minutes). The DO concentration was then reduced. Error bars represent the standard error. Sample sizes for the 10, 52, and 87 day old fish are 8, 16 and 16 respectively.
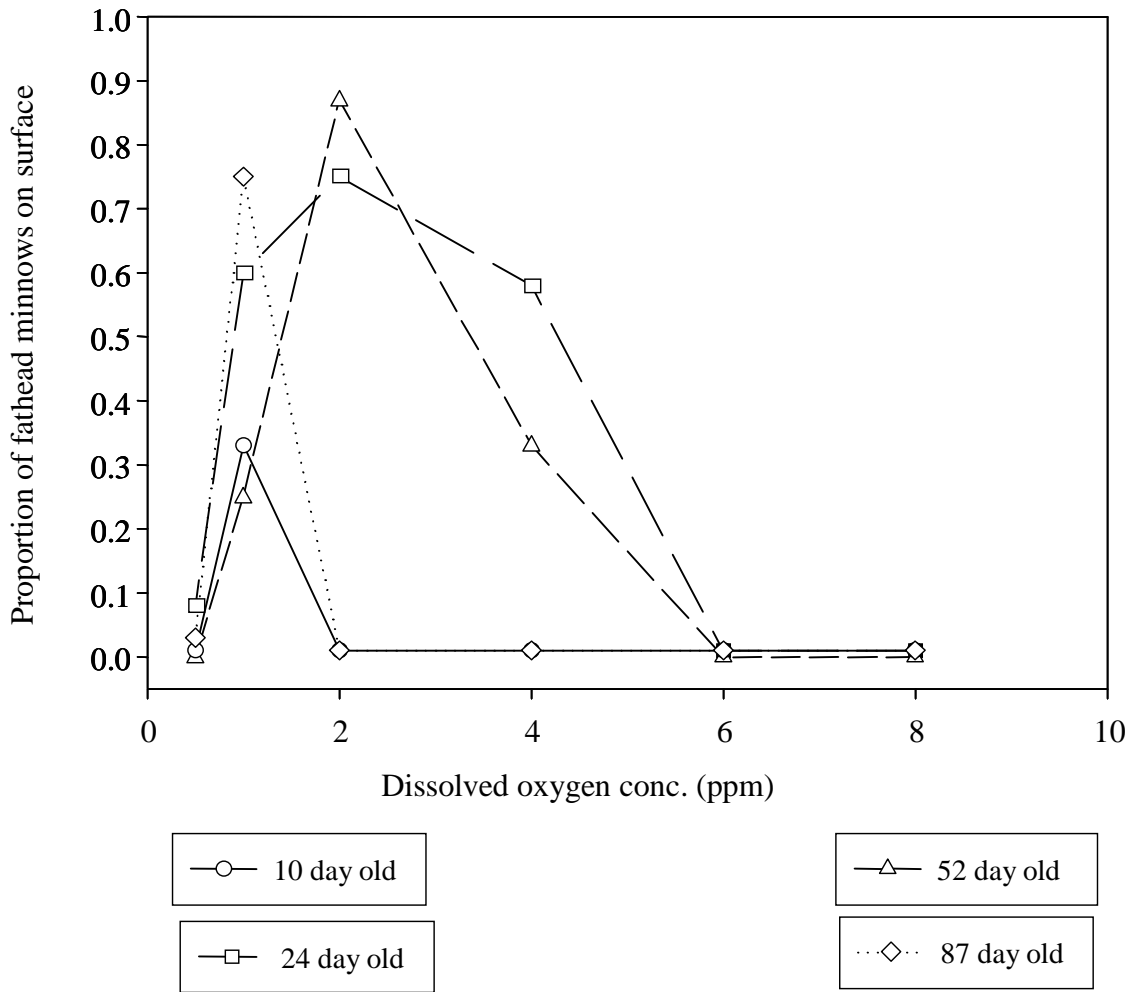
Figure 8. The proportion of fathead minnows at the water's surface as the dissolved oxygen concentrations were reduced. For 10, 24, and 52 day old fish, the symbols represent the proportion of minnows at the surface during 12 observations for each DO concentration tested. For 87 day old fish, the symbols represent the proportion of minnows at the surface during 24 observations for each DO concentration tested.
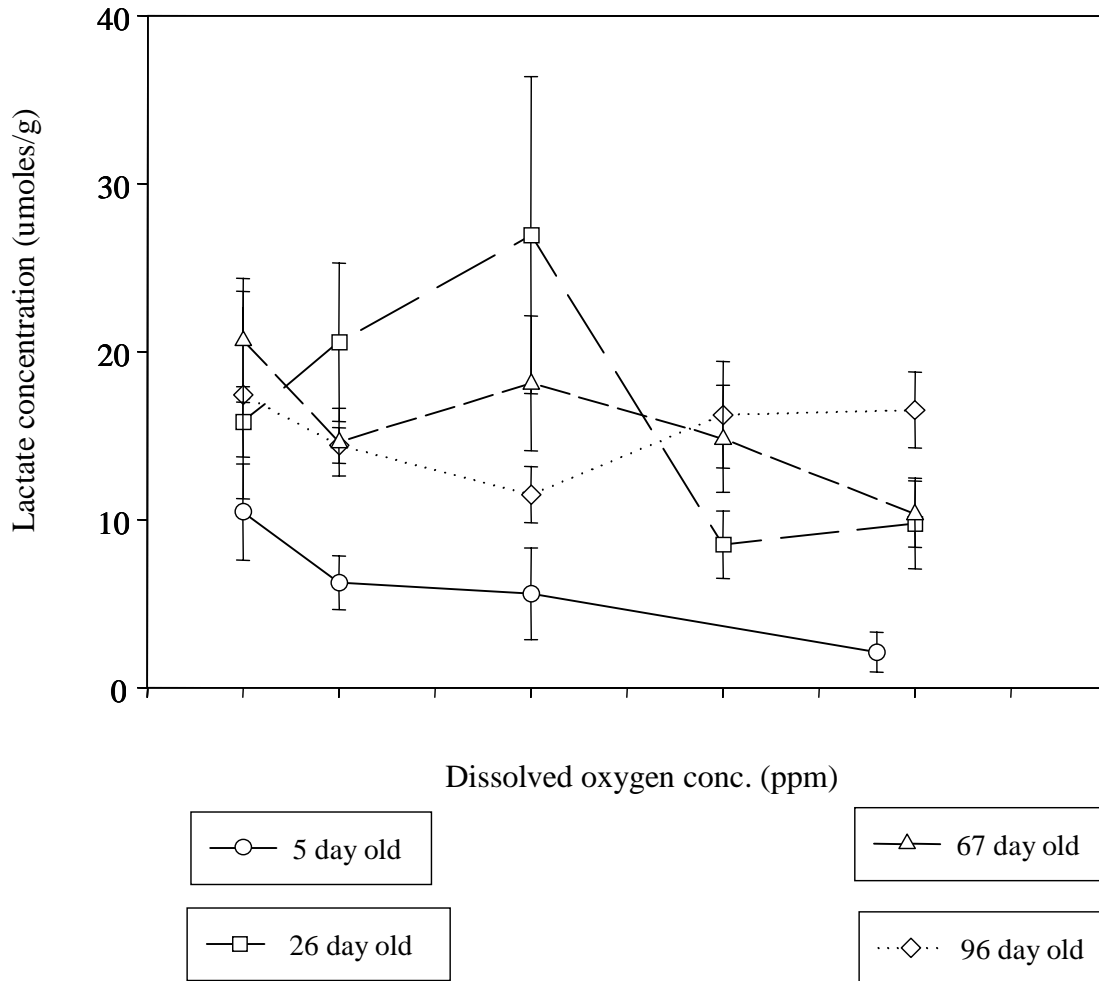
Figure 9. Mean lactate concentrations for fathead minnows of different ages exposed to declining concentrations of dissolved oxygen. The DO concentration was held constant over a 30-minute period and the fish in one of the chambers were sacrificed in liquid nitrogen. The DO concentration was then reduced in the remaining chambers to the next concentration tested. Error bars represent the standard error. Each symbol represents the mean of 5 samples.
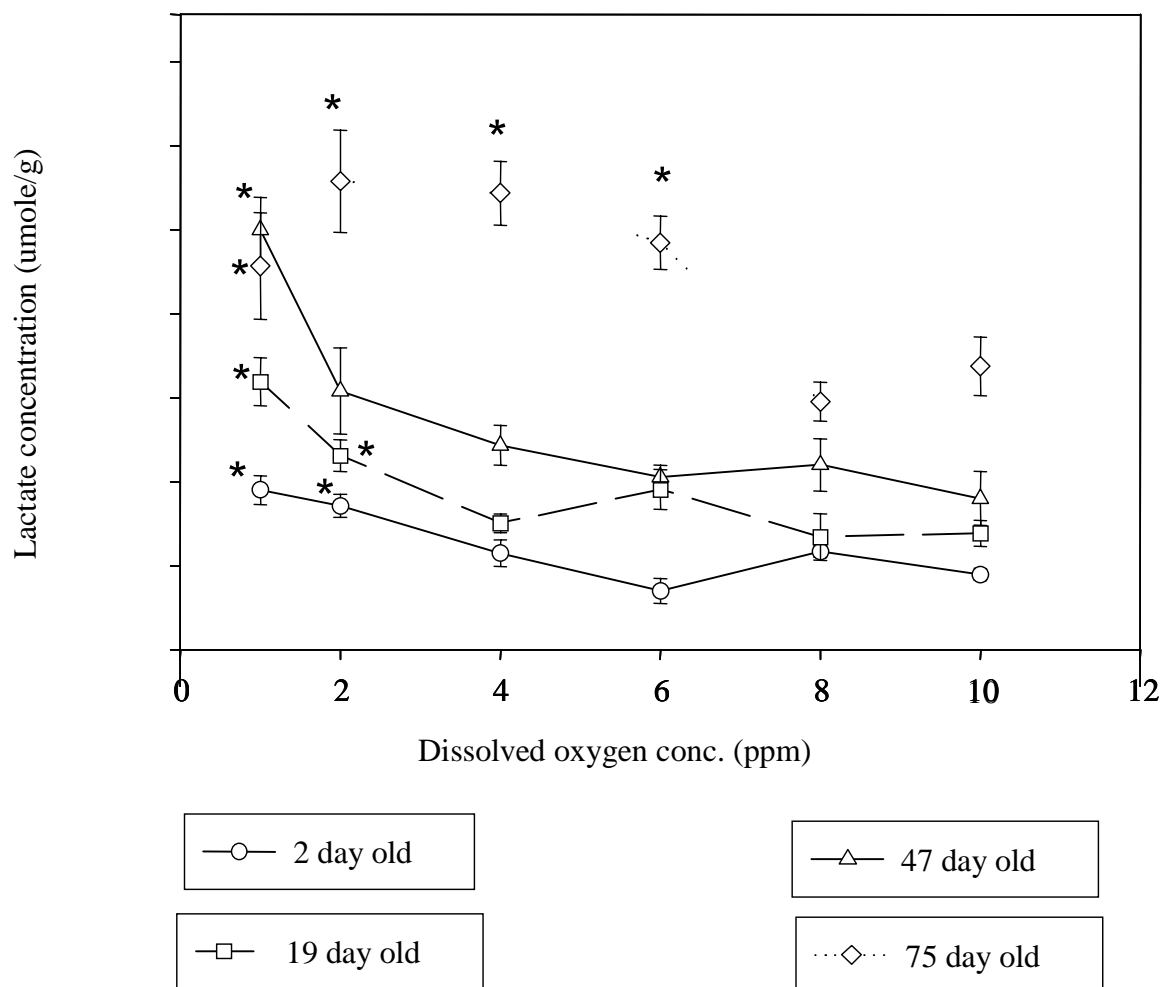
Figure 10. Mean lactate concentrations for rainbow trout of different ages exposed to declining concentrations of dissolved oxygen. The DO concentration was held constant over a 30-minute period and the fish in one of the chambers were sacrificed in liquid nitrogen. The DO concentration was then reduced in the remaining chambers to the next concentration tested. Error bars represent the standard error. * indicate that the lactate concentration at that particular DO concentration varied significantly (p<0.05) from the lactate concentration of fish in the normoxic (DO=10 ppm) group. Each symbol represents the mean of 5 samples.
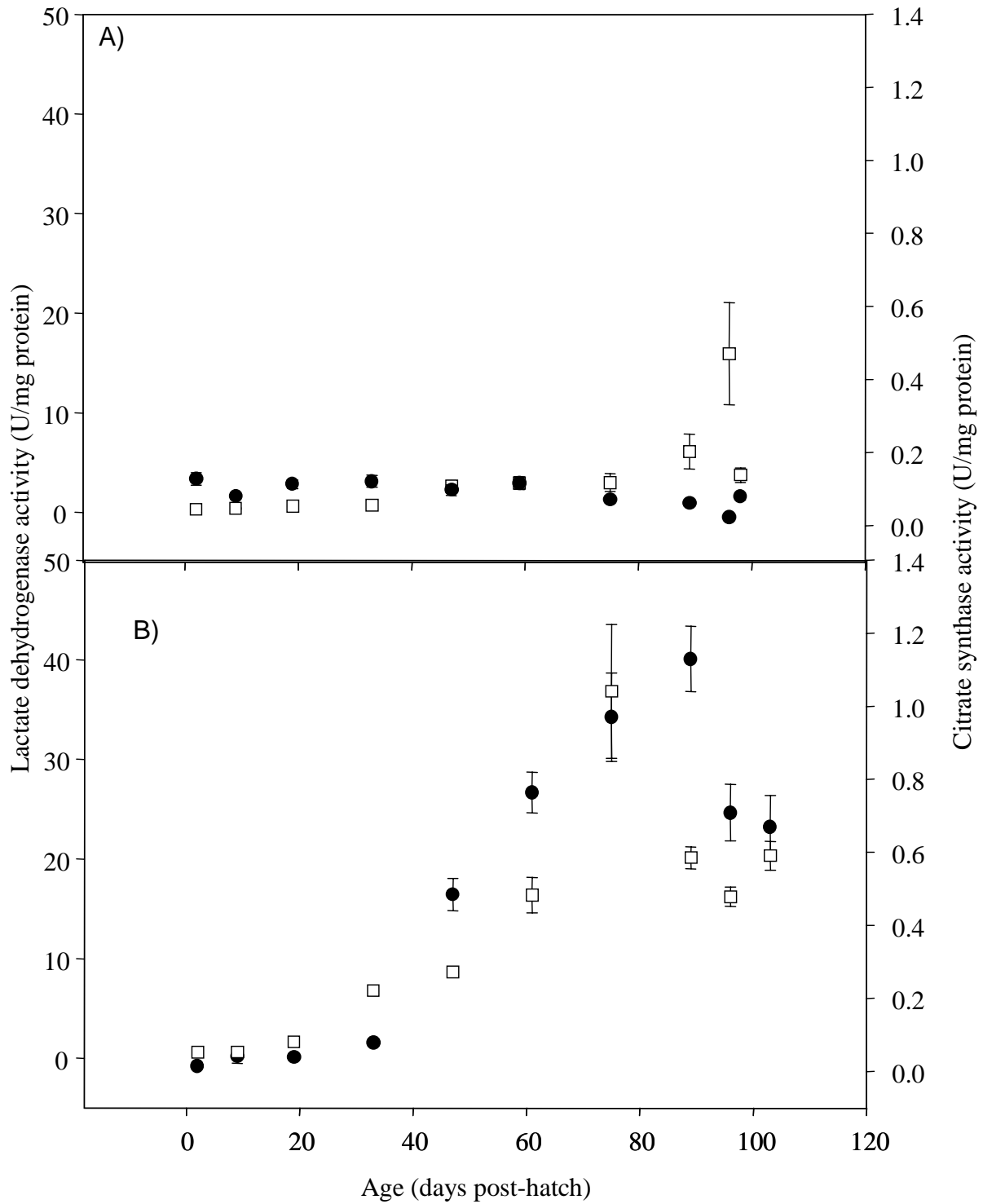
Figure 11. Mean lactate dehydrogenase and citrate synthase activities for fathead minnows (A) and rainbow trout (B) at selected ages over the first 100 days post-hatch. ● represents the mean citrate synthase activity in the fishes, while □ represents the mean lactate dehydrogenase activity at each age. Each symbol represents the mean of 10 samples.

Discussion

Oxygen Consumption Patterns

In several cases, fish of the same age and species have been reported to be metabolic conformers by one research group and metabolic regulators by a different group (reviewed by Heath 1995). These differences in findings may be attributed to variations in testing conditions such as temperature, physical activity of the fishes, experimental holding facilities, etc. My results, however, consistently indicated that physically and biochemically, respiration patterns did not change with development for the rainbow trout. Although the fathead minnows appeared to be undergoing a gradual transition towards becoming metabolic regulators, they did not appear to have the ability to regulate oxygen consumption when exposed to hypoxic conditions during the first 100 days post-hatch. According to Beamish (1964), adult brook trout act as metabolic regulators in situations in which the DO concentration is 5-10 ppm. It is therefore possible that at a period of time past 100 days post-hatch, the rainbow trout larvae become metabolic regulators. As the larvae develop, increased respiratory muscle mass, more developed gills, and the presence of hemoglobin may permit the fishes to function as metabolic regulators. However, it appears that the young larvae of both species tested operate as metabolic conformers because they lack the physiological "machinery" necessary to regulate oxygen consumption during periods of hypoxia.

Conforming oxygen uptake to oxygen availability may allow the larvae to survive brief periods of hypoxia by simply lowering metabolic energy demand. The reduction in metabolic rate, however, may place the larvae at increased risk of predation. Larvae operating on reduced energy may not be able to escape from predators that are more tolerant of hypoxia. The results of the oxygen consumption experiments indicate that the early larval stages of both of these species seem to be at least as tolerant of short-term exposure to low dissolved oxygen concentrations as their older, more developed, conspecifics.

Breathing Rate

In the youngest trout, breathing rates increased as DO concentrations declined. However, older trout did not show this response. Although breathing amplitude was not quantitatively measured, it was observed to increase in response to low DO concentrations as the trout aged. Rainbow trout older than 10 days may increase breathing amplitude in lieu of increasing breathing rates. This conforms to the patterns exhibited by the adults (Smith and Jones 1982). It is unlikely that the youngest trout are capable of greatly increasing the amplitude of breathing, because their mouth and gills are extremely small (Holeton 1971 *b*). The results of the current study concur with Holeton's (1971 *b*) finding that the breathing rates of trout in normoxic water increased during the first 18 days after hatching. It is likely that these results can be attributed to the increasing size and efficiency of the breathing apparatus of the fish. The ability to change breathing rate in response to hypoxia appears gradually, suggesting that this ability is the result of anatomical mechanisms that develop as the fish matures.

The observation that older fathead minnows increase breathing rate, rather than amplitude, in response to low DO concentrations may be indicative of a structural limitation. The minnows may lack the musculature needed to support large changes in breathing amplitude. As a result, it may be necessary for the minnows to increase their breathing frequency to maintain an adequate ventilation volume.

There are several possible explanations why the youngest fathead minnows did not greatly increase their breathing rates as DO declined. One possibility is that the musculature needed to support such rapid movements had not yet developed. Alternatively, as suggested by Holeton (1971 *b*), cutaneous respiration may have been sufficient to supply the needs of the smallest fish. In this situation, gills would not be highly utilized until a critical size was reached. Holeton (1971 *b*) and De Silva (1974) reported that larval trout and plaice may use their pectoral fins as accessory ventilatory organs to move currents of water in rhythmic motions. De Silva (1974) hypothesized that such movements may be typical of all larvae, which could further explain the fathead minnows' extended reliance on cutaneous respiration.

The fathead minnows that swam to the surface as DO declined were probably attempting to reach more highly oxygenated water. This type of oxygen-seeking behavior has been documented in nature. Congleton (1980) observed that adult woolly sculpin (*Clinocottus analis*) moved to the surface as oxygen concentrations declined nocturnally in small tidal pools. According to Kramer (1987), aquatic surface respiration may allow some fishes to survive hypoxic conditions through their utilization of the oxygen in the surface film, where the highest air-water oxygen exchange would occur through diffusion. However, as a consequence, surfacing behavior to facilitate oxygen uptake may lead to increased exposure of the individual fish to predation.

In several instances, small bubbles were accidentally introduced into chambers containing hypoxic water. It was observed, however, that the fathead minnows congregated near the bubbles. In these situations, the experiments were aborted. These fish survived lower DO concentrations than the fish that did not have access to gas bubbles. These observations agree with the findings of Klinger et al. (1982) that suggest that the fathead minnows are capable of obtaining oxygen from bubbles, presumably through aquatic surface respiration.

Hemoglobin

In the youngest minnows, the similarity of the minimum oxygen concentrations for those exposed to CO and those that were not exposed implies that hemoglobin was not vitally important in oxygen transport/supply in minnows of this age. In trout, even the youngest fish exposed to carbon monoxide had a higher minimum oxygen requirement than fish that were not exposed. Therefore, it can be concluded that hemoglobin is playing a larger role as an oxygen-carrier in young rainbow trout than in young fathead minnows. Oxygen affinities for hemoglobin have been demonstrated to differ between species of freshwater fishes (Weber and Jensen 1988) and with age in the same species. Iuchi (1973) found that the hemoglobin from trout larvae one day post-hatch has a higher oxygen affinity than the adult forms. Wilkins (1968) reported a distinctive juvenile form of hemoglobin in Atlantic salmon. In fathead minnows, it is unknown whether hemoglobin differs with developmental age. The small size of these fish would make determinations using the techniques previously employed difficult, but would be possible using molecular techniques. Developmental differences in the affinity of hemoglobin for oxygen as described by Iuchi (1973) may contribute to the ability of the young rainbow trout to tolerate lower DO concentrations.

The impact of carbon monoxide pretreatment on the viability of younger trout compared to the fathead minnow of the same age during the oxygen consumption experiments may be related to body size. The very young, small, fathead minnows were probably capable of using cutaneous respiration for direct diffusion of oxygen. The larger rainbow trout would be more reliant on blood transport of oxygen throughout the body. As both species of fish aged, the

importance of hemoglobin continued to increase as older individuals showed increased difficulty dealing with hypoxia after exposure to carbon monoxide. As demonstrated in the oxygen consumption experiments, the minimum oxygen concentration for survival was elevated as the fishes aged. This indicates that hemoglobin becomes increasingly important as fathead minnows and rainbow trout age. As the fishes grew larger, both species became more dependent on gill respiration for oxygen delivery through the blood than on cutaneous respiration. This transition was probably more marked in the minnows, which were likely dependent on cutaneous respiration early in larval development as compared to the larger trout of the same age.

Lactate concentration

The primary indicator of anaerobic metabolism in vertebrates is the accumulation of lactate (Bartholomew 1982). Klinger et al. (1982) proposed that adult fathead minnows survived in a partially frozen lake by utilizing primarily anaerobic metabolism. The current study does not support this hypothesis since fathead minnows did not show a great increase in lactate concentrations under hypoxic conditions. Experimental temperature may have been a confounding factor. The fish in the current study were reared at 25 ^{o}C, whereas fishes in overwintering ponds would be subjected to much colder water. However, since metabolic rates would be expected to be greater at higher temperatures, the lack of a lactate buildup in the current study suggests that the fathead minnow larvae do not "switch over" to anaerobic metabolism under hypoxia but continue to rely on aerobic mechanisms.

In both species and at all DO concentrations, lactate concentrations were lowest in the youngest groups. The youngest trout appear to gradually increase their use of anaerobic metabolism under severe hypoxia. This suggests that young trout are capable of utilizing anaerobic metabolism to meet energy demands.

Even at very low DO concentrations, the youngest trout tested (age 2 days) accumulated low concentrations of lactate in contrast to the older trout. Therefore, while the youngest trout appear to gradually increase their use of anaerobic metabolism under severe hypoxia, they do not utilize it to the same degree as the older fish. Rainbow trout hatch in redds and rely on water flow between the sediment to provide oxygen. This water may be poor in oxygen (Doudoroff and Shumway 1970, Sowden and Power 1985). As a result, very young rainbow trout emerging from these areas may be adapted to dealing with hypoxic conditions through other physiological mechanisms, such as oxygen transport via hemoglobin.

The rainbow trout larvae gradually started to utilize anaerobic metabolism at DO concentrations between 2 and 4 ppm. Burton and Heath (1980) reported that adult rainbow trout held at the same temperature, "switched over" to anaerobic metabolism at a DO concentration of 4.5-5.5 ppm. Trout < 100 days post-hatch continued to utilize aerobic metabolism at lower DO concentrations than did their adult counterparts, implying that they are more capable of handling hypoxic conditions. As discussed above, it is known that the hemoglobin of larval trout has a greater oxygen affinity than that of older fish (Iuchi 1973), permitting larvae to more easily acquire oxygen in hypoxic situations. In trout, reductions in oxygen consumption during hypoxic events, increased breathing rates by the youngest trout, and the higher oxygen affinity demonstrated by larval trout may all work in sequence to allow the fish to use primarily aerobic respiration during brief hypoxic conditions. The data indicate that the trout larvae are capable of utilizing anaerobic means as well.

White muscle lactate concentrations have been found to increase approximately two-fold in adult rainbow trout exposed to severe hypoxia (Dunn and Hochachka 1986). In the current

study, whole body lactate concentrations approximately doubled in the trout. This similarity may be purely coincidental as the exposure regime was different in the two studies, but this may suggest that anaerobic capacity in white muscle does not change greatly during ontogeny in rainbow trout. Forstner et al. (1983) suggested that rainbow trout larvae use predominately anaerobic white muscles to move prior to swimup, and gradually develop red aerobic muscles during the later stages of larval development. The larval trout may be using white muscles for the additional purpose of supplying needed energy under hypoxic conditions.

Anaerobic and Aerobic Metabolism

The pattern of LDH activities in both species suggests that anaerobic capacity is low in the youngest fishes and increases later in development. The general trend of increasing lactate concentration with age in both species of fish under normoxic conditions is probably a reflection of the increase in LDH activity. This may indicate that the fish are using anaerobic metabolism to provide the energy for at least some of their swimming activity. The increased LDH activity exhibited by older trout and the concomitant increase in lactate concentrations suggest that the larvae are also utilizing their increased anaerobic capacity to provide energy during brief periods of hypoxia. However, the low LDH activity and small lactate concentrations in minnows exposed to low DO concentrations imply that they do not utilize anaerobic metabolism to cope with hypoxia. As a result, it appears that the minnows are relying on other mechanisms to survive short-term hypoxia. By reducing energy demand during hypoxia, the minnow larvae may be able to maximize the contribution of aerobic metabolism.

Citrate synthase activity did not increase with age in the fathead minnow, suggesting that aerobic capacity develops slowly in these fish. In the rainbow trout larvae, changes in musculature have been observed during development, leading to a gradual increase in red muscle mass (Nag and Nursall 1972, and Wieser et al. 1985). Hinterleitner et al. (1987) reported a similar increase in citrate synthase activity for trout larvae of approximately the same age (day 50). They attributed the change to the increased red muscle that develops after swimup, as the trout start actively swimming. The increased amount of red muscle probably allows the fish to swim in a manner that is more reliant on aerobic respiration. In contrast, the fathead minnows did not demonstrate a sudden surge in the activity of this enzyme. It is possible that the fathead minnows lag behind similarly aged trout larvae in the development of aerobic (red) muscle.

General Conclusions

My findings suggest that larval fishes use a suite of physiological mechanisms to tolerate short-term exposure to hypoxia. Hemoglobin, biochemical mechanisms, alterations in ventilation patterns, and changes in behavior may all act in concert to allow the fish to survive brief periods of hypoxia. The importance of each mechanism may vary with the species of fish. The observation of larval hypoxia tolerance challenges the assumption that larval fishes are more sensitive to low oxygen than their adult counterparts. At the population level, while it would appear that exposure to short term hypoxia may have a lesser effect on the larval stages than previously believed, hypoxia may still be detrimental to the population if the less tolerant adults die during low dissolved oxygen events.

The transitions in hypoxia tolerance are gradual in nature as evidenced by the lack of an abrupt change in the physiological mechanisms examined at different ages. The larvae of both species were observed to act primarily as metabolic conformers during the first 100 days post-hatch, although the oxygen consumption patterns indicated that the fathead minnows older than

30 days undergo a gradual transition towards becoming metabolic regulators. This switchover may be a result of the more complete development of the mechanisms, such as respiratory muscles and gills, necessary to regulate oxygen consumption in the face of hypoxia.

Fathead minnows < 17 days old likely utilized cutaneous respiration for the majority of their oxygen uptake, while older minnows increased breathing rates as dissolved oxygen declined. In contrast, rainbow trout larvae increased breathing amplitude under hypoxic conditions. The results of oxygen consumption profiles from fishes exposed to carbon monoxide indicate that larval fishes are using hemoglobin to survive during hypoxic events, and suggest that hemoglobin plays a more vital role at an earlier age in the rainbow trout than in the fathead minnow. Fathead minnow larvae were observed to surface as DO concentrations declined, implying that this species may behaviorally change the depth of their position in the water column to compensate for hypoxia.

In the rainbow trout larvae, increased lactate concentrations associated with declining DO indicate that there was a threshold oxygen concentration below which the fish utilized anaerobic metabolism. The observation that increases in lactate dehydrogenase activity corresponded with increasing age in the trout suggest that these fish develop an anaerobic capacity which may be used to provide energy under conditions of low dissolved oxygen. Fathead minnows do not appear to develop this anaerobic capacity during the first 100 days post-hatch. The rise in citrate synthase activity in rainbow trout larvae after swimup suggests that the fish are developing aerobic means to supply energy. The fathead minnows, conversely, do not appear to greatly develop aerobic metabolism during the first 100 days post-hatch.

Ecological implications of the results of this study must be considered with the understanding of the multiple environmental factors that lead to the hypoxic event, many of which are compounding stressors. During the summer, drought conditions may cause a decrease in water levels and a probable rise in water temperature. The integration of both the effects of temperature stress and hypoxia on the larval fishes may be increasingly detrimental to the organism. If the hypoxic event is caused by organic pollution, an interaction between a pollutant and the fish may cause a response that is distinct from exposure to hypoxia alone. While the effects of chronic hypoxia exposure were not examined, the rapid ventilatory movements (or greater amplitude in the case of the trout) observed in the current study would be expected to increase metabolic demands. This could have serious implications for long-term survival and growth of the fish. Factors such as water quality, temperature, time of day/year, and condition of the fish may all affect the response of the larvae to hypoxia, and could therefore impact the ability of the fish to cope with the situation and ultimately reduce its survival.

CHAPTER 2:
BEHAVIORAL AVOIDANCE RESPONSE OF TWO FISH SPECIES TO LOW OXYGEN CONCENTRATIONS AT EARLY LIFE STAGES
(ABSTRACT)

The ability of fathead minnow (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*) larvae (up to 100 days post-hatch) to detect and avoid areas of hypoxia was examined in a behavioral study. Fish were observed in a flow-through chamber that allowed movement between areas receiving hypoxic water of various dissolved oxygen (DO) concentrations, normoxic, and a mixture of the two waters. Logit regression indicated that both species exhibited an overall trend to avoid low DO concentrations at all ages. Although the youngest individuals ($< \sim 30$ days) appeared to show the strongest avoidance tendencies, the more limited ability of these individuals to move may have caused them to remain in the more highly oxygenated area longer than the older, more mobile fishes. Both species seemed to utilize deliberate movements to leave hypoxic areas, suggesting that their movements were directed by an oxygen sensor system. I hypothesize that this oxygen sensing mechanism becomes active almost immediately post-hatch in both of these species and may play a vital role in allowing the fish to avoid hypoxic regions.

Introduction

In aquatic systems, a variety of natural phenomena and anthropomorphic factors may result in the reduction of dissolved oxygen content. During winter in cold climates, ice prevents contact between air and water (Klinger et al. 1982). In the summer, algal biomass decays and oxygen is consumed by microorganisms (Nicholls et al. 1980). Eutrophication may be exacerbated by sewage spills (Birtwell et al. 1983) and industrial and agricultural runoff (U.S. Department of the Interior 1968). Hypoxia may also occur in localized regions at night as photosynthesis stops and oxygen is consumed by the respiration of microorganisms and animals (Suthers and Gee 1986). In fish, exposure to low oxygen concentrations may decrease growth (Bejda et al. 1992), reduce survival in a year class (Siefert and Spoor 1973), and ultimately change species assemblages or abundances (Smale and Rabeni 1995). Adult fishes are known to employ a variety of physiological mechanisms to cope with hypoxic conditions (Kramer et al. 1978). However, the role of behavior in surviving hypoxia has not been studied extensively. It has been debated whether fish can detect and avoid areas where dissolved oxygen (DO) concentrations are low. Although there has been some research on low-oxygen avoidance by fish, the overall results are conflicting. With the exception of Bishai (1962) and Deubler and Posner (1963), very few studies have examined the abilities of larval fishes to detect and avoid areas of low DO.

Jones (1952) observed that fry of stickleback (*Gasterosteus aculeatus*) and trout (*Salmo trutta*) repeatedly swam into hypoxic areas, and escaped only by thrashing about in wild movements caused by respiratory distress. It has been reported that postlarval flounders (*Paralichthys lethostigma*), minnows (*Phoxinus phoxinus*), and goldfish (*Carassius auratus*) avoided areas of low DO (Deubler and Posner 1963, Stott and Buckley 1979, and Ogilvie 1982). Matthews and Hill (1979) found that adult red shiners (*Notropis lutrensis*) did not change habitats in response to reduced DO concentrations. In their study, however, the oxygen concentrations used were not exceptionally low (>5ppm), and would likely be suitable for normal survival for many fish species. Gent et al. (1995) reported that adult largemouth bass (*Micropterus salmoides*) avoided areas of a backwater channel where DO concentrations were below 6ppm. Similarly, Suthers and Gee (1986) found that the summer distribution of juvenile yellow perch (*Perca flavescens*) changed when DO concentrations fell below 3ppm. The distribution of fish during the winter months is also influenced by DO concentrations as fish may avoid hypoxic areas (Klinger et al. 1982) or move up in the water column in search of higher DO concentrations (Petrosky and Magnuson 1973). Largemouth bass move towards the surface soon after hatching, presumably in part to seek higher DO concentrations (Spoor 1977). Whitmore et al. (1960), indicated that juvenile chinook (*Oncorhynchus tshawytscha*) and coho salmon (*Oncorhynchus kisutch*) avoided areas where the DO concentration was below 4.5 ppm and 6 ppm, respectively, at summer temperatures (20 $^{\circ}$C). However, the avoidance response was greatly reduced at cooler temperatures that roughly approximate those used for the trout experiments in the current study. Therefore, temperature should be recognized as a confounding factor when making comparisons between studies.

To complement the aforementioned studies that have involved adult or juvenile fishes, my study examined the responses of two species of fish larvae exposed to low DO concentrations over the first 100 days post-hatch. I investigated the ability of fishes to detect and avoid hypoxic conditions throughout larval development. Rainbow trout (*Oncorhynchus mykiss*) and fathead minnows (*Pimephales promelas*) were used for testing since the adults of

both of these species are generally considered to represent opposite ends of the hypoxia tolerance spectrum (Doudoroff and Shumway 1970). These two species also differ greatly in their developmental patterns and in their hatching environments.

Fathead minnows lay their eggs on the underside of logs, branches, and rocks in the shallows (<1 meter deep) of ponds and pools in slow moving riverine systems (Nelson 1992). The male fathead minnow guards the egg mass and agitates the surrounding water in a likely attempt to keep the eggs well oxygenated (Wynne-Edwards 1932). After an incubation period of approximately 5 days, the eggs hatch into free-swimming larvae. Spawning occurs from May-August, and rising water temperatures and drought conditions during this time may cause the eggs and larvae to encounter periodic hypoxic events (Markus 1934).

Adult rainbow trout spawn from February to early May and use their tails to create shallow depressions in loose gravel areas in which they lay eggs. The eggs are covered with the substrate and the nest, termed a redd, is left unguarded. Depending on water temperature, the incubation time for the eggs ranges from 44-101 days (Jenkins and Burkhead 1994). The larval trout remain in the redd for a period of 2-4 weeks after hatching, consuming only their yolk sacs to meet nutritional needs. After the yolk sacs are consumed, the trout larvae perform swimup, and emerge from the redd to rest near the bottom in hollows between stones. While in these areas, the larvae are protected from being washed downstream and can feed exogenously (Sedgwick 1982). While in the redd, the trout larvae are dependent on the interstitial flow of water between the substrate particles for the delivery of oxygen (Wickett 1954). Because interstitial flow rates may be slower than the flow rates of the water column, DO concentrations may be relatively low in the redd. As a result, trout larvae may encounter hypoxic conditions, especially before swimup (Sowden and Power 1985).

Although fathead minnows and rainbow trout eggs hatch in different environments, it is likely that the larvae of both organisms face periodic hypoxic events during early development. While it is probable that some of the mechanisms these fishes use to deal with hypoxia are similar, environmental and developmental differences may cause the fishes to respond with different behaviors. This paper focuses on the behavioral response of these larval fishes to hypoxia.

Materials and Methods

Organisms and Holding Methods
Rainbow trout larvae < 24 hours post hatch were obtained from the state fish hatchery in Paint Bank, Virginia in July 1997 and 1998. Fathead minnow larvae < 24 hours post hatch were obtained from Sachs Systems Aquaculture in Florida in March, 1998. Both species of fish were reared, and all experiments were conducted, in dechlorinated Blacksburg, Virginia municipal water (average pH~8.0 and hardness ~50mg/L $CaCO_3$). Upon arrival at Virginia Tech, fish were divided into roughly equal numbers and placed in modified 1-L Nalgene[TM] beakers that served as holding tanks. A large hole was cut in the sides of each beaker and was covered with Nytex[TM] mesh that allowed the circulation of water without permitting the fish to escape. The beakers were suspended in a 500-L Min-o-cool tank[TM], and a constant flow of water was pumped through the beakers. Airstones were placed in each beaker and the dissolved oxygen concentration within the beakers was maintained near saturation. At the start of the study, each beaker contained approximately 150 larvae, but the numbers dropped daily as fish were used in experiments and died at an expected attrition rate. Fathead minnows were held at a water

temperature of 22 $^{\circ}$C $\pm$ 2 $^{\circ}$C, and rainbow trout were held at a water temperature of 14 $^{\circ}$C $\pm$ 1 $^{\circ}$C. The fathead minnows were fed a mixture of *Artemia* and ground TetraMin^{TM} flake food twice a day for the first three weeks, and once a day thereafter. Because the yolk sac supplied their nutritional needs, the rainbow trout were not fed for the first 10 days post-hatch. The trout were then fed a mixture of *Artemia* and ground trout chow twice a day for the next two weeks, and then once a day thereafter.

Behavior Test

To observe avoidance behavior, the fish were sealed in a Plexiglas^{TM} chambers and the their movements recorded as a part of the chamber received hypoxic water. Two plastic 4.2-L tanks with tightly fitting lids served as the water sources for the behavior experiments. Water was added to the two tanks that were then placed in a water bath for temperature control. Below the water bath were two stir plates that drove stir bars (length = 2.5 cm) in the tanks to keep the water constantly circulating. A YSI^{TM} probe, (model 5750) connected to a YSI^{TM} DO meter (model 54A and 57), was inserted in a hole in the lid of the tanks to keep a continuous record of the DO concentrations within the source tanks. Water from these tanks was gravity-fed through Tygon^{TM} tubing (size 14) to the inlets on the fish test chamber.

The test chamber was constructed of Plexiglas^{TM} in a modified "Y" design (17.5 cm L x 8 cm W x 2.2 cm H). It was divided into three sections of equal volume (2 receiving lanes and 1 mixing area, ~76 cm^3 each) with a clear piece of Plexiglas^{TM} separating the lanes receiving oxygenated and deoxygenated water. An 18-gauge syringe needle located at one end of the chamber served as an inlet for each receiving lane and allowed an equal flow rate of 17 ml/min/lane. After mixing with water from the other lane, the water was allowed to overflow through small scour marks at the top of the other end of the chamber (Figure 1).

Each week, five fish of similar size were placed in the mixing zone of the test chamber and the top of the chamber was sealed with a piece of clear Plexiglas^{TM}. The entire chamber was placed in a 28x29x2.2 cm Plexiglas^{TM} tray with graph paper underneath to allow easier tracking of the fish movements. The chamber was tilted at a slight angle (~ 5 degrees) in order to achieve better water flow from the inlet areas to the overflow area. After setup, the fish were left to acclimate for approximately 15 minutes. An Olympus^{TM} color video camera (Model VX-302) was placed approximately 0.35 m above the chamber, and the movements of the fish were recorded on videotape for the duration of the test. After an initial 20-minute period during which both lanes received water that was saturated with oxygen at the test temperature, the DO concentration in the tank supplying one lane was lowered using a mixture of nitrogen gas and air regulated through a Cameron ^{TM} gas-mixing flow meter. This reduced DO concentration was maintained for 20 minutes, while the other lane continued to receive water saturated with oxygen. Every 20 minutes, the DO concentration in the one lane was lowered in this stepwise fashion, while the other lane was maintained at a high DO concentration. In order to keep the flow rates equal throughout the experiment, the source tanks were topped off with a small amount of oxygenated water at the end of each 20-minute interval. The DO concentrations tested in the experiments involving rainbow trout were 10, 8, 6, 4, 2, 1, and 0.5 ppm, while those for the fathead minnow were 8, 6, 4, 2, 1, and 0.5 ppm.

Videotapes were reviewed and the distributions of the fish were noted every 2 minutes during each of the 20-minute exposures. As a result, a total of 10 readings of the distribution of the fish in the high oxygen lane, low oxygen lane, and the mixing area were obtained for each

DO concentration. The average proportion of fish in the lane receiving hypoxic water was plotted at each DO concentration.

Data analysis

To determine if the fish were avoiding the hypoxic areas, a logistic-regression model was used to test for differences in the distribution of fish in the lane receiving low DO water at each of the DO concentrations tested. The logistic regression model is useful for data expressed as proportions and has been used in similar binomial situations (Sowden and Power 1985, Greene 1993, Fjellheim 1996, Salvanes and Hart 1998). The data concerning the number of fish in the mixing lane were not used in the analysis since it was not possible to determine the exact DO concentration in the mixing area or whether the fish in that area were preparing to enter, or had just left the other lanes. As a result, the remaining data on fish positions provided a binomial data set that allowed the analysis to yield a "yes or no" answer as to whether the fishes were exhibiting an avoidance response.

The probability of fish avoiding an area in the chamber was estimated as a factor of the DO concentration of the hypoxic lane, and the age of the fish by fitting a generalized linear model to logit-transformed data (Hamilton 1992) with SAS (SAS Institute, Inc. 1989). The choice variable, which was the response to hypoxia, was converted into a binary variable, which had a value of 3 if the fish avoided the low DO lane and a value of 1 if it remained in the hypoxic lane. $Y_i=2$ represented the data from the mixing area and was not included in the analysis. The formula used to determine the logarithm of the odds favoring avoidance (logit (L)) is:

$$L = \ln\{O(Y_i = 1)\} = \ln \left\{ \frac{P(Y_i = 1)}{1 - P(Y_i = 1)} \right\} \tag{1}$$

where $O(Y_i=3)$ represents the odds favoring hypoxia avoidance, which is the ratio between the probability of hypoxia avoidance ($P(Y_i=3)$) and hypoxia preference ($P(Y_i=1)=1-P(Y_i=3)$). L depends on (a) DO concentration (D_o), (b) fish age (A_{fish}), or (c) DO concentration and fish age together:

$$L = \gamma_0 + \gamma_1 D_o; \qquad\qquad L = \gamma_0 + \gamma_1 A_{fish};$$

$$L = \gamma_0 + \gamma_1 D_o + \gamma_2 A_{fish} + \gamma_3 (D_o * A_{fish}) \tag{2}$$

The logarithmic transformation of calculation (1) was reversed and the estimated coefficients were used to calculate the probability that the fishes would avoid the lane receiving hypoxic water ($P(Y_i = 3)$ is referred to as P) based on DO concentration (D_o), and age (A_{fish}):

$$P = \frac{1}{1 + e^{-L}} \tag{3}$$

Results

The results of the logit regression indicated that regardless of age, the fish of both species gradually exited the lane receiving hypoxic water as the DO concentration decreased in that lane ($p<0.0001$ for both minnows and rainbow trout). In addition, the youngest fish of both species tended to be located in the lane receiving highly oxygenated water regardless of the DO concentration in the lane receiving hypoxic water ($p< 0.0001$). The interaction term between DO

concentration and fish age indicated that as DO concentrations decreased, the younger fishes were even more likely to avoid the hypoxic lane and move to the highly oxygenated side of the chamber than the older fishes (p =0.0134, p= 0.0052 for the minnows and rainbow trout, respectively). The avoidance response exhibited by representative groups of fathead minnows and rainbow trout to declining DO concentrations are shown graphically in Figures 2 and 3, respectively.

While not quantified in the current study, it was observed that the fathead minnows and rainbow trout avoided the hypoxic water with deliberate motions. The fathead minnows would enter the lane receiving hypoxic water periodically and exit in a deliberate manner after exploring the lane for a short time. While the trout "re-tested" water from the hypoxic lane throughout the experiment by swimming into it periodically, they usually immediately retreated from the zone in a deliberate fashion.
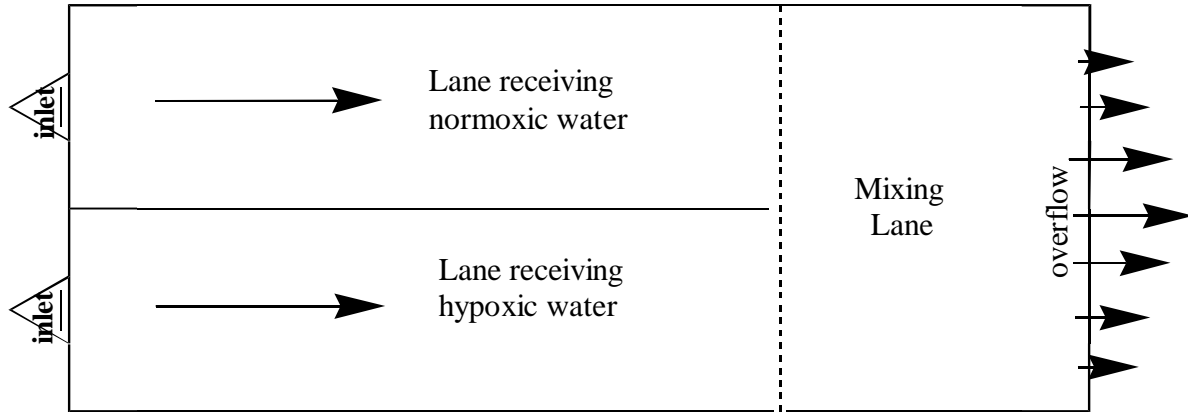
Figure 1. Design of behavioral chamber used in the current experiment. On the diagram, the top lane received a continuous supply of highly oxygenated water. The bottom lane received water in which the DO concentration was lowered over time. Water entered the chamber through inlets on the left and, after mixing with water from the other lane, exited the chamber by overflowing through scour marks at the top of the far end of the mixing area. The chamber was sealed with a piece of clear Plexiglas^TM placed over the top. Arrows represent the direction of water flow.
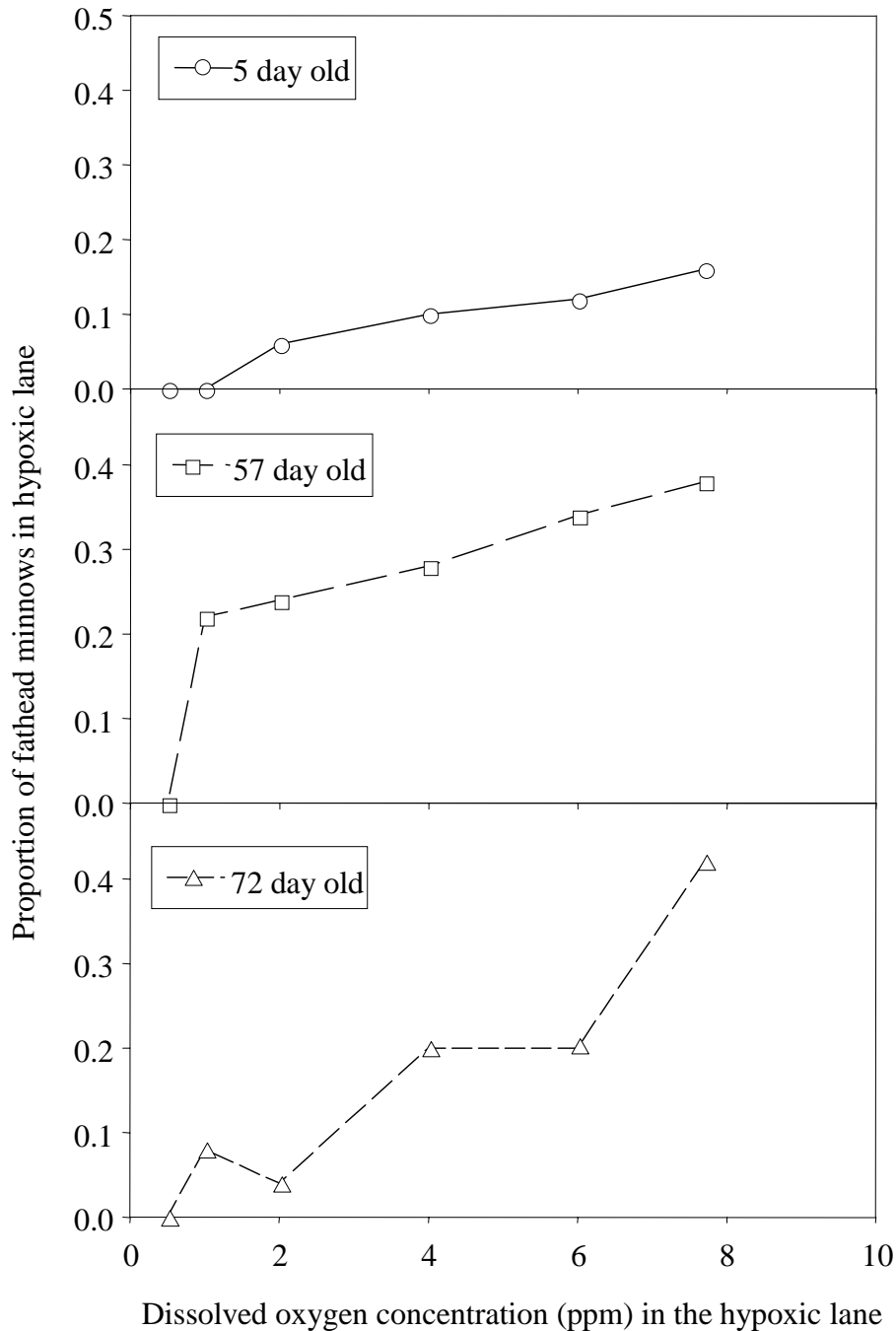
Figure 2. Relationship between the oxygen concentration and the avoidance reaction of fathead minnow larvae of different ages. The data points give the mean proportion of larvae present in the area of the chamber receiving hypoxic water, during the test, compared with the number of larvae in the experimental chamber. Each data point represents the mean proportion of 5 larvae observed every 2 minutes for 20 minutes. Groups displayed are representative of the trends established in the logit regression.
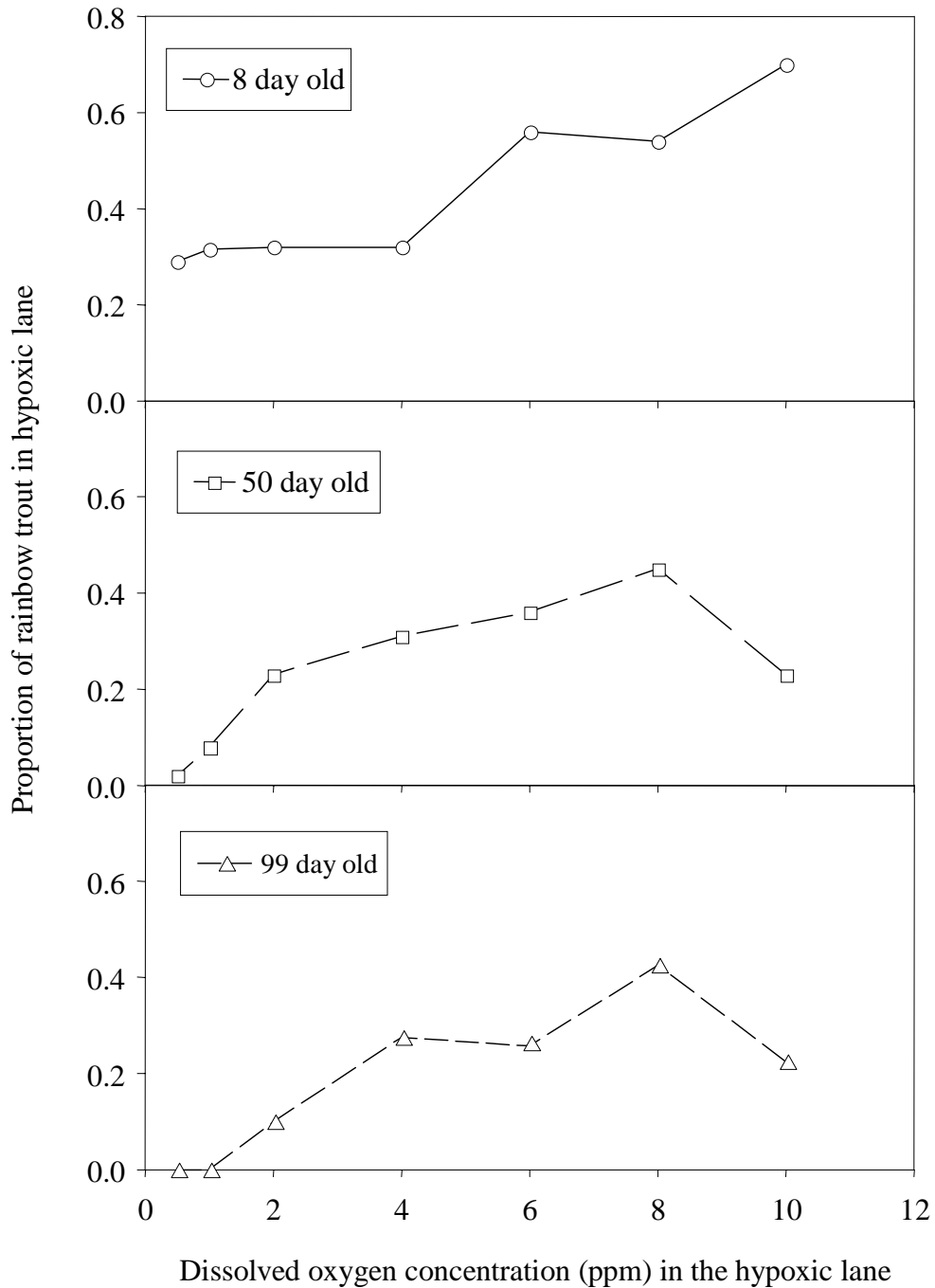
Figure 3. Relationship between oxygen concentration and the avoidance reaction of rainbow trout larvae of different ages. The data points give the mean proportion of larvae present in the area of the chamber receiving hypoxic water, during the test compared with the total number of larvae in the experimental chamber. Each data point represents the mean proportion of 2 replicate tests of 5 larvae observed every 2 minutes for 20 minutes. Groups displayed are representative of the trends established in the logit regression.

Discussion

The observation of an avoidance response by the larval fathead minnows was not unexpected given that these fish are common in lakes and slow moving streams in which hypoxic conditions may be encountered (Klinger et al. 1982, Tompkins and Gee 1983, Gee and Ratynski 1988). Klinger et al. (1982) reported that adult fathead minnows in ice-covered lakes altered their behavior to utilize oxygen from the water around air bubbles trapped under the ice. Balfour and Heath (unpublished data) observed larval fathead minnows in the lab moving to the surface of sealed chambers as DO concentrations declined. These observations offer further evidence suggesting that fathead minnows may alter their behavior under hypoxia. Furthermore, the observation of surfacing behavior in a controlled setting suggests that in nature the minnow may alter its vertical position in the water column upon encountering hypoxic conditions. Although the experimental setup in the current study did not monitor the vertical movements of the larvae, an additional camera angled from the side of the chamber could be used to determine whether the larvae show surfacing behavior as the DO concentration declined. Stratification of water of different conditions (e.g. temperature, DO) may occur in some lakes and ponds. This allows an area, such as a deep pool, to become and remain hypoxic until some movement causes the layers of water to mix. As a result, vertical movement in the water column may allow a fish to escape from hypoxic regions with less effort than horizontal movements, since the area of the hypoxic region could be quite large and would require the fish to swim a great distance. Because larval fish are not as strong swimmers as most adult fish, the vertical movements by the larvae may be the best chance to avoid hypoxic areas.

The results of the current study contradict the findings of Jones (1952) who reported that minnows (*Phoxinus phoxinus)* did not appear to have the ability to detect or avoid hypoxic areas. In contrast, Stott and Buckley (1979) indicated that adults of the same species exhibited such abilities and concluded that the observations by Jones may have been the result of a lack of a sufficient oxygen gradient in his testing chamber. The current study did not incorporate an oxygen gradient as long as that presented to the fish in the study by Stott and Buckley (1979) (~8 m), since a long gradient seemed to be less likely to elicit a response than a shorter, more abrupt, gradient. Despite the fact that a short gradient was used in the test chamber in the current study, an avoidance response was observed. This indicates that gradient length may not be a compromising factor in Jones' experiments.

The statistical figures indicated that the youngest fishes (<~ 30 days) were entering the highly oxygenated lane regardless of the DO concentration in the hypoxic lane. The relevance of this result is questionable since the interaction term indicated that both species avoided the hypoxic lane when the DO concentration declined. It is possible that the youngest individuals, being the least mobile of any stage tested, entered the lane receiving highly oxygenated water and then remained there for a longer period of time compared to the older fishes.

Rainbow trout have a limited ability to move before they undergo swimup between their second and third week post-hatch. As a result, the avoidance response exhibited by the very young rainbow trout (<15 days) is noteworthy because, in nature, the fish would not be likely to move a great distance. However, because the trout are dependent on interstitial flow for their oxygen supply at this time (Wickett 1954), it is possible that even slight movements within the redd may allow the trout access to more oxygenated water. It would be interesting to investigate the variability of DO concentrations within the interstitial flow of a redd to determine whether large differences exist.

The ability of the larval fishes to both detect and avoid hypoxia may increase their chances of survival. In contrast to the current study, Bishai (1962) noted that larval salmon (*Salmo salar L.*) and brown trout (*Salmo trutta*) were unable to effectively detect and avoid water with low DO concentrations until after the absorption of the yolk sac. However, the overall trends for the fishes in the current study indicated that, regardless of age, the larvae avoided the hypoxic region.

The avoidance response demonstrated by both species implies that the physiological mechanism for oxygen detection may develop almost immediately following hatch. Balfour and Heath (unpublished data) have observed that the physiological and biochemical mechanisms (e.g. hemoglobin, increased breathing amplitude, increased enzyme activity) for coping with hypoxia may take some time to develop in these species. If the larvae are not physiologically capable of handling hypoxic stress, then the primary reaction may be to leave the area. As a result, the behavior response may be a major mechanism for very young fish to cope with hypoxia.

The deliberate manner in which the rainbow trout exited the low-DO lane in the current study supports the findings of Bishai (1962) and contradicts Jones (1952) and Höglund (1961) whose research indicated that the fish were only swimming randomly and the struggling caused by hypoxia induced stress merely allowed them to exit a hypoxic area. Observation of this deliberate response offers further evidence that the trout are capable of detecting hypoxic areas through the use of an oxygen sensing mechanism. Bamford (1974) and Holeton (1977) provided evidence that trout have oxygen sensors located either in the medulla of the brain or pseudobranch of the gill. Research by Burleson and Milsom (1993) indicates that oxygen-sensitive chemoreceptors are present on the first gill arch in adult rainbow trout, and that the receptors are capable of responding to both internal and external hypoxia. However, given my findings, more work concerning hypoxia avoidance behavior and the development of oxygen sensing mechanisms in young fish is warranted.

Summary

Fathead minnows and rainbow trout less than 100 days post-hatch appear to actively avoid areas of low DO, with the youngest fishes more likely to exit a hypoxic region. Both fish species appeared to leave the hypoxic areas with deliberate motions indicating that a directed sensor system allowed them to detect oxygen gradients. Random thrashing activity was not likely responsible for their exiting the area. I have hypothesized that oxygen sensing receptors in both of these species become active almost immediately post-hatch and that the structures allow the fish to detect and avoid hypoxic regions.

CHAPTER 3: SUMMARY AND FUTURE DIRECTIONS

Summary

The results of the current experiments suggest that a suite of physiological, biochemical, and behavioral mechanisms allow larval fish to cope with brief exposure to hypoxia. The observation of larval hypoxia tolerance in fathead minnows (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*) challenges the assumption that larval fishes are more sensitive to low oxygen conditions than their adult counterparts. The two species may employ different mechanisms, or utilize them at different stages in order to survive hypoxic events. Alterations in breathing rate or amplitude allow the fathead minnow and rainbow trout larvae to increase oxygen uptake in hypoxic areas. Rainbow trout appear to begin a reliance on hemoglobin as an oxygen carrier at an earlier age than fathead minnows. This is probably a result of the trout becoming too large at an early age to fully supply their oxygen needs through cutaneous respiration. The results of lactate and lactate dehydrogenase assays suggest that rainbow trout larvae utilize anaerobic metabolism when faced with hypoxia. Fathead minnows do not appear to use anaerobic metabolism in similar situations. Both species of fish appear able to detect and behaviorally avoid hypoxic areas almost immediately post-hatch. Although the relative importance of each of these physiological, biochemical, and behavioral responses may vary with the stage of development, it appears that a combination of all of these mechanisms may be necessary for fathead minnows and rainbow trout to survive episodes of hypoxia.

General overview

Hypoxia may occur in a variety of aquatic environments as a result of both natural and anthropomorphic events. As human populations expand, increased industrial and agricultural use of water resources may result in runoff and localized areas of hypoxia. The waters in third world countries are especially vulnerable because these areas will likely be the least able to afford pollution reduction equipment. Fish populations in these regions may be greatly affected if they are not tolerant of short-term exposure to hypoxia. Human populations, dependent on many fish species for food, may see changes in fish populations with a concomitant decline in catch yields of some species. As a result, it is likely that information on the effects of hypoxia on fish will be highly useful to resource managers. Determination of sensitive stages or periods of the year when sensitivity may be higher could lead to changes in policy that could protect certain fish species at their most vulnerable time. More information concerning the habitats and tolerances of different life stages of fish to hypoxia is necessary.

Future directions involving field research

Laboratory settings provide a controlled environment for experiments, however, there are a variety of factors that may affect the dissolved oxygen concentration in a natural setting. Wave action, depth, pollutant load, and temperature are just several of the factors that influence the concentration of dissolved oxygen in an area. Many of these factors alone may cause stress in fishes, and the additive effects cannot easily be simulated in the lab. As a result, information concerning the effects of hypoxia from laboratory experiments must be used cautiously. Field experiments, with continuous monitoring of organisms in cages, may provide more information on the synergistic effects of these factors on fishes. Experiments conducted during different seasons may produce valuable data on the effects of winter ice cover and summer droughts on

certain fish species. Hatchery programs could utilize such information to alter stocking times or locations and thus increase survivorship.

Future directions in behavioral research

Rainbow trout and other salmonids hatch buried under the substrate and therefore depend on interstitial flow for delivery of oxygen (Wickett 1954). While relatively few studies have attempted to measure dissolved oxygen concentrations of the interstitial flow, there is some evidence that suggests that a large percentage of the larvae die while the fish are still buried (Sowden and Power 1985). Although the increased mortality may be a result of other extrinsic or intrinsic factors, hypoxia cannot be ruled out as a possible cause. Further experiments on the water chemistry of these nesting sites could provide valuable insight into the role of hypoxia and the survival of the early life stages of salmonids. If hypoxia is determined to be a major problem in these sites, legislation banning certain industrial and agricultural processes during the season the nesting sites exist could prevent hypoxic events and increase spawning success rates.

The results of the current study suggest that oxygen sensors allow the fathead minnows and trout to detect and avoid areas of hypoxia, however, little information exists on the mechanism by which these sensors function. Although Burleson and Milsom (1993) reported that the oxygen receptors are capable of responding to both internal and external hypoxia, it is unclear whether the fishes are following an increasing oxygen gradient out of the hypoxic region. This hypothesis is supported by the manner in which individuals of both species retreated from low dissolved oxygen concentrations. However, it is also possible that the fishes were simply leaving an area with low oxygen concentrations, and not following any gradient. A different chamber design without a mixing area and with longer gradients may provide a better answer to this question, although a more detailed examination of the structure and function of the oxygen receptors is also necessary.

Future directions in biochemical research

The different oxygen affinities for hemoglobin of different aged salmonids reported by Iuchi (1973) and Wilkins (1968) suggests that some fish species may have specialized forms of hemoglobin that are used during developmental periods when low oxygen concentrations could be encountered. Further research investigating the timing of the appearance of these different hemoglobin forms would be informative. The examination of a wide variety of fish species from different evolutionary backgrounds may also provide information about differences between species-specific forms of hemoglobin.

In addition to having hemoglobins with varying oxygen affinities, it is possible that multiple forms (isozymes) of lactate dehydrogenase (LDH) may exist at different life stages. Different muscle types (e.g. red, white, and cardiac) are known to contain different forms of LDH (Clayton and Franzin 1970). If the relative proportion of a muscle type changed during development, it is possible that there could be changes in the relative activity of an LDH isozyme as well. Changes in the activity of an LDH isozyme could have repercussions on anaerobic capacity. The current study examined the overall activity of LDH in the fishes and did not address isozymes. Histological information on the relative proportion of each muscle type, as well as assays sensitive to a specific isozyme, may allow a more detailed exploration of the relative importance of each isozyme during development. The studies on the above topics could provide valuable information on the abilities of fish species to tolerate hypoxia. Such

information could be used to predict the chance of survival of some species and maintain catch yields in areas exposed to brief periods of hypoxia.

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- Provided introductory lectures prior to each laboratory
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- Served as substitute lecturer for general biology lecture class and upper-level mammalogy class

Graduate Teaching Assistant – Stream Ecology, Department of Biology, Virginia Commonwealth University, Richmond, Virginia, Spring 1994
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Research Interests

Freshwater mussel ecology including life history and physiology

Biology education – integration of cross disciplinarian fields in the classroom, innovative teaching techniques

Ecology and physiology of aquatic insects

Fish behavior

Hypoxia

Achievements

Awarded $350 grant from Sigma Xi for dissertation research – 1999

Best student paper award in Agriculture, Forestry and Aquaculture section of the 1998 meeting of the Virginia Academy of Science meeting.

Awarded $2,500 fellowship from Waste Policy Institute -1998

Member of Sigma Xi Research Honor Society – inducted 1997

Awarded $500 grant from Graduate Research Development Project - 1997

Member of Phi Sigma Biological Honor Society – inducted 1991

Member of Phi Kappa Phi National Honor Society – inducted 1991

Service

Science Fair Judge – Biology/Bacteriology Section, Virginia's Governor's School Science Fair at New River Valley Community College in Dublin, Virginia Spring 1999.

Assistant Instructor at Virginia Tech Outreach Workshop conducted at Lord Fairfax Community College in Front Royal Virginia. Fall 1998. Answered questions relating to use of molecular laboratory techniques in freshman biology labs.

Publications	Balfour, D.L. and L.A. Smock. 1995. Distribution, Age Structure, and Movements of the Freshwater Mussel *Elliptio complanata* (Mollusca: Unionidae) in a headwater stream. Journal of Freshwater Ecology. 10:255-268.
Presentations	Balfour, D. L. Distribution, Age Structure, and Movements of the freshwater mussel *Elliptio complanata* (Mollusca: Unionidae) in a headwater stream. Biology Departmental Seminar. May 1999. Virginia Tech, Blacksburg, Virginia.
	Balfour, D.L., and A.G. Heath. Ontogenetic changes and environmental hypoxia: Responses of two fish species to low oxygen levels at early life stages. Presentation at the Virginia Academy of Science meeting. May 1998. George Mason University, Fairfax, Virginia.
	Rein, S., D. L. Balfour, and L. A. Smock. The Estimation of Age Dependence in the Seasonal Depth Distribution of Mussels. Presentation at the Virginia Academy of Science meeting. May 1996. Virginia Commonwealth University, Richmond, Virginia.
	Yeager, M. M., D. S. Cherry, R. C. Cavender, D. L. Balfour, and J. C. Scott. 1995. The Development of In-situ Testing Methods for Juvenile *Villosa iris* (Bivalvia: Unionidae) with Higher Sensitivity than Laboratory Sediment Tests. Poster presentation at the national meeting of the Society of Environmental Toxicology and Chemistry. November 1995. Vancouver, British Columbia.
	Balfour, D. L. and L. A. Smock. Distribution, Age Structure, and Movements of the Freshwater Unionid Mussel *Elliptio complanata* in a Headwater Stream. Poster presentation at the national meeting of the North American Benthological Society. May 1995. Keystone, Colorado.
	Rein, S., D. L. Balfour, and L. A. Smock. The estimation of age dependence in the seasonal depth distribution of mussels. Presentation at the Virginia Academy of Science meeting. April 1995. Virginia Military Institute, Lexington, Virginia.
Professional Societies	North American Benthological Society
	Sigma Xi Research Society
	Virginia Academy of Science

References Available upon request.

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David L. Balfour