Sublethal effects of diel fluctuations in dissolved oxygen
saturation on freshwater fishes from tropical Queensland

Thesis submitted by

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in August 2005

For the degree of Doctor of Philosophy

in Zoology and Tropical Ecology

within the School of Tropical Biology

James Cook University

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ABSTRACT

The effects of diel fluctuations in DO saturation were investigated for four species of tropical freshwater fish at various life history stages. Fluctuating hypoxia was achieved by gradually lowering DO saturation to a minimum level (minimum level differed between treatments), then allowing DO to return to normoxia each day for the duration of experiments. A range of oxygen regimes were tested on juvenile *Lates calcarifer, Melanotaenia splendida splendida* and *Hephaestus fuliginosus*; adult *Melanotaenia utcheensis*; and embryonic *M. s. splendida, M. utcheensis* and *H. fuliginosus*. Immediate lethal limits after gradual oxygen reductions were recorded for each species/life history stage where possible, as well as various effects on the sublethal level, including effects on growth (for juveniles), ventilation (for juveniles), reproduction (for adults) and viability (for embryos).

The four fish species tested were found to be surprisingly tolerant to the oxygen regimes they were exposed to during the study. Species/life history stages that are frequently exposed to hypoxia in natural situations were found to be the most tolerant during experiments. The rank order of resistance of each species/life history stage from highest to lowest was: eggs of *M. s. splendida* and *M. utcheensis* (no immediate lethal level identified), juvenile *L. calcarifer* (immediate lethal level ~2% DO saturation), juvenile *M. s. splendida* and adult *M. utcheensis* (immediate lethal level 6-7%), and juvenile *H. fuliginosus* (immediate lethal level ~7%).

L. calcarifer, *M. s. splendida* and *M. utcheensis* were all capable of aquatic surface respiration at the juvenile and adult stages tested. Juvenile *H. fuliginosus* did not display this adaptive behaviour. Growth and feeding behaviour of juvenile *L. calcarifer* were affected in treatments reaching 5% and 10% minimum DO saturation daily; as was food consumption of some *H. fuliginosus* individuals in the treatment reaching 10% DO saturation daily (5% treatment was lethal for the species).

Eggs of *M. s. splendida* and *M. utcheensis* were completely resistant to the oxygen regimes tested, and more tolerant to hypoxia than juvenile and adult stages of the same species. Reproduction of surviving adult *M. utcheensis* was largely unaffected by exposure to diel fluctuations in DO saturation, although one of two broodgroups

treated with a minimum DO saturation of 10% daily ceased egg production after 18 days of oxygen cycling; and in the same aquarium one of the two female fish was found to have a high percentage of atretic (degenerative) eggs in her ovary.

Although the results suggested that species of fish tested were remarkably tolerant to the sublethal DO regimes imposed during the study, some effects on reproduction, growth and feeding were apparent and may be much more detrimental in natural situations where food must be caught, and mates located. Additionally, longer durations of daily minimum DO saturation, or longer duration of the fluctuating hypoxia regime may increase effects. The results have implications for water quality guidelines for wetlands and waterways of tropical north Queensland, and provide a broad baseline for more targeted research into the effects of hypoxia on fish from the region.

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1.1 Introduction

This thesis examines the effects of hypoxia – defined as low levels of dissolved oxygen (DO) – on selected fish species from waterways in tropical Queensland. Typically, comparable studies have examined lethality of hypoxia under conditions of static or diminishing levels of dissolved oxygen. This study took a different approach by simulating the fluctuating hypoxia that usually occur in tropical waterways, and investigating its sublethal effects on fish.

This chapter reviews the characteristics of hypoxia and how it affects fish and fish communities, and outlines how this thesis addresses these issues.

1.2 Hypoxia and fluctuating hypoxia

Hypoxia is a common cause of fish kills around the world (e.g. Whitfield & Paterson, 1995; Hamilton *et al.*, 1997). For this reason, hypoxia has been rigorously tested as a factor affecting fish health in laboratories from the Americas to Europe and Asia (e.g. Hunn, 1969; Oseid & Smith, 1971a; 1971b; Davis, 1975; Carlson & Herman, 1978; Brooke & Colby, 1980; Pouliot & de la Noue, 1989; Chapman *et al.*, 1994; Papoutsglou & Tziha, 1996; Chabot & Dutil, 1999; Buentello *et al.*, 2000). Because freshwater fish biology is still very much a developing science in northern Australia, this intense gathering of information has not occurred here and very few studies on the effects of hypoxia on species from the region have been carried out. Moreover, there is limited information available on the effects of hypoxia on Australian fish generally. This lack of information is certainly not due to lack of issues with hypoxia in Australia, especially in the tropics. On the contrary, hypoxia is one of the most common causes of fish kills in northern Australia (e.g. Bishop, 1980; Townsend *et al.,* 1992; Pearson *et al.*, 2003).

In tropical Australian rivers, DO levels are affected naturally by seasonal floods, and run-off from nutrient rich soil. Increases in nutrients, light availability and lack of

competition all contribute to growth of aquatic plants (including macrophytes, algae and cyanobacteria). Aquatic plants contribute to high DO saturation during the day, producing about six times more oxygen through the process of photosynthesis than they consume (Hunt & Christiansen, 2000). During the night, or on cloudy days, plants are net consumers of oxygen as requirements for respiration outweigh oxygen production, and carbon dioxide is also released. For this reason, waterways with high plant biomass can show a daily pattern of DO cycling, where oxygen levels are high in the evening but drop during the night to a minimum at dawn (Davis, 1975). For example, in Lagoon Creek, in the Herbert catchment of north Queensland's wet tropics, DO levels can fluctuate between 2% and 80% daily for several weeks (Pearson *et al*., 2003).

Plants are not the only consumers of oxygen in aquatic ecosystems. Oxygen is also used in respiration by animals, in decomposition of organic matter by microorganisms, and in certain chemical reactions (Hunt & Christiansen, 2000). Because the solubility of oxygen is lower at higher temperatures and because of increased metabolic demand by aquatic organisms at high temperatures (Davis, 1975; Cech *et al*., 1990; Smale & Rabeni, 1995), aquatic ecosystems in the tropics are under particular threat of experiencing hypoxia, predominantly at times when water levels are low and lack of water movement reduces oxygen diffusion at the air-water interface.

Aside from natural causes of increased nutrient levels, which may occur after forest fires and high rainfall, there are several major anthropogenic processes that may lead to hypoxia in aquatic systems. Practices such as grazing and cropping (Oliveira & Kjerfve, 1993; Bonsdorff *et al.*, 1997; Martin & Saiki, 1999; Tucker & Burton, 1999; Collins *et al.*, 2000; Pearson *et al*., 2003); urban run-off (Tucker & Burton, 1999); industrial effluents (Winn & Knott, 1992) and aquaculture (Hargrave *et al.*, 1993; Bonsdorff *et al.*, 1997) can influence DO saturation. Increases in these activities in sensitive and diminishing wetlands of the Australian tropics indicate a need for concern for the survival of aquatic ecosystems and highlight the importance of gathering more information on how hypoxia may ultimately affect fish health.

1.3 Fish and hypoxia – sublethal effects and learning to live with hypoxia

When dissolved oxygen saturation drops below a tolerable level, single or multi-species fish kills are likely to occur. When oxygen drops to or fluctuates between levels that do not cause death, sublethal effects on fish and invertebrate health may result. Fish exhibit changes in behaviour, growth and reproduction during and after long-term exposure to sublethal levels of oxygen depletion as a result of metabolic, ventilatory and physiological changes (Ruggerone, 2000). In the long term these sublethal effects are likely to be similarly detrimental to the health of the system (Kramer, 1987).

Typically, when fish are exposed to hypoxia, physiological responses commence in an effort to maintain normal rates of oxygen consumption. As a result of, or in conjunction with, these physiological changes, fish behaviour, performance, growth and reproduction are altered. It is not possible for fish to regulate rates of oxygen uptake indefinitely, and at a critical oxygen pressure, uptake becomes dependent upon available ambient levels (Jobling, 1994). The range of oxygen levels over which a fish can maintain oxygen consumption varies between species (e.g. Pearson *et al.,* 2003; McNeil, 2004), among age or size groups (e.g. Kalinin *et al.,* 1993) and according to natural habitat (e.g. Kramer, 1987; Chippari-Gomes *et al.,* 2003).

Fish exposed to hypoxia display changes in ventilation, circulation and activity levels. But rather than indicating a negative effect of DO depletion, these changes indicate the ability of the fish to acclimatize to it. Ventilation rates frequently increase in response to hypoxia, allowing greater diffusion of oxygen into the bloodstream (Jobling, 1994). Heart rate may also increase to augment blood flow through the gills (McDonald & McMahon, 1977), or it may decline to allow more time for increased oxygen diffusion from blood into cardiac muscle (Jobling, 1994). Under hypoxia additional alterations in circulation occur, including decreased oxygen pressure in blood cells, redirection of blood flow to important organs or locomotory muscles, and release of red blood cells previously stored in the spleen (Jobling, 1994). Activity levels of fish under hypoxia may increase, indicating an attempt to move away from poor conditions, or decrease in an effort to reduce oxygen requirements for locomotion, and redirect energy to vital functions (Kramer, 1987).

The stress caused to fish by hypoxia results in a reduced ability of animals to cope with other potential hazards. Fish exposed to hypoxia are less able to deal with other forms of pollution (McClosky & Oris, 1991; Wajsbrot *et al.*, 1991; Kreuger, 1994), and are more likely to contract diseases (Virgona, 1992; Caldwell & Hinshaw, 1995) and parasitic infections (Mqolomba & Plumb, 1992; Fukuda *et al.*, 1997a; 1997b). Alterations in fish physiology and general health result in alterations to condition and fitness, including but not restricted to: food consumption; feeding behaviour and growth; ventilatory behaviour; histology of major organs; and reproductive viability and egg survival.

Some fish species have developed high tolerances for hypoxic conditions, reducing the impacts of hypoxia on condition and fitness. The ability to acclimatize to low oxygen conditions tends to be most prevalent in species that are commonly exposed to hypoxia in their natural environment (Kramer, 1987). The various ways fish deal with hypoxia may include obligatory or facultative air breathing (Stevens & Holeton, 1978; Randall *et al.*, 1981; Kramer, 1987; Wells *et al.,* 1997), use of a buccal air bubble (Thompson & Withers, 2002), aquatic surface respiration (Gee *et al.*, 1978; Kramer & McClure, 1982; Chapman & Liem, 1995), reduced metabolism (Rantin *et al.*, 1992), low requirement of water convection to allow respiration (Rantin & Johansen, 1984), large surface area of respiratory organs (Chapman & Hulen, 2001), increased oxygen carrying capacity of the blood (Val *et al.*, 1998) or higher capacity for anaerobic metabolism (Rantin *et al.*, 1992). Such adaptations to reduce the risk of damage under hypoxia are poorly described for fish from the Australian tropics.

For adaptations to hypoxia of tropical fish species, perhaps the best-known fauna is that of the Amazon. In the tropical and subtropical waters of the Amazon, hypoxia is commonplace (Chippari-Gomes *et al*., 2003), and DO may fluctuate between zero oxygen overnight to super-saturation at midday (Almeida-Val *et al*., 1993). Amazon fishes have developed adjustments to many levels of biological organization including ethological, morphological, anatomical, physiological, metabolic and molecular levels (Chippari-Gomes *et al*., 2003). These alterations have resulted in a diverse suite of fish species with various respiration patterns, life history strategies and preferential habitats, well suited to the prevailing environmental conditions.

1.4   Ecological effects of hypoxia on fish communities

Because coastal wetlands are a critical habitat in the life-cycle of various fish and invertebrate species, including species of commercial value, the threat of hypoxia in these systems is of extremely high importance to the overall survival of individual animals, populations and, ultimately, aquatic ecosystems. Hypoxia affects abundance and distribution of fish, the outcome of interactions among organisms, and hence community dynamics (Keister *et al*., 2000). Fish will avoid normally preferred locations if conditions are sub-optimal (Kramer, 1987), and several field studies have revealed correlations between oxygen availability and distribution of fish both vertically and horizontally in the water column (Gehrke, 1991; Pihl *et al*., 1991; Knights *et al*., 1995; Sellers *et al*., 1998; Araujo *et al*., 1999; Barrow & Peters, 2001; Fontenot *et al*., 2001; Richardson *et al*., 2001). Severely hypoxic waters are thought to be similar in effect to an absence of water for some species, creating an obstacle to movement and dispersal (Chapman & Liem, 1995).

On the Ovens River floodplain of Victoria (Australia), there is a positive relationship between laboratory tested tolerance estimates of hypoxia and the distribution and abundance of fish species in their environment (McNeil, 2004). The high tolerance of exotic species such as carp, gambusia, goldfish and weatherloach to hypoxia has no doubt contributed to their success in Australian waterways where hypoxia is common (McNeil, 2004). The implications of these findings are quite serious given the extremely negative impact that such invasive species have had and will continue to have on native fish populations in Australia. Invasive species are an increasing threat in the tropics and are dominated by species such as tilapia (*Oreochromis mossambicus*) that are tolerant of poor conditions, such as hypoxia (Webb, 2005).

Changes in the way fish utilise their habitat under hypoxia are also likely to affect the risk of predation and food availability, impacting upon the complex trophic web of aquatic ecosystems (Pihl *et al.*, 1992; Pihl, 1994; Rahel & Nutzman, 1994; Nestlerode & Diaz, 1998; Tallqvist *et al.*, 1999; Sagasti *et al.*, 2001). The necessity for water-breathing fish to increase time spent at the surface during hypoxic episodes also has ramifications for the trophic structure of an ecosystem. When forced to tolerate hypoxic conditions, fish are more likely to move away from cover, either to search for

habitats with higher oxygen availability, or to perform aquatic surface respiration (Wolf & Kramer, 1987). In this way, hypoxia can increase the risk of both aquatic and aerial predation.

1.5   Thesis outline

The aim of this thesis was to investigate how sublethal exposure to diel fluctuations in DO saturation (hereafter referred to as fluctuating hypoxia) affects selected freshwater fishes of the Australian tropics. The results have implications for how fish populations and aquatic ecosystems may be affected by hypoxia in the long term, and should assist in the development of water quality guidelines based on regional knowledge. The study also allows some speculation on the nature of the tropical Australian fish fauna, and its evolutionary, biogeographic, and ecological history.

Objectives of the thesis are to:
1. identify the effects of fluctuating hypoxia on juvenile stages of selected fish species
    - barramundi – *Lates calcarifer* (Bloch); sooty grunter – *Hephaestus fuliginosus* Macleay; eastern rainbowfish – *Melanotaenia splendida splendida* (Peters, 1876) were used;
2. investigate the effects of fluctuating hypoxia on reproduction on a tropical Australian fish species
    - Utchee Creek rainbowfish – *Melanotaenia utcheensis* McGuigan, 2001 were used;
3. determine viability of eggs of several fish species, following exposure to fluctuating hypoxia
    - sooty grunter, eastern rainbowfish, Utchee Creek rainbowfish were used.

The basis of selection of these species and stages is discussed in Chapter 2.

The thesis is divided into chapters on the basis of parameters measured and life stages tested. Chapter 2 describes general methods and the biology of the species considered, as well as known habitats of these species. Chapter 3 presents results of three experiments that investigate the effects of fluctuating hypoxia on the respiratory

system of juvenile barramundi, sooty grunter and eastern rainbowfish, by identifying ventilatory alterations and histopathological changes to gills. Chapter 4 discusses further aspects of the same experiments, particularly how hypoxia affected the growth, feeding and condition of juvenile fish. Chapter 5 describes the results of an experiment on the effects of fluctuating hypoxia on reproduction of Utchee Creek rainbowfish and effects of parental exposure to hypoxia on embryos. Chapter 6 investigates effects of hypoxia on egg viability and larval health of Utchee Creek rainbowfish, eastern rainbowfish and sooty grunter. Finally, Chapter 7 discusses the implications of these results and suggests areas for future research.

CHAPTER TWO: GENERAL METHODS

2.1   Pilot studies of methodology, and using nitrogen to displace oxygen in an aquarium environment

Experimental studies on the effects of hypoxia on fish have used a variety of methods to achieve depleted oxygen levels, including addition of nitrogen gas, vacuum degassing, addition of sodium sulphite, insertion of cages or mesocosms into low-oxygen environments in the field, and sealing the experimental containers so the fish's own respiration removes oxygen from the water (e.g. Nitrogen - Cech & Massingill, 1995; Pichavant *et al*., 2001; vacuum – Scott & Rogers, 1980; Pouliot & de la Noue, 1989; sodium sulphite – Chapman *et al*., 1994; field experiments – Dunson & Dunson, 1999; Ruggerone, 2000; fish respiration – Hunn, 1969; Thetmeyer *et al*., 1999; Waller *et al*., 2000). Each of these methods has advantages and drawbacks, and the decision on which method to use must allow for constraints such as size of the organisms being used and available space, equipment and funding.

Initially, I trialed the concept of using aquatic plants as a natural oxygen-reduction agent, by placing plants into the experimental tanks and sealing them so that respiration by the plants was sufficient to remove large quantities of oxygen from the experimental aquaria (Appendix 1). Although the technique worked, it required a great deal of further development as in its current form the resulting oxygen cycling was too variable. Consequently, for this study I chose to bubble nitrogen gas into the experimental aquaria to remove oxygen from the water by displacement.

The nitrogen bubbling method has been extensively used in previous studies (e.g. Fry, 1951; Downing, 1954; Kramer & Mehegan, 1981; Cech & Massingill, 1995; Chabot & Dutil, 1999; Taylor & Miller, 2001; Pearson *et al*., 2003), is known to be efficient, and is capable of producing extremely low levels of dissolved oxygen (DO) saturation. The advantages of using nitrogen gas are that it is easy to control, biologically inert and readily available. It does, however, have some drawbacks, including the presence of a "nitrogen atmosphere" above the water's surface (which prevents fish from breathing air, or performing aquatic surface respiration) and unnatural cycling of pH when compared to field situations.

2.2 Design of experiments

2.2.1 Location

With the exception of the experiment on sooty grunter eggs (Chapter 6), all experiments were carried out in an aquarium room in the School of Tropical Biology, James Cook University, Townsville. The experiment on sooty grunter eggs was carried out at the QDPI Research Station at Walkamin, near Atherton.

2.2.2 Holding tanks

Prior to experiments, fish (excluding embryos) were held in 200 L and 1000 L tanks in the experiment room. All fish were housed with others of the same species and similar size. Density in holding tanks was low, and never rose above one fish per 5 L. Holding tanks were fitted with Fluval canister filters, and water quality in the holding tanks was monitored regularly. Fish were fed daily on either Nutrafin tropical fish flake (in the case of eastern rainbowfish and Utchee Creek rainbowfish) or commercially prepared aquaculture-grade pellet food (in the case of sooty grunter and barramundi). Adult rainbowfish used in the egg and reproduction experiments were also fed with frozen bloodworms and live *Artemia* nauplii to maximise their reproductive output. All fish were held and acclimatized to laboratory conditions for at least two weeks prior to transfer to experimental aquaria. Fish were allocated to experimental tanks using random numbers to prevent bias.

2.2.3 Experimental aquaria and associated equipment

Apart from the sooty grunter experiment (Chapter 6), all experiments were carried out in 30 L glass aquaria with an internal PVC shelf around the perimeter and a PVC lid (Figure 2.1), filled with 25 L of water. This design allowed tanks to be completely closed when necessary, and no access to the air:water interface was available to fish once lids were in place. Two air tubes were housed permanently inside each tank, one carrying air, and the other nitrogen. Each tube could be turned on or off with a tap. Industrial nitrogen was delivered from cylinders fitted with a gas regulator through a manifold, with plastic tubes running from the manifold and delivering equal pressures of nitrogen gas to each experimental tank. Aquaria were kept on three levels of open

shelving, and allocation of DO treatments to aquaria was randomized and maintained for all experiments (Figure 2.2, 2.3). Circulation and gas exchange within aquaria were maintained during most experiments by use of a small submersible pump (Resun SP-600: 5 watts; 220 volts; 60/50 hertz; 250 L/hr delivery) in each. Pumps were not used in the experiments involving juvenile sooty grunter or in any of the experiments on eggs because pilot trials showed sooty grunter and hatching fish larvae to be distressed by the water current. Further detail is included in Chapters 3 and 4. Water temperature was maintained at 28°C (+/- 2°C) using temperature controls for the room, to simulate summer-time conditions in the field, when depleted dissolved oxygen levels are likely to be more detrimental to fish (Martin & Saiki, 1999).

Figure 2.1 30 L glass experimental aquaria with specially designed PVC lids and access holes.
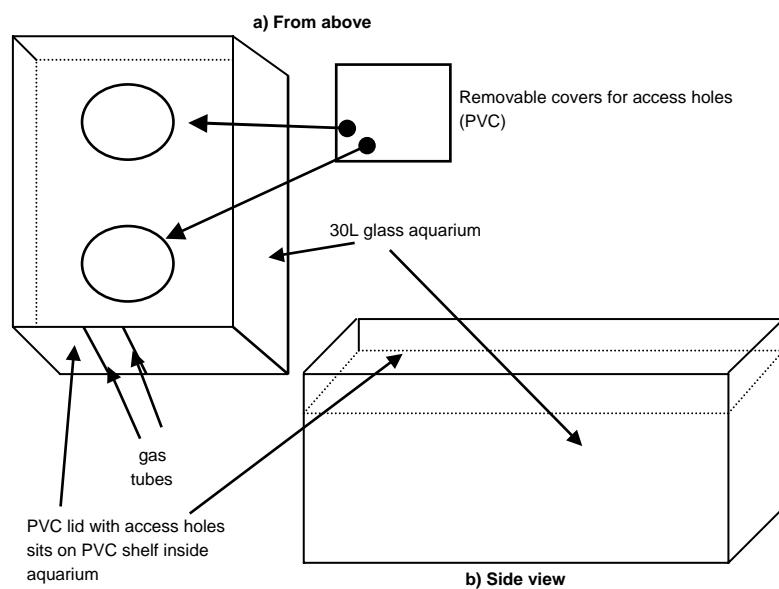
2.2.4   Treatments

In all experiments, 18 aquaria were used. Four of these were reserved as normoxic (>85% DO saturation) controls, with the remaining fourteen divided into seven treatments, two aquaria to a treatment. The seven treatments had minimum dissolved oxygen levels of 5%, 10%, 20%, 30%, 40%, 50% and 60% with maximum oxygen levels being normoxic (>85%) in all cases. Oxygen levels were cycled daily (Figure 2.4), with depletion taking place from 8.00 a.m. until 1.00 p.m., with

dissolved oxygen levels recorded half-hourly. Depletion of oxygen was achieved by bubbling nitrogen gas into the water column until the desired level was reached, at which time nitrogen supply was turned off, and re-aeration began using compressed air.

During oxygen reduction, lids were placed on the aquaria so that a nitrogen atmosphere formed between the surface of the water and the lid. This meant that although aquatic surface respiration (ASR – described further in Chapter 3) was attempted by fish, it was not advantageous, as higher oxygen areas were not able to be accessed. Thus, fish could not supplement dissolved oxygen available in the experiment with oxygen gained at the air:surface interface. This situation is somewhat different to what may occur in the wild, but does allow determination of an absolute level of DO saturation that causes harmful effects to the fish.

Figure 2.2 Configuration of experiment tanks on shelving units (view from back of tanks). Tanks are labeled with treatment (number) and allocation within treatment (letter). All tanks except controls had both a nitrogen bubbling tube and an air bubbling tube. Lines dropping from the manifold to the tanks carry nitrogen gas. Nitrogen flow could be stopped by separate taps on each gas line. Treatments reached a minimum DO saturation each day of: treatment 1, 5%; treatment 2, 10%; treatment 3, 20%; treatment 4, 30%; treatment 5, 40%; treatment 6, 50%; treatment 7, 60%; treatment 8, control (>85% at all times).
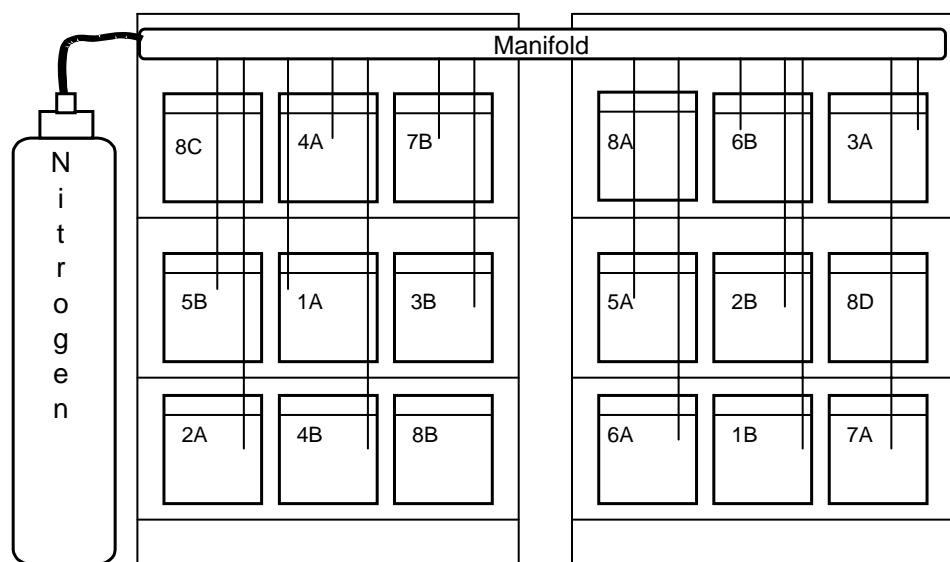
2.2.5 *Lighting*

During the 5-hr oxygen depletion period, the experiment room was in darkness, with the exception of photographic quality red globes (Philips 240 volt 15 watt RED). This situation simulated nocturnal oxygen depletion, as usually occurs in the field. Because fish metabolism may differ diurnally it was important to replicate photoperiod in this way. Room lights switched on automatically at 1.00 p.m., to simulate daybreak and switched off again 14 hours later, at 3.00 a.m., producing a 14-hr light : 10-hr dark photoperiod. During the experiment on reproduction of rainbowfish (Chapter 5), an additional set of "dawn" lights came on at 12.00 p.m., which cast a gentle light on the room for one hour until "daybreak" at 1.00 p.m. This adjustment was made because rainbowfish prefer to produce eggs during the early morning when light is dim (B. Sambell, pers. comm. 2004[1]).

Figure 2.3 Photo of experimental set up in the aquarium room taken from front of tanks, and showing nitrogen gas cylinders with regulator attached to the cylinder in use. PVC lids on each aquarium have access holes, and are depressed about 2 cm below the rim of the aquaria.

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[1] Mr Bruce Sambell, President Aquaculture Association of Queensland, Inc., PO Box 415, Childers, Queensland 4660.

Figure 2.4 Example of daily DO fluctuations for each treatment. Note minimum level reached for each treatment before returning to normoxia.
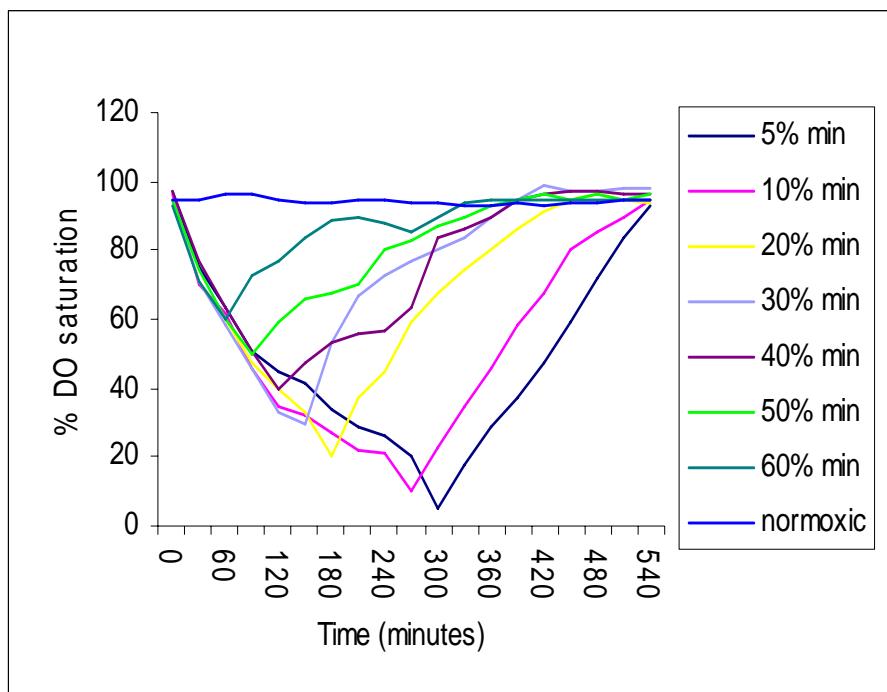
2.2.6   Water quality

The water used for holding tanks and experimental aquaria was tap water filtered using a series of three Raindance filters (5 μm sediment, 1 μm sediment, 0.5 μm carbon). Testing by the Australian Centre for Tropical Freshwater Research (ACTFR) laboratory demonstrated that this filter series removes chlorine, heavy metals, sediments and bacteria from the water (B. Butler, pers. comm. 2001[2]).   During experiments, aquaria received a daily 50% water change, and excess food and waste were siphoned off. Temperature and pH were recorded at 8.00 a.m., and at the time at which minimum oxygen level was reached for each aquarium (this time varied between treatments). All dissolved oxygen, pH and temperature readings were taken using a WTW (Wissenschaftlich-Technische Werkstatten) pH/Oxi 340i meter, in combination with a CellOx 325-3 dissolved oxygen probe and SenTix pH probe. Both probes were calibrated daily. Nitrogenous wastes including nitrate, nitrite and ammonia were monitored throughout the experiments using test kits.

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[2] Mr Barry Butler, Australian Centre for Tropical Freshwater Research, James Cook University, Townsville, Queensland 4811.

2.2.7   *Experiments*

Seven experiments were carried out. The aim was to cover several species, and several life history stages to identify a variety of sublethal parameters and measure the effects of hypoxia on these (Table 2.1).

Table 2.1 Experiments carried out for the project in chronological order with lists of parameters measured in each.

1.1 *Experiment*	Species	Age class	Average weight of fish and (range) (g)	Date commenced & duration	Parameters measured in response to hypoxia
1	*2* *Lates calcarifer*	Juvenile	2.65 (1.43 – 3.97)	Feb 03 21 days	Ventilation Gill condition (histology) Feeding behaviour Food consumption Growth Body condition
2	*Melanotaenia splendida splendida*	Juvenile	0.28 (0.12 – 0.60)	May 03 28 days	Ventilation Gill condition (histology) Feeding behaviour Food consumption Growth Body condition
3	*Melanotaenia splendida splendida*	Embryos	N/A	Oct 03 18 days (collection & cycling)	Viability Mortality Deformities Incubation time Size of hatching larvae

1.1 *Experiment*	Species	Age class	Average weight of fish and (range) (g)	Date commenced & duration	Parameters measured in response to hypoxia
4	*Hephaestus fuliginosus*	Embryos	N/A	Jan 04 5 days (cycling & hatching)	Viability Mortality Deformities Incubation time Size of hatching larvae
5	*Hephaestus fuliginosus*	Juvenile	Set 1: 1.45 (1.18 – 1.86) Set 2: 1.48 (1.11 – 2.09)	Mar 04 28 days each, two sets	Ventilation Gill condition (histology) Feeding behaviour Food consumption Growth Body condition

1.1 *Experiment*	**Species**	**Age class**	**Average weight of fish and (range) (g)**	**Date commenced & duration**	**Parameters measured in response to hypoxia**
6	*Melanotaenia utcheensis*	Adults (& resulting embryos)	Male: 2.71 (1.39 – 5.53) Female: 2.04 (1.46-3.43)	July 04 28 days (& 9 days for embryos)	Brood size Egg size Total eggs over duration Body condition GSI Gonad condition (dissection & histology) Resulting embryos: viability, mortality, deformities, incubation time, size of hatching larvae
7	*Melanotaenia utcheensis*	Embryos	N/A	July 04 18 days (collection & cycling)	Viability Mortality Deformities Incubation time Size of hatching larvae

2.3   Test species

Fish species were in part chosen opportunistically for inclusion in this study. Logistical restraints in keeping animals alive in a captive environment, sourcing the numbers of individuals required for each experiment and the availability of human resources all contributed to the selection of species. As the project could only attempt to cover a sample of the general conditions experienced in the real world, the opportunistic sampling of all possible treatments might arguably be as good as any systematic but restricted approach in providing a cross-section of fish responses. The inclusion of key species such as the important predators barramundi and sooty grunter and the ubiquitous and common eastern rainbowfish, as well as the range-restricted Utchee Creek rainbowfish, lends a level of variety and interest to this approach.

As many life history stages of each species as possible were tested. Juveniles of barramundi, sooty grunter and eastern rainbowfish were easily accessible, so all were tested. This life history stage was thus the most thoroughly examined within the thesis. I was able to test eggs of eastern rainbowfish, as only a small number of adults were available to work with. However, insufficient adult eastern rainbowfish were available to allow additional experiments on them – and, given the choice, it was more relevant to consider the effects of hypoxia on adults of Utchee Creek rainbowfish as they are relatively unknown. Sufficient adults of Utchee Creek rainbowfish were provided by a commercial hatchery to allow the effects of hypoxia on both adults and eggs to be tested. Juvenile Utchee Creek rainbowfish were unable to be sourced, and it was not possible to collect them from the wild for experiments, given their restricted range and low numbers. Eggs of barramundi were not available for testing, and the small size of the experimental aquaria precluded the testing of mature barramundi or sooty grunter – in any case it would have been very difficult (and possibly meaningless) to assess the effects of hypoxia on reproduction of these species as they have to be artificially induced to spawn in captivity.

The fish used were a mixture of wild caught and farmed fish. Barramundi were hatchery raised in an intensive aquaculture facility. Sooty grunter juveniles were grown out in an extensive aquaculture facility, as were all Utchee Creek rainbowfish. Juvenile eastern rainbowfish were wild caught, and eastern rainbowfish eggs were

spawned in aquaria by wild-caught adult eastern rainbowfish. Sooty grunter eggs were also artificially spawned by a wild caught adult fish, this species requires hormonal induction to spawn in captivity.

The use of farmed rather than wild caught fish was preferred for this study where possible, particularly for Utchee Creek rainbowfish which are not abundant in the wild. However the use of farmed rather than wild fish may add another level of complexity to interpretation of results, especially as the differences in behaviour, physiology and tolerance to environmental conditions of wild caught versus farmed animals are not well documented.

Studies on masu salmon (*Oncorhynchus masou*) have shown that farmed fish exhibit reduced predator avoidance compared to wild fish, and also show higher feeding aggression (Yamamoto & Reinhardt, 2003). Similar behaviour differences have been identified in wild and farmed Atlantic salmon (*Salmo salar*) (Huntingford & Adams, 2005). In salmon, which have been farmed for many years, the continuing selection of fish which are better suited to an aquaculture environment, particularly fast-growing individuals, has resulted in an inadvertent selection of fish with bold personalities who take greater risks when foraging (Huntingford & Adams, 2005). For this reason it is necessary to consider the results of each experiment in the context of the animals used, and to note that some animals were hatchery-raised fish and thus may show different behavioural traits to their wild counterparts. Any differences in behaviour of the fish used in this study are likely to be less pronounced than those found between wild and cultured salmon, as fish used in this study were only first or second generation farmed fish.

Pusey *et al.* (2004) provide a general description of fish species of north-eastern Australia, from which the following details on the four study species are derived, unless otherwise indicated.

Barramundi (*Lates calcarifer*) Family Centropomidae

Barramundi for the experiment were purchased from a commercial fish hatchery in far north Queensland. They are highly valued in commercial and recreational fisheries and as a profitable aquaculture species. They are common and widespread in coastal

drainages from Shark Bay in Western Australia around the northern parts of Australia and as far south as the Mary River in southeastern Queensland (Allen *et al*., 2003). Barramundi also have an important role as predators at the top of the aquatic food chain, consuming microcrustaceans, fish and aquatic insects (as postlarval fish), macrocrustaceans, fish and aquatic insects (as juvenile fish), and fish and macrocrustaceans (as adult fish).

Adults are protandrous hermaphrodites; they commence life as males, mature as males at approximately three years of age, and undergo a rapid sex change to females as early as six years of age. The females are extremely fecund and may lay up to 20 million eggs per year. The life-cycle is semi-catadromous, and adult fish migrate to estuarine waters to spawn during the first wet season of their adult lives, but do not return to freshwater following this. In field situations, larvae develop rapidly after a short embryonic stage (< 24 hrs) and move into supralittoral habitats until they are about 20cm long, at which stage they migrate upstream into freshwater for three to four years prior to adulthood (Allen *et al*., 2003). During the young juvenile stage, whilst living in wetlands, barramundi are regularly exposed to harsh environmental conditions including high salinities (up to 50 ppt) and high water temperatures (up to 38°C). In an aquaculture or aquarium situation, juveniles may be successfully raised in fresh water from about 2 cm in length (L. Rodgers, pers. comm. 2004[3]).

Despite being a victim of hypoxia-related fish kills from time to time, barramundi are generally thought to be moderately hypoxia-tolerant. They have been recorded living in the wild in waters with surface level DO concentrations down to 1.1 mg/L (13-15% saturation at 25-30°C, Pusey *et al*., 2004). However this is very difficult to interpret without accompanying time of day data, due to potentially massive diel cycling in oxygen levels in tropical water bodies (Pearson *et al*., 2004). That is, if the reading was taken from mid-morning onwards, the fish may have been exposed to much lower levels of oxygen at dawn than have been recorded.

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[3] Mr Les Rodgers, Queensland Department of Primary Industries, Freshwater Fisheries and Aquaculture Centre, Kennedy Highway, Walkamin Qld 4872.

Sooty grunter (*Hephaestus fuliginosus*) Family Terapontidae

Sooty grunter were obtained from the Tablelands Fish Stocking Association, based in Atherton. The species occurs in tropical fresh waters of Australia and New Guinea. In Australia their range is fragmented, and extends from the Northern Territory to central Queensland, but it is unclear in some cases whether the species occurs naturally or has been introduced as a game fish (Pusey *et al*., 2004). Sooty grunter are not part of a commercially recognized fishery, but are a popular game fish for recreational fishers and have recently been quite successfully grown as an aquaculture species. They are a voracious predator, and consume a variety of invertebrates, fish and plant materials. Fish are quite territorial and in aquarium situations may aggressively attack fish including those much larger than themselves (pers. obs.).

Sooty grunter are found in moderately to well-oxygenated running waters, and have been recorded only from waters that are almost completely fresh. The minimum dissolved oxygen level recorded for wild fish is 3.7 mg/L (about 45-50% DO saturation at 25-30°C, Pusey *et al*., 2004). However, as described for barramundi above, the lack of information on time of day at which this DO saturation was measured makes it difficult to interpret in terms of a level of tolerance of the species.

Adults have been observed to spawn in shallow water in aggregations of up to 50 individuals during summer[4]. They lay demersal eggs, with average diameter 2.1 mm (Hogan, 1990) that sink to the substrate and lodge in cracks and crevices. In one spawning session a female may lay hundreds of thousands of eggs, and within several weeks she is ready to spawn again (A.E. Hogan, pers. comm. 2004[5]). Eggs of sooty grunter take from 1-3 days (at 28°C; Hogan, 1990) to 3-4 days (Allen *et al*., 2003) to hatch, with larval hatch size being about 5.5 mm (Hogan, 1990). Embryology of the closely related coal grunter (*Hephaestus carbo*) has been described and is likely to be very similar to that of sooty grunter. Coal grunter take from 48 to 98 hrs to hatch, with most hatching occurring around 60 hrs post-fertilisation. Larval size on hatching is about 4.2 mm (at 25 – 28°C; Close *et al*., 2001).

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[4] www.nativefish.asn.au /sootygrunter.html
[5] Mr Alf Hogan, Queensland Department of Primary Industries, Freshwater Fisheries and Aquaculture Centre, Kennedy Highway, Walkamin Qld 4872.

Eastern rainbowfish (*Melanotaenia splendida splendida*) Family Melanotaeniidae

Eastern rainbowfish were sourced from JCU Campus Creek, Townsville. Eastern rainbowfish are small schooling fish that are widely distributed along the east coast of Queensland from the Lockhart River in the north to at least the Boyne River in the south, although the exact distribution is confused due to similarities between subspecies within the *Melanotaenia splendida* group (Pusey *et al*., 2004). Eastern rainbowfish are abundant wherever they occur, frequently making up greater than 20% of a fish assemblage, and are tolerant to environmental degradation, including removal of riparian zones and low DO saturations.

Eastern rainbowfish are omnivorous feeders that consume primarily small aquatic invertebrates and terrestrial insects from the water's surface. In aquarium situations they consume commercially prepared flake foods or pellets, supplemented with fresh or frozen foods such as *Artemia* or bloodworms (pers. obs.). Their ease of care in artificial situations has made rainbowfish (Melanotaeniidae) popular occupants of home aquaria in Australia as well as around the world. Rainbowfish are also easy to breed in artificial situations, which adds to their importance as a subject for home aquaria as well as for biomonitoring and laboratory testing (Humphrey *et al*., 2003).

Although eastern rainbowfish appear to prefer well-oxygenated waters, they are known to sometimes experience naturally occurring hypoxia in the wild. The species has been recorded in wetland habitats where oxygen levels dropped to 0.2 mg/L (2-3% DO saturation at 25-30°C) in the bottom water layers. However, as described for barramundi above, the lack of information on time of day at which this DO saturation was measured makes it difficult to interpret in terms of a level of tolerance of the species. Pusey *et al*. (2004) suggest a dissolved oxygen level of 4 mg/L (50-55% DO saturation at 25-30°C) would probably be sufficient to protect most populations of the species.

Rainbowfish typically become reproductively mature after their first year (Allen *et al*., 2003). During the summer breeding season, when water temperatures are higher and photoperiod longer, eastern rainbowfish lay and fertilise eggs daily on submerged macrophytes. Adult eastern rainbowfish may produce up to several hundred eggs each day (Humphrey *et al*., 2003), which become attached to vegetation with thin adhesive

filaments. Hatching may take 4–8 days, and larvae hatch at a size of about 3.7 mm standard length and swim at the surface of the water (Humphrey *et al*., 2003).

Utchee Creek Rainbowfish (*Melanotaenia utcheensis*) Family Melanotaeniidae

Adult Utchee Creek rainbowfish (*Melanotaenia utcheensis*) were donated by the Aquaculture Association of Queensland and provided by Ausyfish commercial fish hatchery in Childers, Queensland. The Utchee Creek rainbowfish has only recently been reclassified as a distinct species, having previously been considered a morph of *M. trifasciata* or *M. s. splendida* (McGuigan, 2001). In fact, *M. utcheensis* is likely to be an older species of rainbowfish than the well-known and widespread *M. s. splendida*. The species has a restricted range, and is found only in the wet tropics bioregion of north Queensland, specifically in the Johnstone River Basin (Pusey *et al*., 2004). Its name comes from one of the lowland creeks it inhabits, Utchee Creek, a tributary of the South Johnstone River. Very little is currently known about the Utchee Creek rainbowfish, but it may qualify as vulnerable under criteria of the World Conservation Union if future studies show it is being excluded from its natural range by *M. s. splendida* (McGuigan, 2001).

The lowland habitats populated by *M. utcheensis* are likely to experience hypoxia as the South Johnstone River is surrounded in part by agricultural lands and nutrient levels are possibly increased through runoff from rich fertilized soils. However the only available data on oxygen levels experienced by wild populations range from 5.7 mg/L (70-77% DO saturation at 25-30°C) to 9.2 mg/L (supersaturated waters of about 112-124% DO saturation at 25-30°C). As described for barramundi above, the lack of information on time of day at which these DO saturations were measured makes it difficult to interpret in terms of a level of tolerance of the species.

No published information is available describing reproduction or embryology of the species, but it is likely to be very similar to other fish of the family Melanotaeniidae. Typical rainbowfish breeding behaviour and biology is described for *M. s. splendida* above.

CHAPTER THREE: THE EFFECTS OF FLUCTUATING HYPOXIA ON VENTILATION OF JUVENILE FISHES

3.1   Introduction

The respiratory system has traditionally been a dynamic focus of study in fish physiology (Perry & Tufts, 1998). It is a system in which the links between an animal and its environment are paramount, and it reinforces the concept that biological fitness is fundamentally dependent upon ambient conditions. Most fish ventilate external gills by creating a flow of water via branchial pumping or by opening the mouth and operculi whilst swimming. Efficient oxygen uptake is enabled by the fine sieve structure of gills, and is vital because of the low concentration of oxygen in water in comparison to air (Moyle & Cech, 2004).

The majority of studies on the effects of hypoxia on fish record changes to cardiovascular and respiratory physiology (reviews include: Fry, 1971; Hughes, 1973; Kramer *et al*., 1978; Kramer, 1987; Bridges, 1988; Jobling, 1994 - Chapter 15; Heath, 1995 - Chapter 2; Val *et al*., 1998). As dissolved oxygen levels drop, fish attempt to increase the rate of gas exchange at the gills to overcome the reduced water-to-blood oxygen gradient (Moyle & Cech, 2004). One way of doing this is to increase the volume of water pumped over the gills by increasing ventilation rate (frequency of opercular pumping) or stroke volume (amount of water pumped with each stroke). For example, while channel catfish (*Ictalurus punctatus*) alter ventilation by changing stroke volume, bluegill (*Lepomis macrochirus*) and carp (*Cyprinus carpio*) increase stroke frequency in response to hypoxia (Heath, 1995).

An increase in ventilation under mild hypoxia allows the oxygen pressure in arterial blood to remain similar to that found under normoxic conditions (Heath, 1995). The level of hypoxia at which a fish begins to increase its ventilation rate differs between species, perhaps as a result of the different degrees of adaptation to or tolerance of hypoxia. The issue of control of ventilation rate in fish is one of much contention in the literature. Heath (1995) provides a review of arguments presented by various authors. In summary, the mechanisms of ventilation control have been suggested as receptors responding to blood oxygen pressure, blood oxygen volume or surrounding

water oxygen saturation or catecholamine release, including adrenalin and noradrenaline.

Fish can also use special behavioural mechanisms to increase the amount of oxygen available to them. Aquatic surface respiration (ASR) is a technique used frequently by water-breathing fish to acquire oxygen (Kramer *et al*., 1978; Kramer & McClure, 1982; Saint-Paul & Soares, 1988; Gee & Gee, 1991; Chapman *et al*., 1995; Val *et al*., 1998). During ASR fish utilize the highly oxygenated water at the air:water interface by tilting the body upwards and directing the mouth to the water's surface. ASR can be thought of a "sophisticated version of the escape response" according to Jensen *et al*. (1993). Fish living in frequently hypoxic waters are more likely to perform ASR than those from consistently normoxic habitats (Kramer, 1983). Guppies (*Poecilia reticulata*) utilizing ASR are able to survive for 10 hours under hypoxic conditions that would otherwise be detrimental after 10 minutes (Kramer & Mehegan, 1981). This ability would be extremely useful in an oxygen cycling scenario, when dawn oxygen levels are very low, but last only for a few hours.

The primary sites of gas exchange within the gills are the multitude of tiny lamellae that line the paired gill filaments. The number of lamellae used in respiration increases under hypoxia, as does perfusion of blood in the lamellae (Sollid *et al*., 2003; Moyle & Cech, 2004).

Fish gills are important organs not just for their role in gas exchange, but also for other functions, including ion regulation, maintenance of acid-base balance and excretion of nitrogenous wastes (Hinton & Lauren, 1990). Because the gills are essentially external organs, they are also exposed to ambient conditions, making them vulnerable to damage if conditions are unfavourable (Mallat, 1985). Gills are regarded as a site for potential injury after exposure of fish to pollutants, including: acid waters (Jagoe & Haines, 1983); heavy metals such as copper (Heath, 1991; Cerqueira & Fernandes, 2002) and nickel (Nath & Kumar, 1989); herbicides and pesticides including endosulfan (Cengiz & Unlu, 2002) and trifluran (Poleksic & Karan, 1999); ammonia (Reichenbach-Klinke, 1973; Wedemeyer *et al*., 1976); oil and oil dispersants (Monfils, 1998) and many others (for a review see Mallat, 1985). Because of this, gills are considered to be the most appropriate organs to use as indicators of

water pollution levels (Alazemi *et al.*, 1996). However, few published studies have attempted to identify the effects of hypoxia on fish gills. Exceptions are Scott & Rogers (1980), who identified histopathological lesions on gills of channel catfish following sublethal exposure to hypoxia; and Drewett & Abel (1983), who found in post-mortem examination of fish killed by hypoxia that gill epithelial breakage had occurred.

In this chapter I investigate the effects of fluctuating hypoxia on ventilation, ventilatory behaviour and gill histology of juveniles of three species of fish common to the Australian wet tropics – barramundi (*Lates calcarifer*), eastern rainbowfish (*Melanotaenia splendida splendida*) and sooty grunter (*Hephaestus fuliginosus*).

3.2   Methods

3.2.1   *Experiments*

The barramundi experiment ran for 21 days with no deaths; the rainbowfish experiment ran for 28 days, and seven out of eight fish in the 5% DO treatment (treatment 1) died, as well as one of the eight fish in the 10% DO treatment (treatment 2); and the sooty grunter experiment ran for 28 days, in which all four fish died in the 5% DO treatment (treatment 1), and there was one unexplained death (suspected bacterial gill disease) in the control treatment.

Barramundi

Juvenile barramundi were kept for three weeks before being placed into experimental aquaria (see Chapter 2), which for this experiment were each divided into four equal compartments using 5 mm gutter guard plastic mesh. A total of 72 barramundi were used in the experiment, with four fish in each of 18 tanks. Fish were weighed, and standard length and total length were measured (Chapter 4) before they were placed into the experimental aquaria, where they were acclimated for several days until feeding normally. After acclimation, dissolved oxygen cycling commenced and was carried out on a daily basis from 8.00 a.m. until 1.00 p.m. for 21 d. After cessation of oxygen cycling at 1.00 p.m., fish were left for two hours, then fed at 3.00 p.m. with commercially prepared barramundi aquaculture pellets, and aquaria were cleaned and

a 50% water change carried out at 4.30 p.m. Levels of nitrogenous wastes including ammonia, nitrite and nitrate were measured regularly using test kits. Although some fluctuations in wastes were identified prior to changing the water each day, they were not at dangerous levels and were remedied by the daily water change. It was intended to keep barramundi under fluctuating hypoxia for 28 d (as for all other experiments on juveniles and adult fish) but they quickly outgrew the experimental tanks. Therefore, the experiment was stopped after 21 d to avoid any confounding effects of high stocking density. After 21 d cycling, fish were left for 24 hr with no feeding before being euthanased in an icy slurry, weighed and measured again, and fixed in 4% phosphate-buffered formaldehyde for later histopathological analysis.

Eastern rainbowfish

Before commencement of dissolved oxygen cycling juvenile rainbowfish were anaesthetized using benzocaine (ethyl p-aminobenzoate) then weighed, and standard length and total length were measured (Chapter 4). The anaesthetic was used to prevent fish flipping off the scales and causing themselves damage. The fish were then placed into the experimental aquaria (Chapter 2) and allowed to acclimate for several days until feeding recommenced. For this experiment, the experimental aquaria housed four fly-mesh containers, each of which held one fish. The closed screw-top containers prevented movement of fish between compartments. A total of 72 juvenile rainbowfish were used in the experiment, with four fish in each of 18 aquaria. Of these, seven out of eight fish in the lowest (5%) DO treatment died during the experiment, as did one fish in the 10% DO treatment.

Once fish had acclimated to the aquarium conditions, dissolved oxygen cycling commenced. After cessation of oxygen depletion at 1.00 p.m., fish were left for 2 hr, fed at 3.00 p.m., and their aquaria cleaned and 50% of the water changed at 4.30 p.m. This treatment was repeated every day for 28 d over the course of the experiment. Levels of nitrogenous wastes including ammonia, nitrite and nitrate were measured regularly using test kits. Although some fluctuations in wastes were identified prior to changing the water each day, they were not at dangerous levels and were remedied by the daily water change. After 28 d, fish were left in aquaria for 24 hr with no feeding and euthanased in an icy slurry, weighed and measured again, and fixed in 4% phosphate-buffered formaldehyde for later histopathological analysis.

Sooty grunter

Several hundred juvenile sooty grunter (about three months old) were kept in holding tanks until commencement of normal feeding (five days). A pilot trial found that sooty grunter did not recover well from anaesthesis with benzocaine, and frequently damaged themselves on the plastic mesh partitions used to separate barramundi. Therefore, several changes were required to adapt the experimental design used in the other experiments for this species. Firstly, partitions were removed from aquaria to prevent the fish causing themselves damage. This meant the four fish in each aquarium could no longer be treated as individuals in the collection of growth data. Secondly, the use of anaesthetic before initial size measurements of fish was removed, and so fish length could not be measured. Weight was obtained by carefully blotting water droplets from alert (not anaesthetised) fish and placing them in a pre-weighed container of water on scales. A length:weight relationship was identified by anaesthetizing 20 fish not used in the experiment and measuring their total length, standard length and weight (Chapter 4).

A few days into the experiment on sooty grunter it became clear that a dominant fish had arisen in each tank, and was aggressive towards the other three fish, often to the point of fatality. The severity of the aggressive behaviour was surprising, as no similar event had been observed in the holding tanks. It was decided that the data gathered from the three subservient fish would not be usable, as bullying would be a confounding factor in analysis of fish condition. Hence, all but the one dominant fish were removed from each tank. This reduced the information to be collected from the experiment, and to offset this reduction, a second set of tanks was established, with one (previously unused) sooty grunter in each tank. The second experiment commenced two weeks into the first sooty grunter experiment and continued for two weeks after cessation of the first. Hereafter, fish from the two experiments will be referred to as being form either set 1 (the experiment where dominant fish were retained) or set 2 (carried out using untested fish). Weights were obtained in the same manner as previously, and tank set-up was identical to the first set of tanks and in the same aquarium room. In all 36 tanks used for sooty grunter, four small (~5 cm diameter) rocks were placed in each aquarium, to provide some substrate, as fish seemed unduly stressed in empty tanks.

Submersible pumps were not used to circulate water during this experiment, as fish appeared to be disturbed by the high currents created, and water circulation was naturally greater after removal of the plastic mesh partitions, allowing sufficient mixing of water for uniform de-oxygenation. In each set of tanks, after acclimating fish for several days until they were feeding normally, DO cycling commenced and was carried out on a daily basis from 8.00 a.m. until 1.00 p.m. (set 1) for 28 d. After cessation of oxygen cycling in all tanks, fish were left for two hours, and then fed at 3.00 p.m., and aquaria were cleaned and a water change implemented at 4.30 p.m. At the beginning of the set 1 experiment, there were four fish in each tank and 50% of water was changed, but once fish numbers in each tank were reduced to one, only 25% of water was changed daily. Levels of nitrogenous waste were measured regularly, and no increases from normal were identified. During the experiment, all fish in the lowest treatment (reaching 5% DO saturation) died, as did one fish in a control tank in Set 2.

After 28 d of oxygen cycling, fish were left for 24 hr with no feeding before being euthanased in an icy slurry, weighed and measured (total and standard lengths), and fixed in 4% phosphate buffered formaldehyde for later histopathological analysis.

3.2.2 *Measuring ventilation*

Ventilation rate of juvenile fish was measured, using a stopwatch to record the time for fish to take 50 breaths (in-and-out movements of the operculum). Time to take 50 breaths was then converted to breaths taken per minute (beats per minute). Ventilation rate was measured before commencement of oxygen depletion in the morning (as baseline ventilation rate), as well as at the minimum oxygen level for each aquarium. On some occasions, it was not possible to measure baseline ventilation rate, such as when breathing was so slight it was not observable. In these instances, no ventilation rate was recorded. Due to the aquarium set-up only two fish were visible in each aquarium (of rainbowfish and barramundi), so values are only available for half the fish from each experiment. Ventilation behaviour was recorded at the same time as ventilation rate, and was classified into four stages. These were 0) no opercular movement detectable; 1) mouth closed, opercular movements barely visible, behaviour normal with no erratic movements, but easily disturbed; 2) mouth closed,

opercular movements obvious but not large, behaviour normal; 3) mouth open, fast, large opercular movements, appears to gulp at water, behaviour abnormal, circles aquarium sides, not easily disturbed; 4) aquatic surface respiration behaviour attempted (as described by Kramer & Mehegan, 1981), mouth open, behaviour abnormal, circles aquarium sides and spends large amounts of time at the water's surface.

In these trials, ASR could be attempted by fish as a behaviour, but was not successful, as fish did not have access to oxygen at the air:water interface, where greater oxygen levels would normally be present (see Chapter 2). Instead, the air above the water was largely made up of nitrogen gas, which diffused out of the water as a result of using the gas as a means of displacing dissolved oxygen, creating a nitrogen atmosphere. For this reason no advantage was gained by fish who attempted ASR.

3.2.3   *Histological and pathological methods*

Fish were fixed in 4% phosphate-buffered formaldehyde, and transferred after one week to 70% ethanol. Prior to fixation, juvenile barramundi and sooty grunter were opened with a cut to the ventral surface to allow greater penetration of fixative – this was unnecessary for the juvenile rainbowfish due to their small size. After transfer to ethanol, the upper gill arch on the right side of each fish was dissected out, and placed into a histology cassette. Gills were decalcified overnight in 10% formic acid and the end-point of decalcification was determined using Arnim's test (Arnim, 1935). Decalcified gills were embedded in paraffin wax and cut using a microtome to 5 μm sections, stained with Mayer's Haemotoxolyn & Young's Eosin (H&E stain) and mounted with coverslips on glass slides using dibutyl-phthalate-polystyrene-xylene (DPX) mountant.

To quantify pathological changes to gill filaments, five filaments were analysed for each fish. On each filament several parameters of gill morphology were considered at 400x magnification. These are listed in Table 3.1, and illustrated in Figure 3.1. Gills from every barramundi and every sooty grunter were considered. Damage to

rainbowfish gills during histological preparation precluded detailed analysis of hypoxic effects for this species (see section 3.4.3).

Table 3.1 Parameters of gill morphology considered during histopathological analysis of gill condition of juvenile fish. Superscript numbers refer to illustration in Figure 3.2.

Part of gill	Parameter
Filament	Presence/absence of melanin
	Presence/absence of collagen
	Presence/absence of blood cell congestion
	Presence/absence of parasites
	Location of eosinophilic granular cells (if present)
	Location of macrophages (if present)
	Number of aneurisms
	Width of tip and base of filament and of widest section
Lamella	Level of severity of epithelial lifting and breakage[1]
	Number of lamellae with haemorrages and/or aneurisms[8]
	Level of blood cell congestion in worst affected lamella
	Level of fusion of lamellae in worst affected area[3]
	Level of severity of hypertrophy[4] or hyperplasia[5] if lamellae not fused
	Presence/absence of lamellar disarray
	Presence/absence of leukocyte infiltration of lamellar epithelium[13]

3.3 Results for barramundi

3.3.1 *Ventilation rates at minimum DO saturation and at normoxia*

Ventilation rates increased with decreasing DO saturation, especially between 20% and 50% DO saturation. Three regression lines were fitted to three unique groups within the data (Figure 3.2A, $r^2 = 0.011, 0.655, 0.088$) to give the best fit and interpretation. There was no difference in ventilation rate between control fish and fish in treatments 6 & 7, with respective minimum DO saturations of 50% and 60%. Ventilation rate increased rapidly through treatments of 50% down to 20% DO saturation, but fish in 20%, 10% and 5% treatments showed no difference in ventilation rates. A similar pattern is described by a sigmoidal curve (Figure 3.2B, $r^2 = 0.747$).

At normoxia (measured prior to daily DO depletion during the experiment), baseline ventilation rates showed a trend of being higher in fish that had been exposed to the

lower DO treatments (Figure 3.3, $r^2 = 0.537$, p <0.001). This was most pronounced in the severe hypoxia treatments, which reached 5% and 10% daily.

Figure 3.1 Composite diagram of the common irritant-induced gill lesions reproduced from Mallat (1985). Six respiratory lamellae are shown (a-f), the top one of which is normal (*Salmo gairdneri*, modified from Skidmore & Tovell, 1972). Numbered features as in Table 3.1.
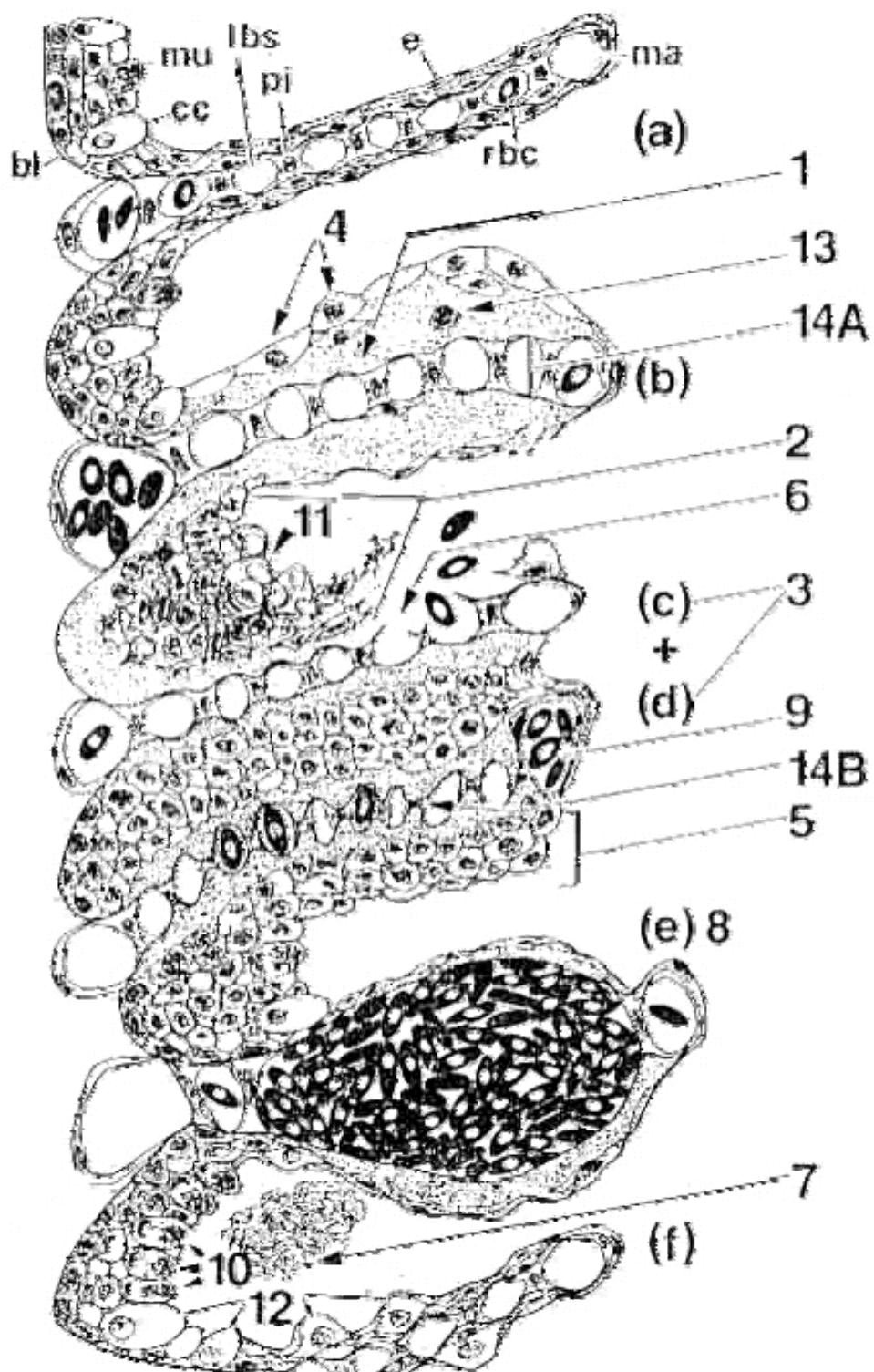
No regular patterns of change were identified in baseline ventilation rates over the three weeks of the experiment, when rates were considered weekly (Figure 3.4). There was some suggestion that baseline ventilation rates of the four fish considered in the lowest treatment (reaching 5% DO saturation at minimum) dropped through the experiment. This may have been a result of acclimation to the hypoxic treatments, but similar patterns were not obvious for other treatments.

Figure 3.2 Ventilation rates (beats per minute) of juvenile barramundi at minimum dissolved oxygen level in each tank, averaged for each fish over 21 days. A) visible patterns in the data indicate a period of no change, followed by a period of rapid change, followed in turn by a period of no change; B) curvilinear (sigmoidal) representation of the data, $r^2 = 0.747$, $p < 0.001$, $f = a/(1+\exp(-(x-x0)/b))$ where $a = 1562.163$, $b = -159.150$.
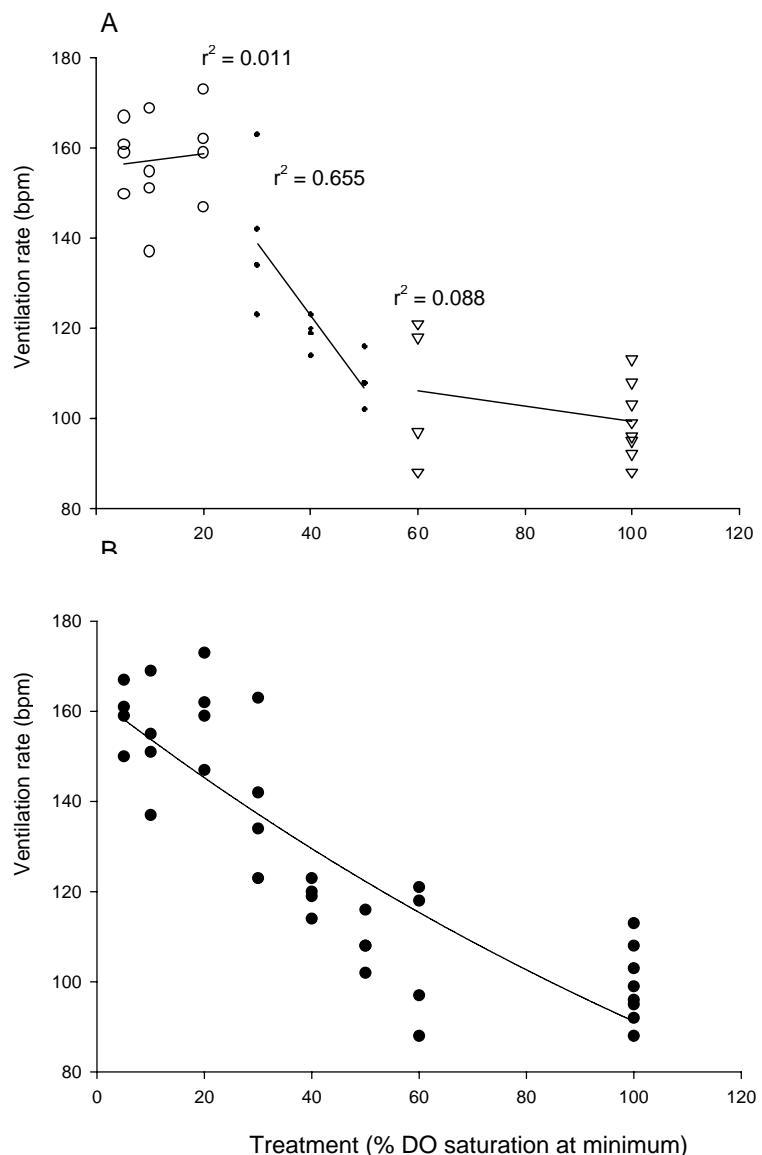
Figure 3.3 Ventilation rates (beats per minute) of juvenile barramundi under normoxic conditions, averaged for each fish over 21 days ($r^2 = 0.537$, $p < 0.001$).
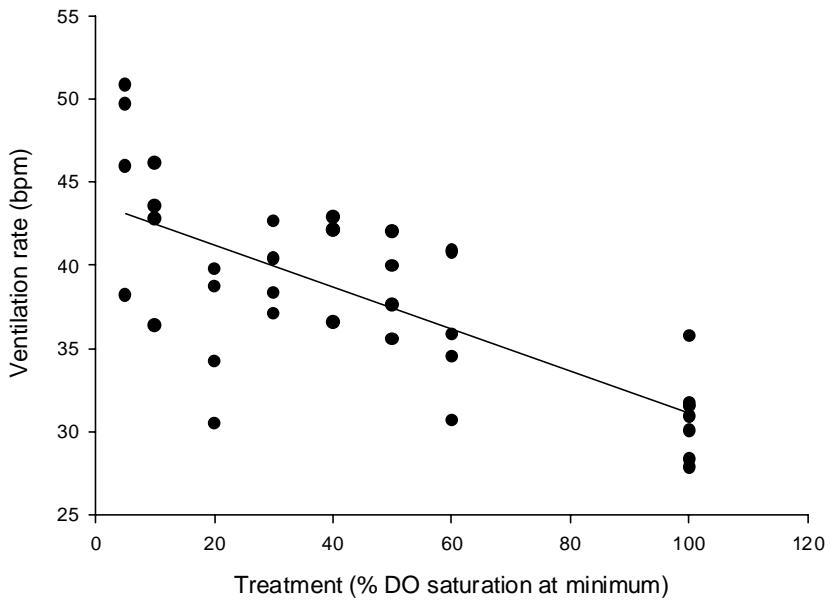
3.3.2   Ventilation behaviour

Stages of ventilatory behaviour are described in section 3.2.2. The proportion of days that fish in each treatment displayed each stage of behaviour at the minimum DO saturation for that treatment is shown in Figure 3.5. Attempts at ASR (stage 4) were only recorded for fish in the treatment reaching 5% DO saturation at minimum. Fish in treatments reaching 10% and 20% displayed predominantly stage 3 (rapid gulping of water with open mouth); fish in the treatment reaching 30% varied between stages 3 and 2 (obvious opercular movements with closed mouth); fish in treatments reaching 40%, 50% and 60% displayed mostly stage 2; while fish in the control treatment displayed stage 1 (opercular movements barely visible) and sometimes stage 2 or stage 0 (no opercular movement visible).

3.3.3   Histopathological analysis of gills

Histology of barramundi gills was considered using factors of gill health as described by Mallat (1985) and additional parameters suggested by an expert in fish pathology (L. Owens, pers. comm. 2003[6]) to identify differences between treatments

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[6] Assoc/Prof Leigh Owens, James Cook University, Townsville QLD 4811.

(Figure 3.1). Exploratory data analysis of the gill histopathology for barramundi, using non-metric multidimensional scaling (NMDS) in the PC-ORD package (McCune and Mefford, 1999), found no relationship between measures of gill histopathology and dissolved oxygen treatment (using Bray-Curtis dissimilarity, 3-axis solution, stress = 4.86, r <0.1 for all axes). Similarly, simple correlation analysis and scatterplots showed no linear or non-linear relationships between individual histopathology measures and dissolved oxygen treatment.

The gills of some fish in all treatments were damaged, but as this damage was not related to hypoxic treatment, or found in all fish or even all fish in one tank, the damage must have occurred prior to the experiment, either preceding their purchase, during transit or during holding in the aquarium room. The source of the damage is unknown. For reference, an undamaged gill is illustrated in Figure 3.6.

Figure 3.4 Average baseline ventilation rates (beats per minute) of juvenile barramundi each week, over the three-week duration of the experiment. Graphs are grouped by treatment (% DO saturation at minimum). Missing values occur where readings could not be obtained.
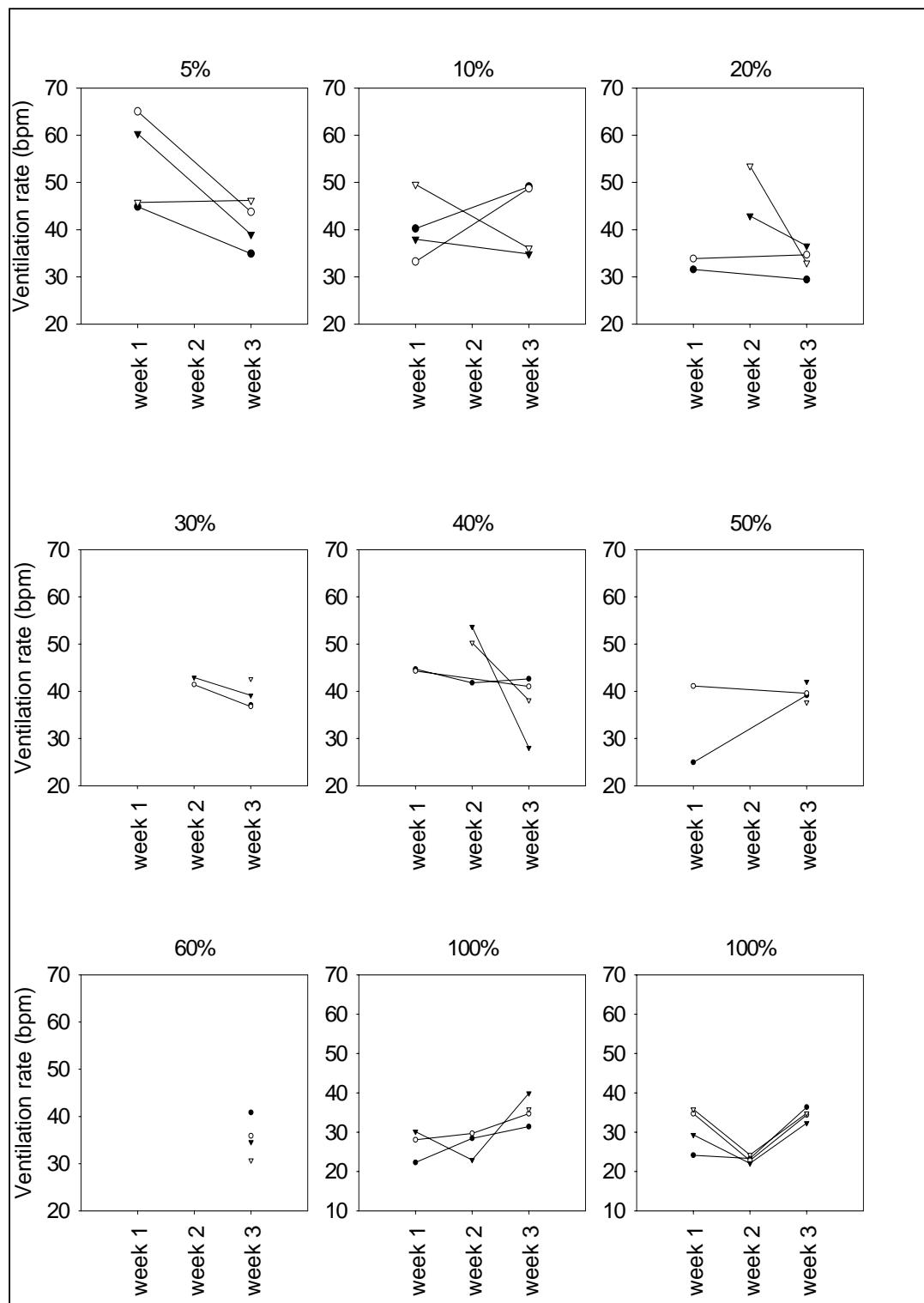
Figure 3.5 Ventilation behavioural stages recorded for juvenile barramundi in each treatment shown as a proportion of days at each behavioural stage.
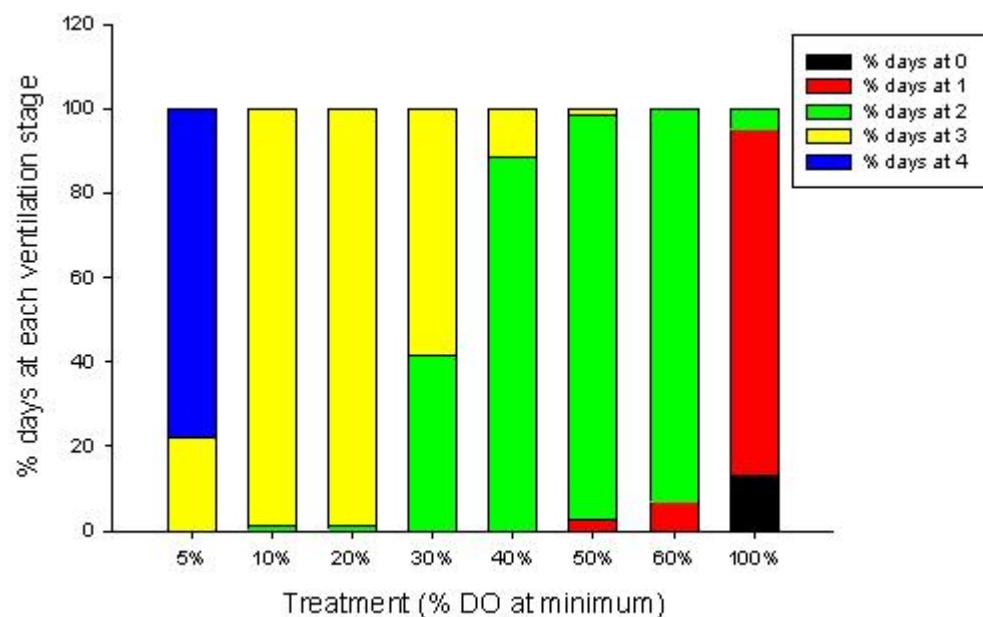
Figure 3.6 Photomicrograph of juvenile barramundi gill from control treatment. All treatments showed evidence of gill damage in some fish, and not others. The gill shown here is in good condition.

3.4   Results for eastern rainbowfish

3.4.1   Ventilation rates at minimum DO saturation and at normoxia

As for barramundi, ventilation rates of eastern rainbowfish at minimum DO saturation for each treatment are best described by splitting the data into discreet sets prior to fitting linear regressions (Figure 3.7A, r^2 = 0.900, 0.582). There was no observed difference between ventilation rates of fish in treatment 6 (50% DO saturation at minimum), treatment 7 (60%) and controls. Ventilation rates increased in a strong linear regression (r^2 = 0.900) between treatment 6 (50%) and the lowest DO treatment, treatment 1 (5%). A similar pattern is described by a sigmoidal curve (Figure 3.7B, r^2 = 0.874, $p < 0.001$).

No strong trend was found for increasing or decreasing baseline ventilation rates of fish exposed to increasingly severe hypoxia treatments (Figure 3.8, r^2 = 0.118, p = 0.050). Although the relationship between ventilation rate and treatment was statistically significant, the low r^2 value indicates there was no strong relationship. Baseline ventilation rates of most individual rainbowfish decreased week by week throughout the four-week duration of the experiment (Figure 3.9). This trend was more marked in the hypoxic treatments than in the controls, which indicates acclimation to hypoxic exposure may have been occurring in these fish.

3.4.2   Ventilation behaviour

Eastern rainbowfish predominantly attempted to perform ASR (behaviour stage 4) in both the treatment reaching 5% DO saturation at minimum and the treatment reaching 10% (Figure 3.10). Fish in treatments reaching 20% and 30% displayed stage 3 most frequently; fish in treatments reaching 40% and 50% most regularly used stage 2; fish in the treatment reaching 60% varied between stages 1 and 2; and fish in the control tanks usually displayed stage 1, but occasionally stage 2 or stage 0.

Figure 3.7 Ventilation rates (beats per minute) of juvenile eastern rainbowfish at minimum dissolved oxygen level in each tank, averaged for each fish over 28 days. A) visible patterns in the data indicate a period of rapid change from severe to moderate hypoxia, followed by a period of little change from moderate hypoxia to control. B) curvilinear (sigmoidal) representation of the data, $r^2 = 0.874$, $p < 0.001$, $f = a/(1+\exp(-(x-x0)/b))$ where $a = 13179.318$, $b = -71.562$.
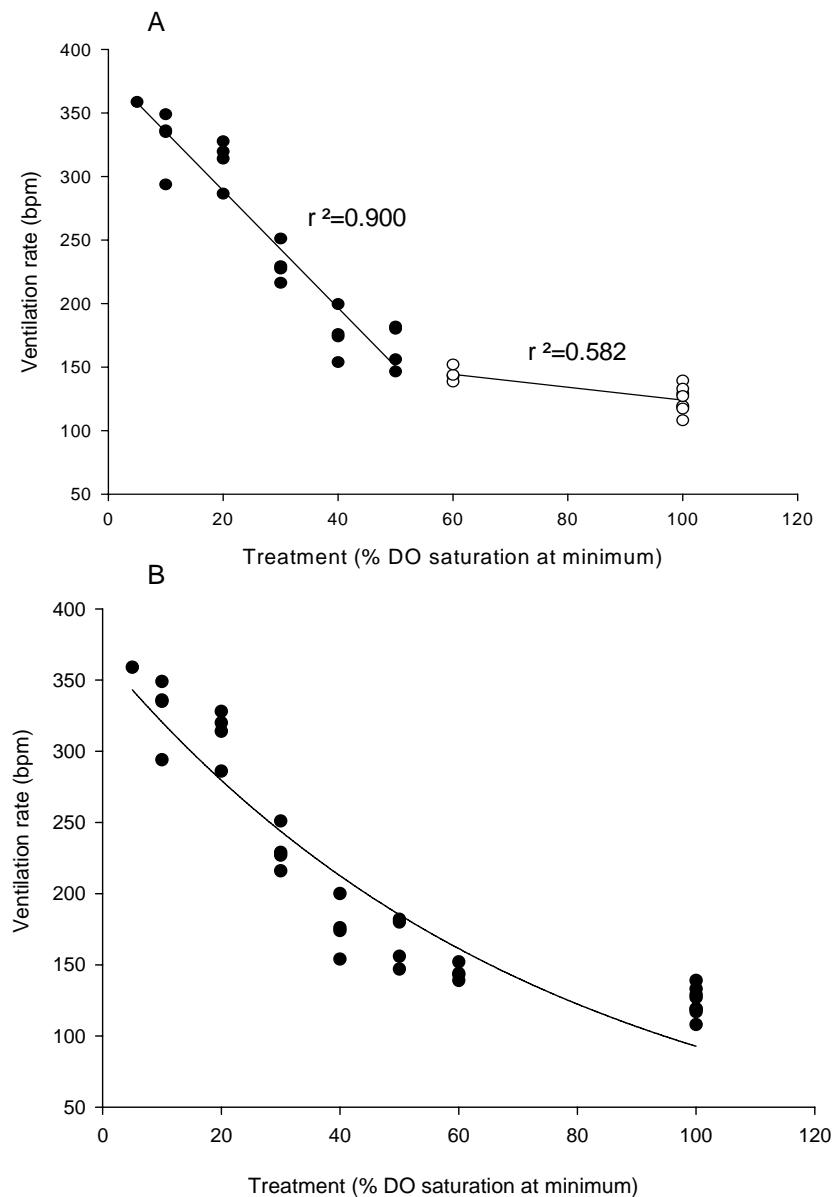
Figure 3.8 Ventilation rates (beats per minute) of juvenile eastern rainbowfish under normoxic conditions, averaged for each fish over 28 days, $r^2 = 0.118$, p = 0.050.
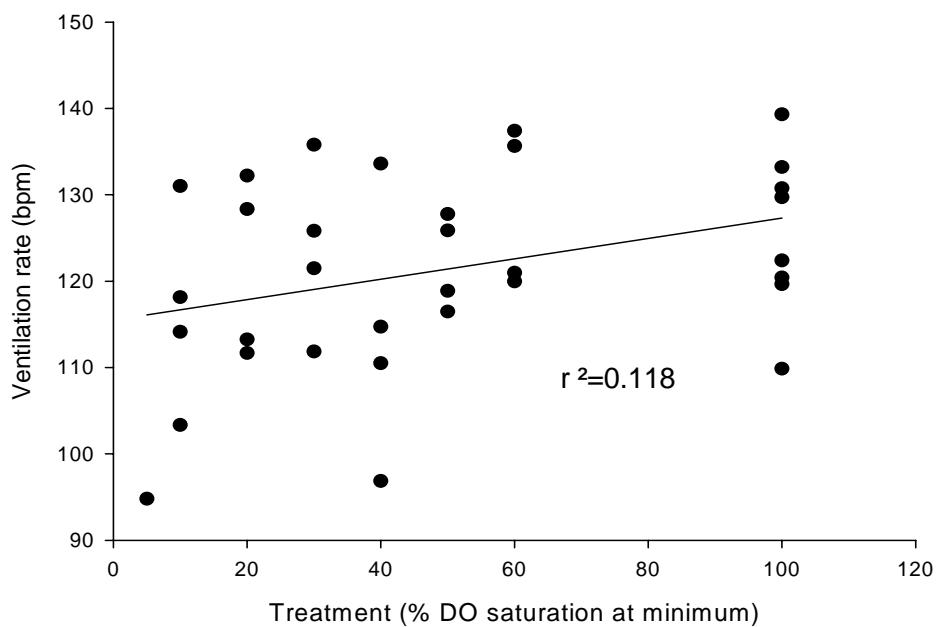
3.4.3 *Histopathological analysis of gills*

Following the results from the other fish species, and a preliminary examination of eastern rainbowfish gills, the gills of eastern rainbowfish were not formally analysed. They were very small and difficult to section using standard techniques, resulting in artifactual tearing of the gills. Several attempts were made to section the gills, including using Arnim's method of decalcification (Arnim, 1935), without success.

Figure 3.9 Average baseline ventilation rates (beats per minute) of juvenile eastern rainbowfish each week, over the four-week duration of the experiment. Graphs are grouped by treatment (% DO saturation at minimum). Missing values occur where readings could not be obtained.
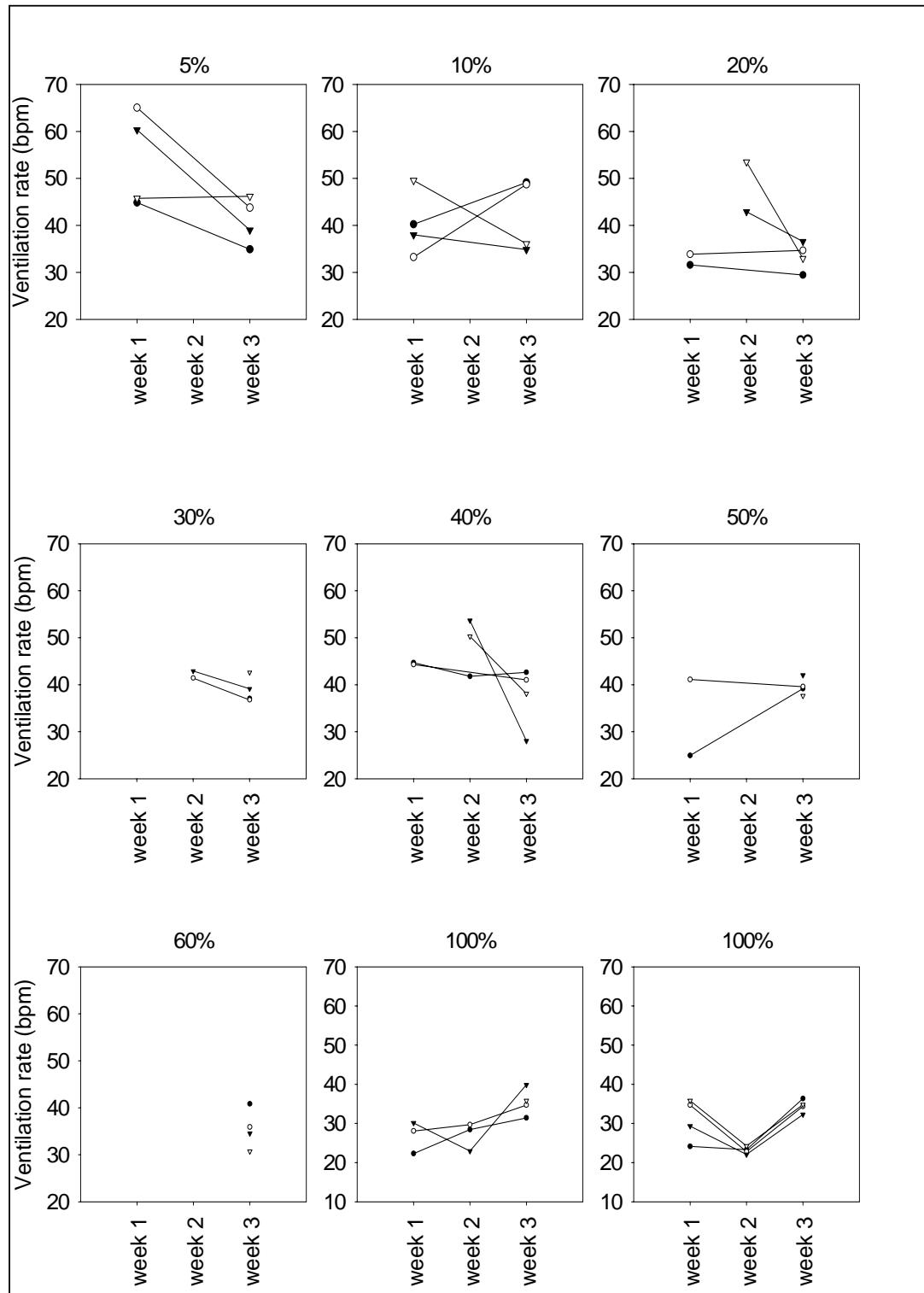
Figure 3.10 Ventilation behavioural stages recorded for rainbowfish in each treatment shown as proportion of days at each behavioural stage.
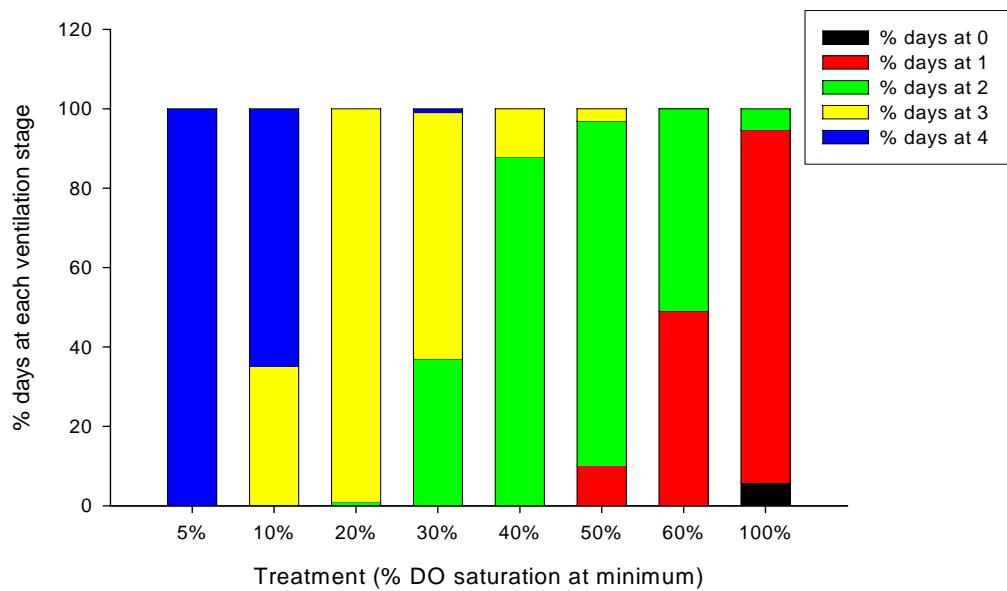
3.5 Results for sooty grunter

3.5.1 *Ventilation rates at minimum DO saturation and at normoxia*

Sooty grunter from the two experiments described in the methods section are treated as two separate sets of fish. Ventilation rates increased linearly with increasing hypoxic stress in both sets of fish (Figure 3.11, set 1 $r^2 = 0.905$, set 2 $r^2 = 0.818$). There was no observed difference in observed baseline ventilation rates between treatments for either experimental set of fish (Figure 3.12, Set 1 $r^2 = 0.076$ p = 0.322, set 2 $r^2 = 0.081$ p = 0.304). Fish in set 2 showed lower average baseline ventilation rates than fish in set 1 (Table 3.2, p = 0.002, Figure 3.12).

Figure 3.11 Ventilation rates (beats per minute) of two sets of juvenile sooty grunter at minimum dissolved oxygen level in each tank, averaged for each fish over 28 days. Set 1 – filled circles, set 2 – empty circles.
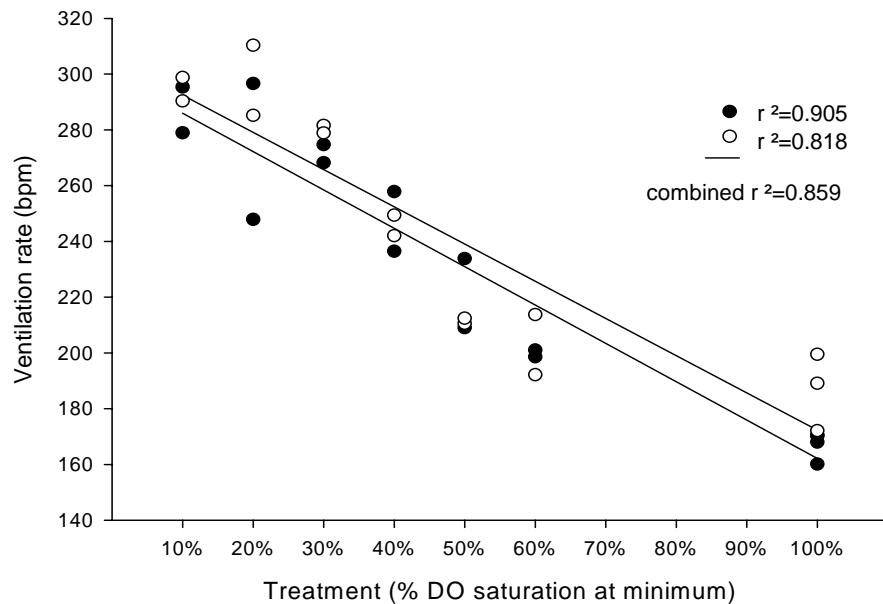
Table 3.2 Independent samples t-test to test for differences between baseline ventilation rates (at normoxia) of sooty grunter from set 1 and set 2. There is a significant difference between sets, at p = 0.002.

		Levene's Test for Equality of Variances		t-test for Equality of Means						95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference		Lower	Upper
VAR00016	Equal variances assumed	.605	.443	3.465	29	.002	1.4455	.4172		.5922	2.2988
	Equal variances not assumed			3.448	27.755	.002	1.4455	.4192		.5864	2.3046

Figure 3.12 Ventilation rates (beats per minute) of two sets of juvenile sooty grunter under normoxic conditions, averaged for each fish over 28 days. Set 1 – filled circles ($r^2 = 0.076$, $p = 0.322$), set 2 – empty circles ($r^2 = 0.081$, $p = 0.304$).
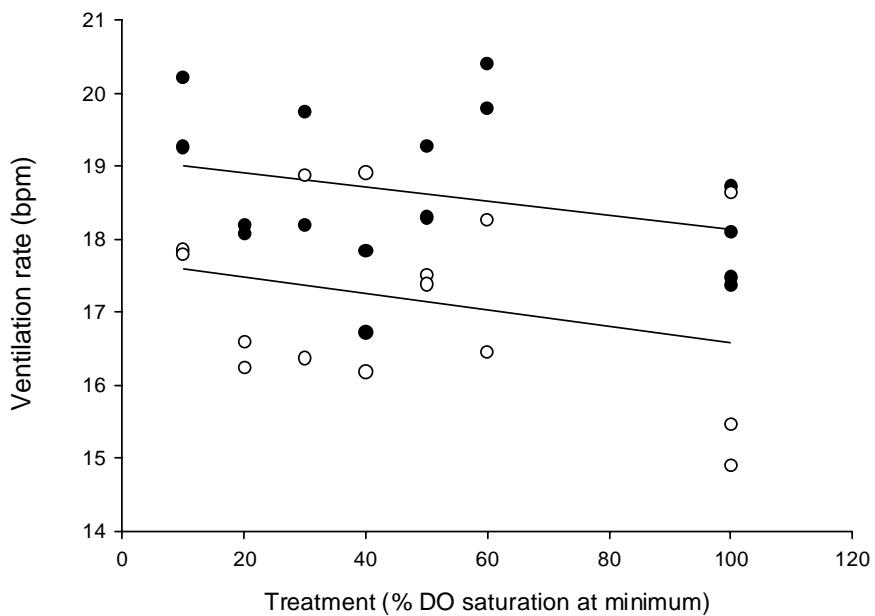
3.5.2   *Ventilation behaviour*

Sooty grunter did not attempt to perform ASR (ventilation behaviour stage 4) at any time. Occasionally fish were found to be close to the water's surface but this did not appear to be related to DO saturation. It is more likely the behaviour was a response to stress as control fish occasionally swam near the surface. One fish in set 2 spent some time apparently attempting ASR (Figure 3.13 B); this was one of the fish in the treatment reaching 20% DO saturation at minimum. It only moved to the surface under DO saturations <25%, but it was not clear whether this was a stress response rather than a deliberate attempt to access oxygen at the water's surface. Fish in set 1 (Figure 3.13 A) and set 2 (Figure 3.13 B) showed similar ventilation behaviours at each treatment. The exception to this was fish in the control tanks, which displayed solely stage 1 in set 1, but one control fish in set 2 also regularly displayed stage 2. Fish 8C (a control fish) in set 2 died on day 21 of the experiment of unknown causes, so it has not been considered in any analyses.

Figure 3.13 Proportion of days that individual sooty grunter reached each ventilation behaviour stage. A – set 1, B – set 2.
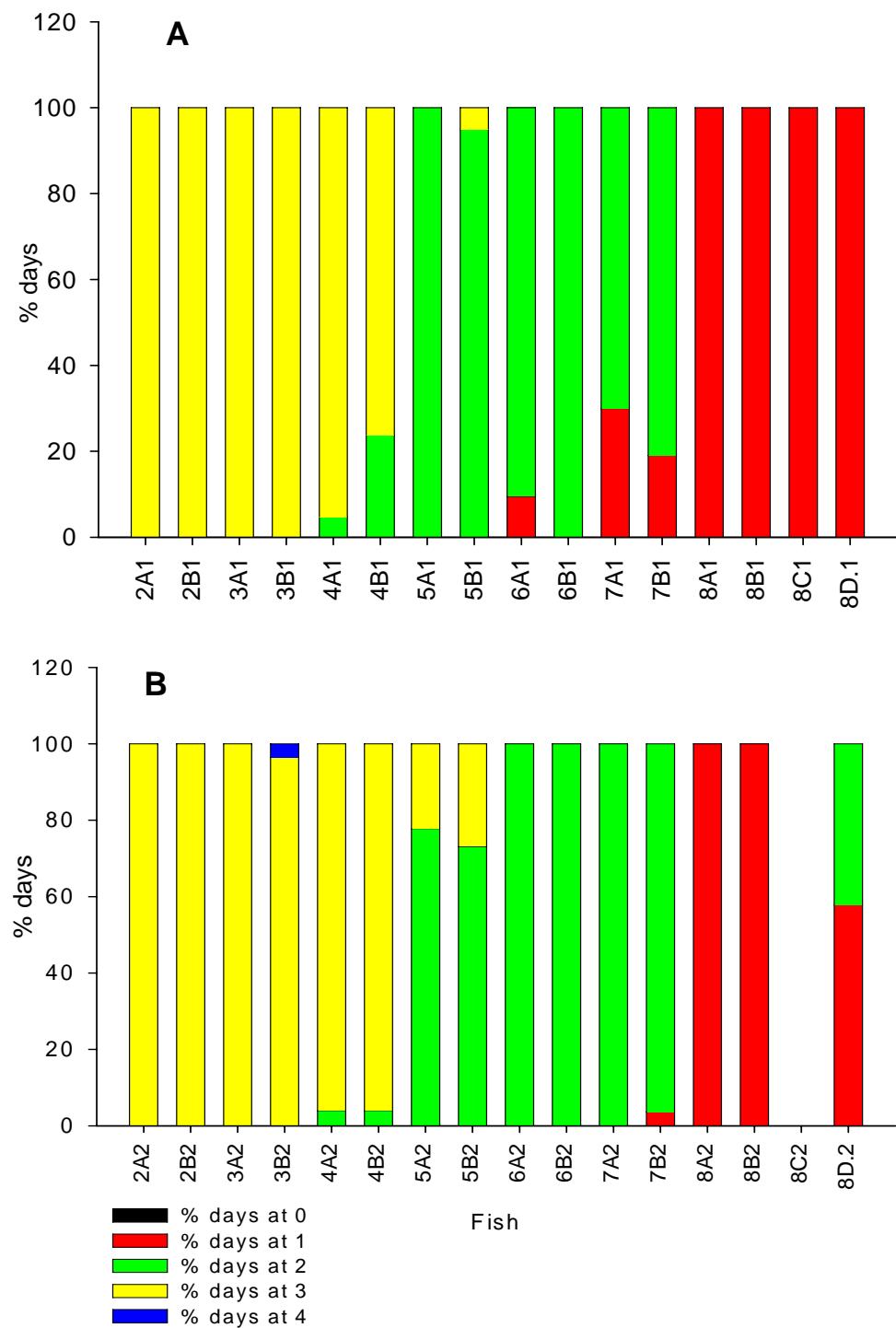
3.5.3 *Histopathological analysis of gills*

Exploratory analysis of the gill histopathology data for sooty grunter, using non-metric multidimensional scaling (NMDS) in the PC-ORD package (McCune and Mefford, 1999), found no relationship between measures of gill histopathology and dissolved oxygen treatment (using Bray-Curtis dissimilarity, 3-axis solution, stress = 8.21, $r < 0.11$ for all axes). Similarly, simple correlation analysis and scatterplots showed no linear or non-linear relationships between individual histopathology measures and dissolved oxygen treatment.

As for barramundi gills, damage was apparent in all treatments including controls. The source of damage is unknown.

The gills of the control fish from set 2 that died of unknown causes prior to completion of the experiment were examined, and were found to be severely damaged, with completely fused gill lamellae, and gill filaments thickly congested with blood. It is possible that the fish had a gill disease which caused its death, or that the tank in question had a problem not experienced in other tanks. Nitrogenous wastes in the dubious control tank were found to be normal during and after the death of the fish, but water appeared slightly cloudy on the morning of the fish's death possibly due to a bacterial or algal bloom.

3.6   Discussion

Ventilation rates of fish exposed to hypoxia increased markedly with decreasing DO saturation. Barramundi showed a maximum increase from normoxia to the lowest surviving treatment (reaching 5% DO at minimum) of 1.6 times the baseline ventilation rate. Sooty grunter showed a similar increase between normoxia and their lowest surviving treatment (10% DO at minimum: set 1 – 1.7 times, set 2 – 1.6 times). Eastern rainbowfish had a much greater increase in ventilation rate, with the average rate at 5% DO saturation being almost three times the average rate at normoxia (although only one fish survived in this treatment); and at 10%, fish had ventilation rates of 2.6 times the rates recorded in normoxic control tanks.

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Similarly high ventilation rates of eastern rainbowfish were recorded by Pearson *et al.* (2003), with a threefold increase in ventilation rates between normoxic and hypoxic treatments (although in this case the hypoxic treatment reached only 35% before ventilation rates tripled). Barramundi were found in the same study to triple ventilation rates between normoxia and about 10% DO saturation. The results differ from those presented here, probably because fish in experiments carried out by Pearson *et al.* were not acclimated gradually to increasing hypoxia, but shifted directly from normoxic waters to the hypoxic treatment tanks. This suggests that the rate of oxygen decline has a large impact on the effects of hypoxia on fish.

The level of hypoxia at which ventilation rate begins to increase differs between fish species, as does the maximum ventilation rate achieved. This may be a result of the adaptation of a species to hypoxia. For example, two species of erythrinids, *Hoplias malabaricus* and *H. lacerdae*, tropical South American fish of the same genus, and with very similar external morphology, have different gill ventilation rates and oxygen extraction efficiencies under hypoxia. *H. malabaricus* exhibits lower gill ventilation rates and higher oxygen extraction efficiency when exposed to low DO levels than *H. lacerdae*, and this is thought to be a result of the different habitats occupied by the two species – *H. malabaricus* is found in frequently stagnant hypoxic waters, whereas *H. lacerdae* inhabits well-oxygenated streams (Rantin *et al.*, 1992).

Higher gill ventilation rates under hypoxia do not necessarily indicate lower tolerance. Flounder (*Platichthys flesus*) are flatfish that inhabit frequently hypoxic shallow water habitats, whereas plaice (*Pleuronectes platessa*) are found in deeper waters and are less tolerant to hypoxia. The flounder's ability to survive deoxygenated waters depends on its greater capacity to increase ventilation rates sufficiently to maintain oxygen uptake across the gills (Steffensen *et al.*, 1982).

Baseline ventilation rate (rate during normoxia) of barramundi showed a trend of being higher in treatments with lower DO saturations at minimum levels, and this was particularly pronounced for fish in the lowest treatments, reaching 5% and 10% DO saturation each morning. This suggests that even up to 18 hours after exposure to hypoxia the previous night, barramundi had not recovered oxygen levels in the blood

sufficiently to slow their ventilation rate to that displayed by fish under conditions of constant normoxia.

No strong evidence was found to suggest that fish might acclimate to hypoxic conditions in such a way that ventilation rates can be reduced for either barramundi or sooty grunter. Baseline ventilation rates of these two species did not show a decline over the course of the experiment even in fish exposed to the most stressful hypoxic treatments. However there was some indication of a reduction in baseline ventilation rate of eastern rainbowfish exposed to hypoxia, for all treatments except the control. This suggests that rainbowfish may have a greater capacity to acclimate physiologically to fluctuating oxygen conditions than the other two species, possibly by altering oxygen-carrying capacity of the blood, or by increasing blood flow to the gills. Fish that live in hypoxic conditions often have better oxygen-binding capabilities of haemoglobin, reducing the need to increase ventilation (Heath, 1995).

Sailfin mollies (*Poecilia latipinna*) acclimated to chronic hypoxia exhibit higher haemoglobin and red blood cell concentrations and lower critical oxygen tension than fish acclimated to normoxia (Timmerman & Chapman, 2004). Mollies acclimated to hypoxia also show decreased use of ASR over time. No similar decline in use of ASR was found in this study, although the period of exposure to hypoxia may not have been long enough, particularly as hypoxia was periodic rather than chronic. The use of ASR by fish in this study is discussed further below.

Studies in Uganda have shown that populations of the cyprinid *Barbus neumayeri* found in low-oxygen papyrus swamps have higher tolerance to hypoxia than populations from nearby river and stream sites with higher oxygen levels (Olowo & Chapman, 1996). It is hypothesized that naturally occurring hypoxic habitats such as papyrus swamps thus promote diversification among populations of fish (Olowo & Chapman, 1996).

The ventilatory behaviour of fish exposed to graded hypoxia can be explained by five behaviour stages. Under normoxia, fish were found to display only very slight opercular movements, or sometimes movements so slight they were not visible to record (except sooty grunter for which opercular movement was never slight enough

to be invisible). When exposed to mild hypoxia, opercular movements became more pronounced, but the mouth remained closed and behaviour was normal. Under moderate hypoxia, fish opened their mouths to allow greater flow of water over the gills, and also produced much stronger opercular movements, and swam more actively in circles within the water column. When DO levels dropped to become severely hypoxic, aquatic surface respiration (ASR) was attempted by both barramundi and eastern rainbowfish, but not by sooty grunter.

During ASR, fish attempt to skim the more highly oxygenated surface layer of the water, with open mouths and strong, rapid opercular movements. In these experiments, ASR was unsuccessful as the air:water interface was not available to the fish. However, the fish showed the same behaviour as that exhibited during successful ASR. They circled around with mouths held to the water's surface and were not easily disturbed by external noises or movements during this time. In these experiments ASR did not allow fish to access higher oxygen levels, as the use of nitrogen gas to remove oxygen from the water in an enclosed aquarium produces a nitrogen atmosphere above the water's surface (Chapter 2). Despite this fact the behaviour persisted. This suggests that ASR is an automatic behavioural response to hypoxia for rainbowfish and barramundi that does not cease if there is no increase in oxygen attainment.

Many non-air-breathing fish species perform ASR under reduced oxygen conditions (Kramer *et al*., 1978; Kramer & McClure, 1982; Saint-Paul & Soares, 1988; Gee & Gee, 1991; Chapman *et al*., 1995). ASR is most common in species that inhabit potentially hypoxic habitats (Kramer, 1983; Verheyen *et al*., 1994), and 80% or more of the fish in such habitats may utilize the behaviour (Gee *et al*., 1978; Congleton, 1980; Kramer & McClure, 1982; Jobling, 1994). Using ASR makes it possible for fish to endure hypoxia for longer periods of time (Kramer & Mehegan, 1981; Kramer & McClure, 1982; Weber & Kramer, 1983; Stierhoff *et al*., 2003), and is considered to be a very important behavioural adaptation in fish species that are unable to breathe air (Chippari-Gomes *et al*., 2003).

Amazonian charcarid fishes exemplify the value of being able to perform ASR effectively, as they have developed dermal lip protuberances to enhance ASR by

moving more of the oxygenated surface water over the gills (Winemiller, 1989). Specialised morphological characters often exist in species adapted to perform ASR, including a relatively flat dorsal surface and pointed heads with upturned mouths (Jobling, 1994). Fish with this shape, and juvenile eastern rainbowfish are a good example, are able to perform ASR without drastic changes to the position of their body within the water column, and to swim along almost normally whilst skimming highly oxygenated water off the surface.

In contrast, fish like sooty grunter, whose dorsal surface is highly curved and whose jaw is low on the head, would have to tip themselves into an almost vertical position in the water column to perform ASR. It is perhaps not surprising then that in this study sooty grunter did not perform ASR, given their body shape and tendency to inhabit oxygenated, lotic waters (Pusey *et al*., 2004). It is interesting to note that some fish species with under-slung jaws perform ASR by inverting their posture (swimming belly-up) to put their mouth into proximity of the oxygen-rich surface layer. Fish species known to exhibit this postural inversion as an aid to ASR include the Mochokid catfish *Synodontis nigriventris* (Chapman *et al*., 1994) and the Mormyrid *Petrocephalus catostoma* (Chapman & Chapman, 1998).

Barramundi have an intermediate body shape to the other two fish species tested here, but at least as juveniles are adept at performing ASR.

Whilst ASR benefits fish by allowing access to higher oxygen levels at the air:water interface, breathing at the surface has disadvantages, including increased susceptibility to predators, both aerial (Kramer *et al*., 1983) and aquatic (Poulin *et al*., 1987; Wolf & Kramer 1987), as well as sunburn. Thus, fish may choose to delay ASR until metabolic requirements force them to move to the surface, if the perceived risk causes them to be hesitant in leaving cover (Yoshiyama *et al*., 1995; Watters & Cech, 2003). This observation is contrary to a theory that fish may use ASR even when it is not necessary for survival, in an attempt to increase the energy available for growth (Weber & Kramer, 1983).

Hypoxia was not found to have deleterious impacts upon gill histology of barramundi and sooty grunter in this study. A study of the histopathological effects of sublethal

hypoxia on channel catfish (*Ictalurus punctatus*) found lesions on the gills as well as other organs. Changes to the gills included haemorrhage, oedema (filling of tissues with liquid), epitheliar hypertrophy (increased cell size), hyperplasia (increased cell numbers), and mucus discharge on the gill lamellae (Scott & Rogers, 1980). Although some of these conditions were identified in fish gills in this study, their presence or absence did not appear to be related to hypoxic treatments.

Hypoxia may cause normally unused gill lamellae to become perfused with blood and project out of the filament to access more oxygen (Jobling, 1994; Heath, 1995). This increases gill surface area and aids diffusion across the gills by decreasing distance from the water to the blood (Heath, 1995). In this study, although gills were observed to project out of the operculum under hypoxia, dissected gills of fish exposed to hypoxia were found to be no more gorged with blood than gills of control fish. The possible explanation for this is that fish were not collected from hypoxic waters for dissection, and had 24 hours to recover from the final bout of hypoxia before euthanasia. These results may be in part due to the "plastic" nature of gills, which may be quickly repaired following stress. If that were the case, gills may have shown the effects of hypoxia if they were collected and euthanased during a period of hypoxia. Crucian carp (*Carassius carassius*) gills undergo gross morphological changes during hypoxia, including reduction in cell mass surrounding the lamellae and lamellar protrusion with increasing exposure to de-oxygenated waters; however, this alteration is reversed after a week in normal oxygen saturations (Sollid *et al.*, 2003).

In channel catfish, identical lesions were observed in fish that were sampled immediately following recovery from 24, 48 and 72 hours of exposure to hypoxic conditions, and again after five days recovery (Scott & Rogers, 1980). This suggests that it is not necessary to collect fish during hypoxia to see the damage it has caused. However, the extent of damage was not as severe in fish that had been allowed to recover (Scott & Rogers, 1980), and the fish in that study, as in the study on crucian carp, were exposed to chronically low DO levels rather than levels that dropped gradually and returned to normal each day as in the experiments presented here. The damage caused to catfish gills during hypoxic exposure was thought to have reduced the oxygen uptake ability of the gills (Scott & Rogers, 1980), and channel catfish have

been shown to display a reduction in gill efficiency when subjected to short-term hypoxia (Gerald & Cech, 1970). No such evidence has been presented for barramundi, eastern rainbowfish or sooty grunter. However if exposure to hypoxia had been longer per day or if experiments had been carried out over longer time periods effects may have been recognised.

Aside from pathological results, the effects of hypoxia on respiratory systems of fish have been extensively reported in the literature. Adaptations to allow survival under hypoxic conditions often culminate in changes to the respiratory and circulatory systems, including ventilation rates, ventilatory mechanisms and behaviours, and changes to circulation and blood chemistry that allow higher diffusion of oxygen into the bloodstream. For this reason it is important to record results from further fish species to allow for comparisons between species and assemblages from various habitats.

Comparison of the ventilatory capacity of the three species presented here supports the theory that fish commonly exposed to hypoxia in their natural habitat are more able to tolerate low DO saturations. Juvenile barramundi had the lowest immediate lethal limit of the three, and were able to perform ASR, which would reduce this limit still further in field situations where surface waters with higher oxygen levels are accessible. Eastern rainbowfish were also able to perform ASR, and showed large increases in gill ventilation rates, which may add to their ability to withstand hypoxia. Additionally, rainbowfish were found to have lower baseline ventilation rates after several weeks of exposure to fluctuating hypoxia, suggesting some form of acclimation such as increased oxygen-carrying capacity of the blood. Both barramundi and eastern rainbowfish, unlike the sooty grunter, are found in habitats that may naturally experience hypoxia. Sooty grunter are seldom found in hypoxic waters, and prefer to inhabit running streams (Pusey *et al*., 2004). They had a higher immediate lethal limit than barramundi, but equal to eastern rainbowfish. However, unlike for the other two species, their inability to perform ASR means that this limit is probably absolute, whether or not more highly oxygenated surface waters were available.

CHAPTER FOUR: THE EFFECTS OF FLUCTUATING HYPOXIA ON FEEDING, GROWTH AND CONDITION OF JUVENILE FISHES

4.1 Introduction

Because of its impact on activity, metabolism, feeding behaviour, food consumption and food conversion, hypoxia can have significant effects on fish growth. Oxygen is a limiting factor on growth (Brett, 1979; Boeuf *et al.,* 1999), causing a reduction in appetite and an increase in energetic costs. Feeding itself has a metabolic cost, and in the laboratory the maximum metabolic requirement may be two to three times the standard rate, which equates to about one-third of active metabolism (Brett, 1979). Several investigations (Stewart *et al.*, 1967; Whitworth, 1968; Brett, 1979) suggest there is added growth depression when oxygen levels are fluctuating rather than constant around a mean low DO concentration (Thetmeyer *et al*., 1999).

There is a very substantial literature on growth and related factors, including hypoxia. Table 4.1 summarises the findings of a selection of papers relating to hypoxia and shows that its effects on growth vary markedly between species. While benthic flatfish such as sole, plaice and turbot are relatively hypoxia resistant, more mobile pelagic fish are less so (Boeuf *et al.*, 1999). This is not always the case – for example, the cichlid *Oreochromis niloticus* (Teichert-Coddington & Green, 1993) is active but anoxia resistant. It is apparent that the major cause of decreased growth in fish subjected to hypoxic conditions is a decrease in food intake, rather than a decrease in food conversion efficiency (i.e. increased food conversion ratio – FCR), although increased FCRs under hypoxia are sometimes recorded (e.g. Papoutsglou & Tziha, 1996; Chabot & Dutil, 1999; Pichavant *et al*., 2000).

The study of condition of fish is often based on analysis of length-weight relationships and assumes that fish in better condition are of heavier weight for a given length (Bolger & Connolly, 1989). Growth is an ultimate expression of fitness as it represents a combination of increases in size, condition and energy concentration within tissues, but individual condition indices are also believed to be a good indicator of the general fitness of the population under consideration (Bolger & Connolly, 1989). One of the most effective uses of condition indices is to compare monospecific

populations living under similar, or different, environment or feeding conditions (Bolger & Connolly, 1989). To my knowledge, no published studies have considered the effects of hypoxia on condition indices of inviduals or populations of fish.

In this chapter, I present the results of three experiments investigating the effects of fluctuating hypoxia on juvenile fishes. The experiments were carried out on eastern rainbowfish, barramundi and sooty grunter, and examined effects on feeding behaviour, food consumption, growth and condition.

4.2   Methods

4.2.1   Experiments

Experimental design and holding conditions were as described in Chapter 3. As reported in Chapter 3, the barramundi experiment ran for 21 days with no deaths; the rainbowfish experiment ran for 28 days, and seven out of eight fish in the 5% DO treatment (treatment 1) died, as well as one of the eight fish in the 10% DO treatment (treatment 2); and the sooty grunter experiment ran for 28 days, in which all four fish died in the 5% DO treatment (treatment 1), and there was one unexplained death (suspected bacterial gill disease) in the control treatment.

4.2.2   Measuring feeding

When fish were fed each day during the experiment an individual index of "appetite" was recorded. Fish were observed to be feeding (a) immediately upon introduction of food to the tank, (b) tentatively following introduction of food, or (c) not at all – meaning fish either did not eat that day or ate following my departure from the room. The index of interest in food was recorded for each individual fish in each of the three experiments every day.

Table 4.1 Effects of hypoxic conditions on growth, food intake and food conversion in fish. Examples from freshwater and marine habitats.

Species	Species Information	Growth	Food Intake	Food Conversion	Reference
Ictalurus punctatus	Freshwater, temperate (10 - 32°C), native to North America, demersal predator, highly commercial fishery, aquaculture and aquarium trade	Growth was significantly reduced at constant mean oxygen concentrations of 3.5 mg/L or less, and at mean diel fluctuations of 3.1 – 1.0 mg/L, but was not impaired at diel fluctuations of 4.9 – 2.0 mg/L.	At concentrations of 5 mg/L and higher fish fed vigorously and ate all of their daily rations. At 3.5 mg/L only a small portion of the ration was consumed, and at 2.0 mg/L fish appeared stressed after feeding, and increased gill ventilation.		Carlson *et al.*, 1980
Perca flavescens	Freshwater, temperate, native to North America, benthopelagic predator, commercial fishery	Yellow perch had higher tolerance for lower oxygen concentrations, and growth was significantly reduced by constant mean concentrations near 2.0 mg/L, but was not affected by mean diel fluctuations of 3.8 – 1.4 mg/L.	For fish reared near 2 mg/L the daily ration of food was not consumed. Fish at 3.5 mg/L consumed more than fish at 2 mg/L, but not all, but ration consumption was complete at higher oxygen concentrations.		Carlson *et al,.* 1980
Poecilia reticulata	Freshwater, tropical, native to South America, benthopelagic omnivore, commercial aquarium trade	If surface access was possible, guppies showed high growth at all concentrations. If access was denied, there was a progressive reduction in growth below 2 – 3 mg/L and death from 1mg/L.	Food intake declined sharply with depleting oxygen resources in fish with no surface access.		Weber & Kramer, 1983

Species	Species Information	Growth	Food Intake	Food Conversion	Reference
Pseudopleuro-nectes americanus	Marine, temperate, native to North America, demersal predator, commercial fishery	Growth rates were significantly reduced after 10 – 11 weeks at low oxygen levels (29%) and fluctuating oxygen levels (30 – 90%) compared to controls (90%). Fish from chronically low oxygen treatments displayed less growth during the test (half of normoxic growth), but faster growth during recovery than fish from fluctuating oxygen conditions.	Food consumption of fish under hypoxia (29% saturation) was 63% that of controls (90% saturation), and under fluctuating conditions (30 – 90%) food consumption was 83% of the control fish consumption.		Bejda *et al*,. 1992
Oreochromis niloticus	Freshwater, tropical (8 - 42°C), native to Africa, benthopelagic omnivore, commercial fishery and aquaculture	Growth was reduced at 0% oxygen saturation, compared to 10%, but there was no significant increase in growth between 10% and 30%.			Teichert-Coddington & Green, 1993
Osmerus eperlanus	Anadromous, temperate, native to Europe, pelagic predator, commercial fishery	Low oxygen conditions (< 4.5 mg/L at 18°C) caused increased variability and decreased increment width in otoliths.			Sepulveda, 1994
Orthodon microlepidotus	Freshwater, temperate, native to North America, benthopelagic		But no difference in food intake was found between normoxic (130 torr) and hypoxic (60 torr) groups.		Cech & Massingill, 1995

Species	Species Information	Growth	Food Intake	Food Conversion	Reference
Oreochromis aureus	Freshwater, sub-tropical, native to Africa, benthopelagic planktivore, highly commercial fishery, aquaculture and aquarium trade	Increase in body weight over 200 days was 2.5 times higher under normoxia (77.5%) than under hypoxic conditions (31.3%) and twice as high under normoxia than under intermediate hypoxic conditions (44.6%).		Food Conversion Ratio was 2.89 under hypoxic treatment, 1.7 under intermediate, and 1.9 under normoxic conditions. The best FCR was under intermediate DO conditions (31.3%).	Papoutsglou & Tziha, 1996
Acipenser oxyrhynchus	Freshwater, subtropical, native to North America, demersal predator, commercial fishery	Mean growth was 2.9 times less under hypoxia than normoxia, and when tanks were sealed, consistently lower growth rates occurred than in tanks where fish had surface access.			Secor & Gunderson, 1998
Gadus morhua	Marine, cold-temperate (0 - 20°C), native to the North Atlantic, benthopelagic omnivore, highly commercial fishery and aquaculture	Growth was significantly less in fish from 45% and 56% saturation than at 65%, 75%, 84% and 93% saturation.	Food consumption decreased under hypoxia, and explained 97% of the variation in growth. Variation was 14.9 g/day/fish at 45% oxygen to 32.6 g/day/fish at 93% oxygen.	Food conversion varied significantly with dissolved oxygen, once the outlying point of 93%, which had lower than expected conversion efficiency was removed.	Chabot & Dutil, 1999
Platichthys flesus	Estuarine/marine, polar, native to Europe, demersal predator, commercial fishery		Predation efficiency of juvenile fish significantly lower at 20 and 30% DO than at 40 and 100% DO.		Tallqvist *et al.*, 1999

Species	Species Information	Growth	Food Intake	Food Conversion	Reference
Dicentrarchus labrax	Migratory – marine → freshwater, subtropical (8 - 24°C), native to Eastern Atlantic, demersal predator, commercial fishery and aquaculture	Growth was reduced most in hypoxic (40%) conditions, and intermediately in oscillating (40-86%) conditions. Growth was correlated with food intake, suggesting reduced growth was due to reduced appetite, and not decreased feed conversion efficiency.	Fish from hypoxic conditions consumed significantly less food. Oscillating groups were intermediate and not significantly different from either hypoxic or normoxic groups.	Feed conversion efficiency was not significantly affected by oxygen conditions.	Thetmeyer *et al.*, 1999
Lepomis macrochirus	Freshwater, temperate (0 - 36°C), native to North America, benthopelagic predator, commercial aquaculture and aquarium trade	RNA-DNA ratios of fish from hypoxic (<2.0 mg/L) habitats were significantly lower than those of fish from normoxic (>4.0 mg/L). Similar results could not be replicated in a laboratory situation, indicating simulation of energetic demands did not adequately replicate the field.			Aday *et al.*, 2000

Species	Species Information	Growth	Food Intake	Food Conversion	Reference
Ictalurus punctatus	Freshwater, temperate (10 - 32°C), native to North America, demersal predator, highly commercial fishery, aquaculture and aquarium trade	Weight gain was greatest under normoxia (100% DO saturation), and dropped at 70%, and again at 30% DO saturation over two six-week trials. Weight gain increments for the three treatments were 100% (hypoxic), 155% (intermediate) and 225% (normoxic).	There was a progressive reduction in food intake as dissolved oxygen declined from 100% to 30% saturation.	Feed efficiency values were gradually reduced with decrease in oxygen saturation in 100% 70% and 30% treatment groups. The reduction in food intake is explained by the effects of slowed stomach evacuation.	Buentello *et al.*, 2000
Scophthalmus maximus	Marine, cold-temperate, native to Europe, demersal predator, commercial fishery and aquaculture	Growth of juvenile turbot was significantly lower under hypoxia than normoxia with no differences between two hypoxic treatments (3.5 mg/L and 5.0 mg/L).	Food intake was significantly lower under hypoxia, with no significant difference between 3.5 and 5.0 mg/L. During the first 2 weeks, intake was halved under hypoxia, following that, food intake increased in hypoxic treatments to about 2/3 – 3/4 of normoxic.	Food conversion ratio increased with decreasing DO saturation (FCR in lowest treatment was 3 x higher than normoxia), but after 2 weeks ratios were no longer affected.	Pichavant *et al.*, 2000

Species	Species Information	Growth	Food Intake	Food Conversion	Reference
Scophthalmus maximus	Marine, cold-temperate, native to Europe, demersal predator, commercial fishery and aquaculture	Fish were divided into four groups – 3.2 mg/L oxygen, fed to satiation; 4.5 mg/L oxygen, fed to satiation, 7.4 mg/L oxygen (normoxia), fed to satiation, and 7.4 mg/L oxygen fed the same amount as was consumed by fish in 3.2 mg/L treatment. Fish under hypoxia grew significantly less than fish in normoxia fed to satiation. Mass gain was similar between 3.2 mg/L fish, and fish from normoxia fed restricted rations.	Food intake was significantly less in fish under hypoxic conditions, than fish fed unrestricted rations under normoxic conditions. *S. maximus* under hypoxia ate 1.7 – 1.8 times less, while *D. labrax* ate 1.5 – 1.7 times less under hypoxia than the same species under normoxia.	Food conversion efficiency was unaffected by oxygen concentration or feeding procedure.	Pichavant *et al.*, 2001
Dicentrarchus labrax	Migratory – marine - freshwater, subtropical native to Europe, demersal predator, commercial fishery and aquaculture				
Paralichthys lethostigma	Marine, subtropical, native to North America, demersal predator, commercial fishery	Lowest growth was under hypoxic treatment (2.8 mg/L), followed by oscillating (2.8-6.2 mg/L), both were significantly different to normoxic treatment. Intermediate treatment (4.8 mg/L) was not significantly different from normoxic.			Taylor & Miller, 2001

Species	Species Information	Growth	Food Intake	Food Conversion	Reference
Sparus auratus	Marine, temperate, native to Europe	Growth was unaffected by hypoxia (55% DO saturation) when food in both hypoxic and normoxic treatments was rationed to the amount consumed by fish under hypoxia.			Henrique *et al.,* 2002

Because sooty grunter were held one fish per tank (for reasons as described in Chapter 3) and fed on pellet food it was possible to accurately record food consumption of individual fish each day of the experiment. The number of pellets placed into each aquarium was counted, as was the number of pellets remaining after one hour, and the number of pellets consumed by each fish was then determined.

4.2.3   Measuring growth

Growth of barramundi and eastern rainbowfish was measured by recording weight, total length and standard length before and after the experiment. Fish were sedated before initial measures were taken, to prevent them damaging themselves during the procedure. Final measures were taken after euthanasia of fish by icy slurry, but before fixation. Fish were weighed on a Sartorius B310S balance, to 0.001 g. Length measurements were made using Kincrome vernier calipers, graduated to 0.02 mm, accuracy +/- 0.02 mm.

In the case of sooty grunter, only initial and final weights were measured (before and after the experiment). The reason for this was that sooty grunter were intolerant of the anaesthetic being used for sedation (Benzocaine, see Chapter 2). To avoid post-sedation deaths, fish were not sedated, and were weighed by quickly blotting them dry and then weighing them in a pre-weighed container of water on the balance. To determine length:weight relationships of sooty grunter, twenty spare fish (not to be used in the experiment) were sedated, weighed, and standard length and total length were measured. The relationship between length and weight of these twenty fish is presented in the results section of this chapter. Growth of sooty grunter in the experiment is presented only for weight data.

Weight change was calculated as a percentage of initial weight, i.e. change in weight divided by initial weight and multiplied by 100 for each individual.

4.2.4 Measuring condition

Condition following the experiment was measured using two indices. Fulton's K (Fulton, 1911) describes the relationship between length and weight, and assumes isometric growth:

$$\frac{\text{Final whole body weight (g)}}{(\text{Final standard length(cm)})^3} \times 100 = \mathbf{K}$$

Hepatosomatic index (HSI) is a ratio of liver weight to whole body weight:

$$\frac{\text{Liver weight (g)}}{\text{Whole body weight (g)}} \times 100 = \mathbf{HSI}$$

HSI compares body weight with the weight of the liver, and is useful for determining whether energy stores (often retained within the liver) have been depleted by a physiological challenge.

4.3   Results for barramundi

The relationship between initial weight, initial standard length and initial total length of barramundi is shown in Figure 4.1. There was a high correlation between the three parameters, with all three r^2 values (representing the measure of proportion of variability in one variable which is accounted for by variability in the other) exceeding 0.8, and all three relationships significant to $p < 0.001$. The strong relationships meant that just one parameter of fish size could be used as an indicator of change in size – the parameter chosen was fish weight, as it was also used in condition analyses.

Figure 4.1 Standard length, total length and weight of juvenile barramundi prior to commencement of the experiment. Weight vs total length: $r^2 = 0.878$, $p < 0.001$; weight vs standard length: $r^2 = 0.869$, $p < 0.001$; total length vs standard length: $r^2 = 0.941$, $p < 0.001$.
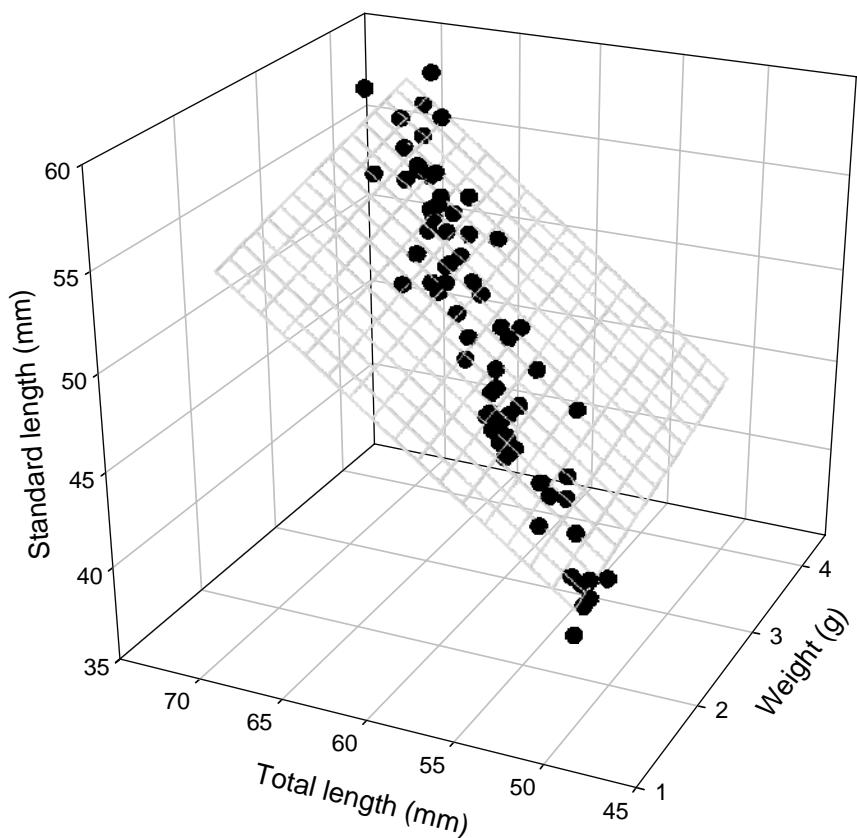
4.3.1 *Feeding*

Barramundi showed consistent feeding behaviours throughout the experiment. Fish in the control (normoxic) treatment fed actively and eagerly 100% of the time throughout the entire 21 d experiment (Figure 4.2). This percentage decreased gradually through the treatments, with the fish in the lowest treatment (treatment 1, minimum 5% DO saturation each day) feeding actively only 70% of the time. The remainder of feeding episodes for fish in treatment 1 was split equally into feeding tentatively and not feeding while I was observing.

Figure 4.2 Feeding behaviour of juvenile barramundi grouped by DO treatment. Data are presented as the percentage of days spent by all fish in a treatment at each index of feeding behaviour. Black represents days feeding actively, light grey is feeding tentatively and dark grey represents days when feeding was not observed.
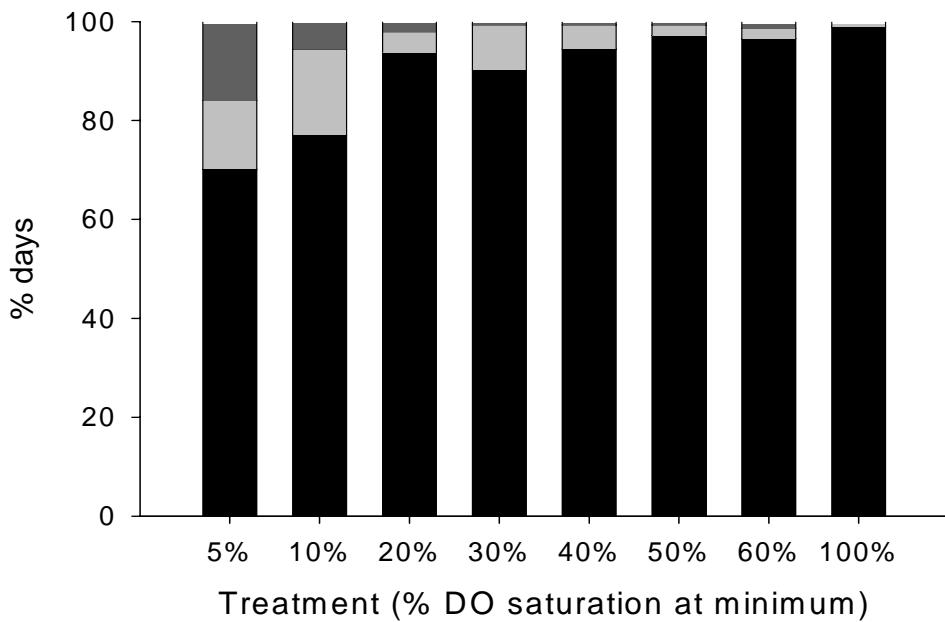
4.3.2   Growth

Juvenile barramundi appeared to have lower growth rates in treatments reaching 5% and 10% DO saturation each day (Figure 4.3). Although the curve that best described this relationship (f=a*(1-exp(-b*x)) with constants a = 430.3 and b = 5.5) was significant, the r^2 value showed that it did not fit the data well ($r^2 = 0.246$, $p < 0.001$). There appeared to be no difference in growth rates between fish in treatments 3 to 6 (with a minimum DO saturation each day of 20% or greater) and the controls.

A One-Way ANOVA was carried out to test the hypothesis that the means of all treatments were equal. The ANOVA showed that there was a difference between groups, with a significance level of $p < 0.001$ (Table 4.2). A Tukey's test carried out post hoc, showed the significance of differences between treatments. Significant differences at the $p < 0.05$ level were present between the 5% treatment and the 20%, 40% and 100% treatments; between the 10% treatment and the 20%, 40% and 100% treatments. Given that there was no difference between the 5% and 10% treatments

and the 30%, 50% and 60% treatments, the differences with the 20% and 40% treatments are unlikely to be true effects of hypoxia. However, when considered in combination with the difference noted in the regression (Figure 4.3), the significant difference between the severely hypoxic treatments (5% and 10%) and the control treatment may be a true effect of hypoxia.

Figure 4.3 Growth of juvenile barramundi in each treatment after 21 days of DO cycling. Change in weight expressed as percentage of initial weight. $r^2 = 0.246$, **p < 0.001**.
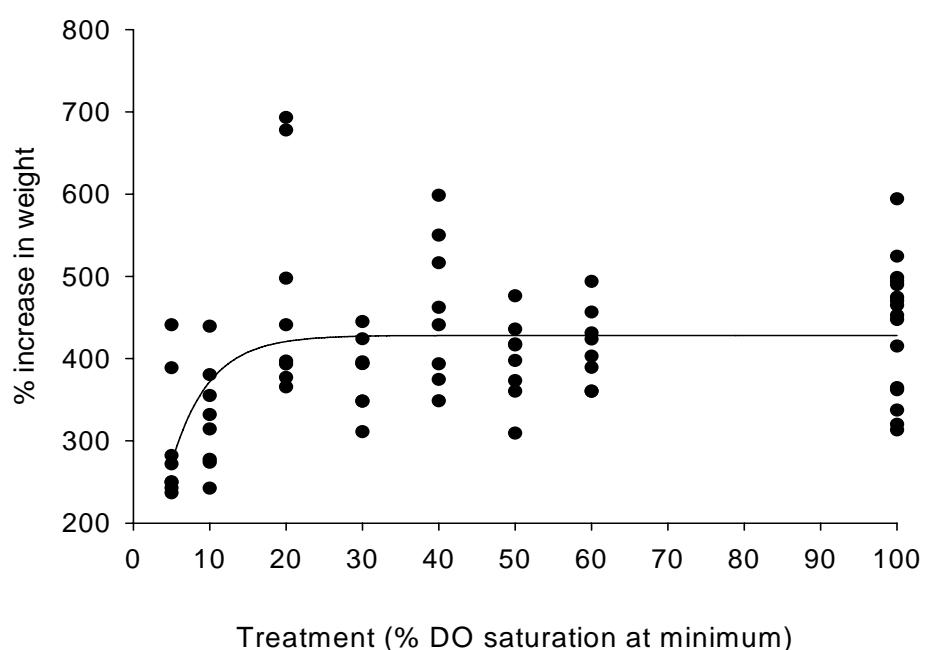
Table 4.2 One-Way ANOVA comparing growth of juvenile barramundi in all treatments.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	238642.7	7	34091.818	5.564	.000
Within Groups	392110.0	64	6126.718		
Total	630752.7	71			

4.3.3 Condition

No difference in condition was apparent between barramundi in different DO treatments using either Fulton's K (Figure 4.4) or HSI (Figure 4.5). One-Way ANOVAs confirmed this statistically (Table 4.3, Table 4.4). HSI was not significantly different between DO treatment (p = 0.857), and neither was Fulton's K (p = 0.986).

Figure 4.4 Fulton's K of each juvenile barramundi following 21 days of DO cycling.

Treatment (% DO saturation at minimum)
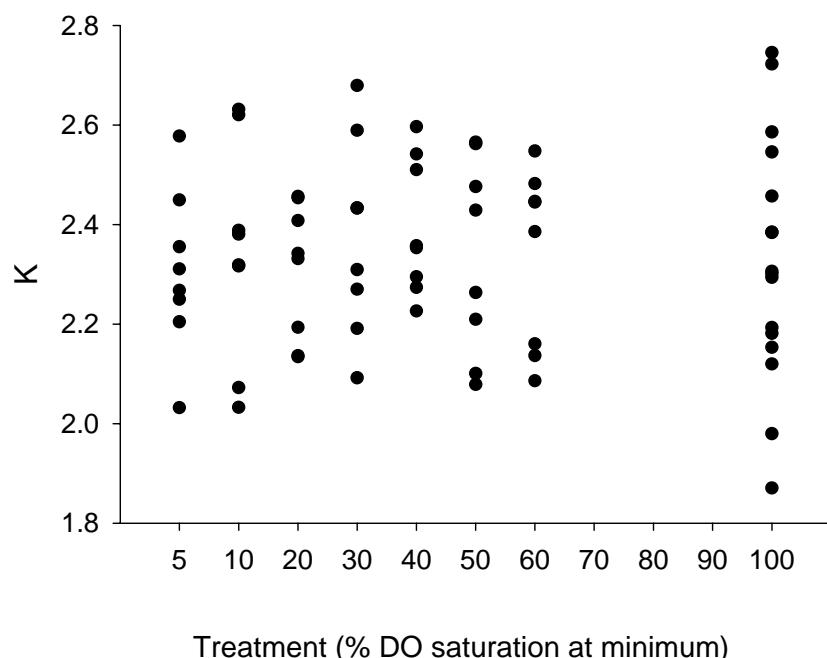
Table 4.3 One-Way ANOVA comparing Fulton's K of juvenile barramundi in all treatments.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.187E-02	7	7.410E-03	.191	.986
Within Groups	2.448	63	3.885E-02		
Total	2.500	70			

Figure 4.5 HSI of each juvenile barramundi following 21 days of DO cycling.
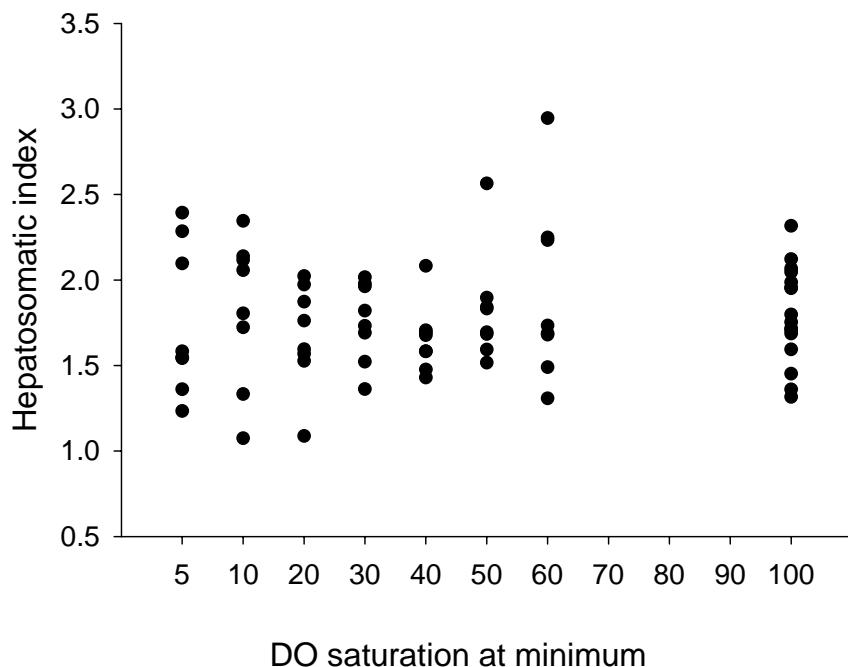
Table 4.4 One-Way ANOVA comparing HSI of barramundi in all treatments.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.404	7	5.771E-02	.463	.857
Within Groups	7.847	63	.125		
Total	8.251	70			

4.4 Results for eastern rainbowfish

A strong relationship was found between initial weight, initial total length and initial standard length of juvenile eastern rainbowfish (Figure 4.6). There was a high correlation between size parameters with all three relationships significant to $p < 0.001$. The strong relationships meant that just one could be chosen for analysis, as was the case for barramundi; again weight was chosen as a measure of fish size.

Figure 4.6 Standard length, total length and weight of juvenile eastern rainbowfish prior to commencement of the experiment. Weight vs total length: $r^2 = 0.769$, $p < 0.001$; weight vs standard length: $r^2 = 0.734$, $p < 0.001$; total length vs standard length: $r^2 = 0.958$, $p < 0.001$.
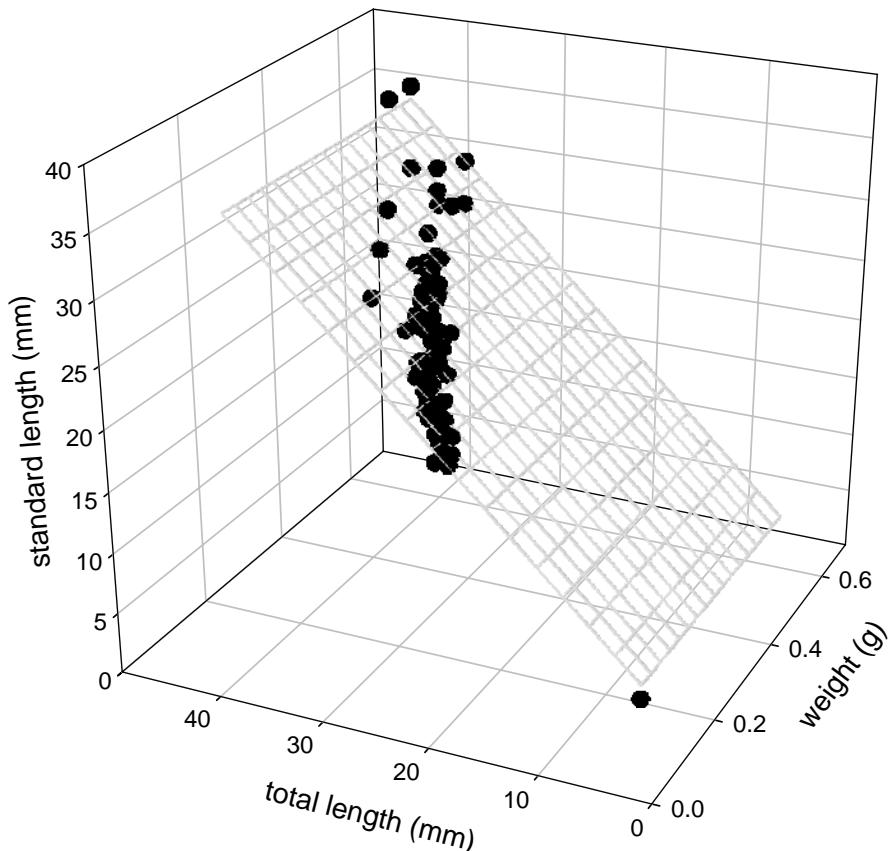
4.4.1   Feeding

Eastern rainbowfish generally did not feed eagerly during the experiments. This was strange for the species, which is normally a voracious feeder even in aquarium situations. It is possible that the confined containers used to keep fish separate caused anxiety and reduced active feeding. Most fish were not observed to feed on most days (Figure 4.7) although it is possible that they fed during the time that I was out of the room. This seems likely given that dissections following the experiment showed most fish to have food in their stomachs, and that growth occurred during the experiment (see Section 4.4.2).

Figure 4.7 Feeding behaviour of juvenile eastern rainbowfish grouped by DO treatment. Data are presented as the percentage of days spent by all fish in a treatment at each index of feeding behaviour (total of 28 x 8 = 224 days per treatment except for treatments reaching 5% & 10% where less than 8 fish survived). Black represents days feeding actively, light grey is feeding tentatively and dark grey represents days when feeding was not observed.
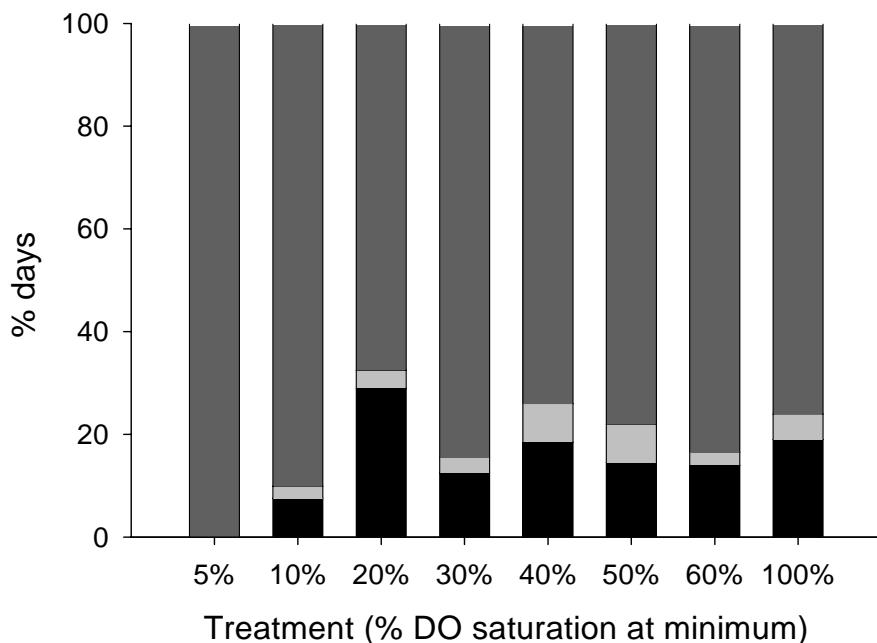
4.4.2 Growth

No relationship between DO treatments and growth was identified for juvenile rainbowfish (Figure 4.8). Although a significant difference between means was found by a One-Way ANOVA (Table 4.5, p = 0.008), post hoc multiple comparisons analysis (Tukey's test) showed the significant difference was due to the 10% treatment differing from the 30% and 40% treatments. This would suggest the result was not entirely related to hypoxia treatment. Note that the 5% treatment was excluded from the One-Way ANOVA, as only one fish survived to allow growth measurements to be taken.

Figure 4.8 Growth of juvenile eastern rainbowfish in each treatment after 28 days of DO cycling. Change in weight expressed as a percentage of initial weight.
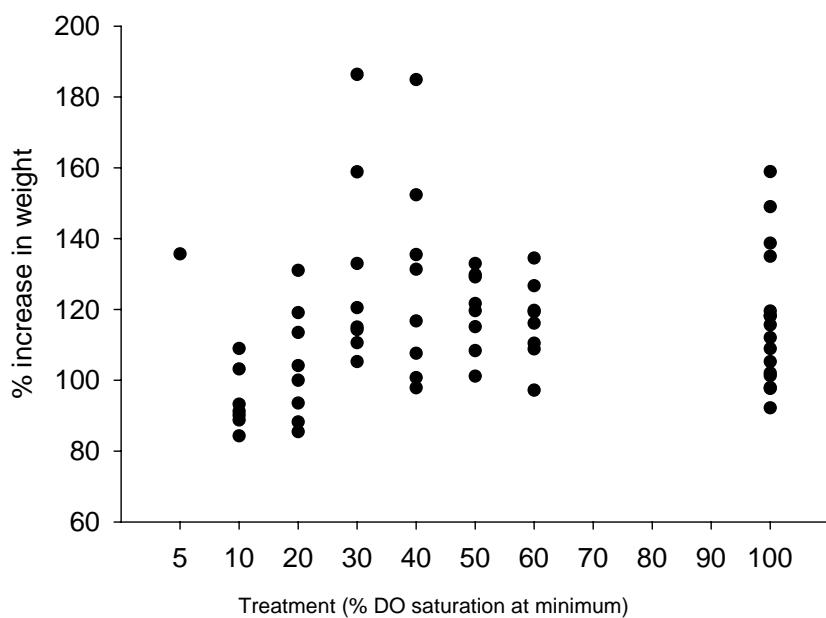
Table 4.5 One-Way ANOVA comparing growth of rainbowfish in all treatments.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7417.963	6	1236.327	3.253	.008
Within Groups	21282.769	56	380.049		
Total	28700.732	62			

4.4.3 Condition

No difference in final condition is evident for juvenile rainbowfish in different DO treatments, as measured by Fulton's K (Figure 4.9). Although a statistically significant difference was found between treatments using One-Way ANOVA (Table 4.6, p = 0.043), post hoc multiple comparisons tests (Tukey's test) could not identify the source of the significance. Once again the 5% treatment was not considered in the analysis as only one fish survived. HSI was not measured for rainbowfish as fish were retained whole for histological processing due to their small size.

Figure 4.9 Fulton's K of each surviving juvenile eastern rainbowfish following 28 days of DO cycling.
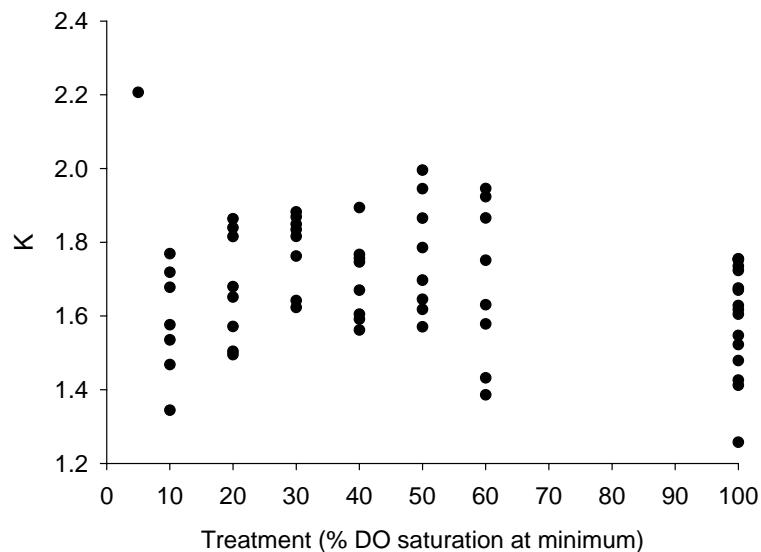
Table 4.6 One-Way ANOVA comparing Fulton's K of rainbowfish in all treatments.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.322	6	5.358E-02	2.352	.043
Within Groups	1.276	56	2.279E-02		
Total	1.598	62			

4.5   Results for sooty grunter

The relationship between length and weight of juvenile sooty grunter was established using 20 fish that were not used in the experiment. The relationship between weight, standard length and total length was strong (Figure 4.10). The significance of all three correlations was high (p < 0.001), and all r^2 values were higher than 0.7. For this reason one of the three parameters could be chosen for analyses. As for barramundi and eastern rainbowfish weight was chosen for the analysis of change in size.

Figure 4.10 Standard length, total length and weight of a sample of juvenile sooty grunter prior to commencement of the experiment. Weight vs total length: $r^2 = 0.902$, $p < 0.001$; weight vs standard length: $r^2 = 0.769$, $p < 0.001$; total length vs standard length: $r^2 = 0.867$, $p < 0.001$.
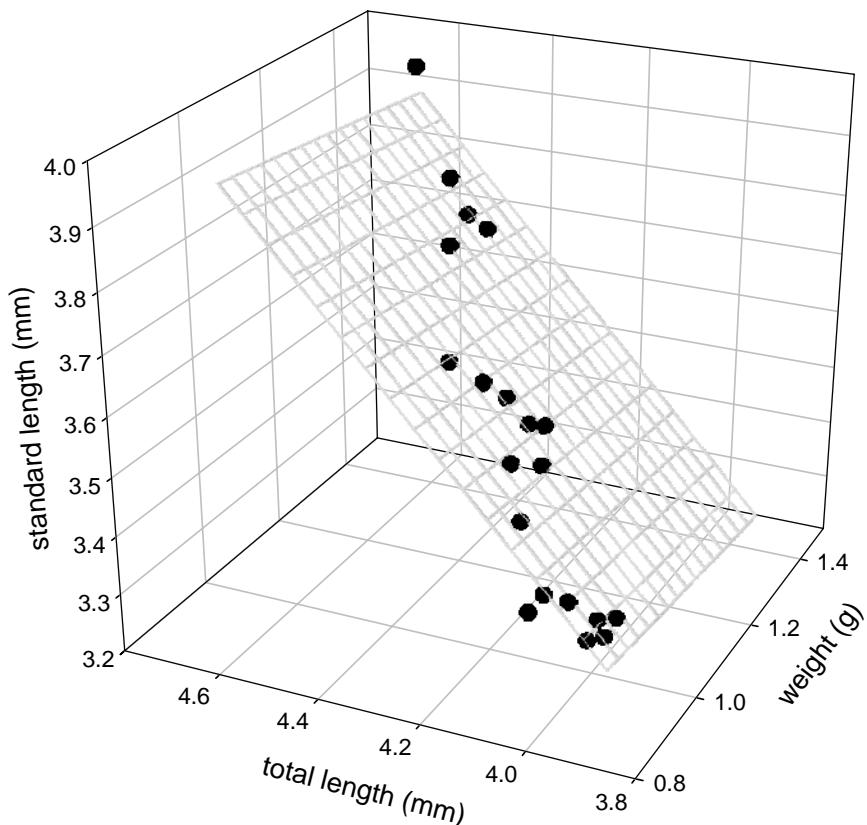
4.5.1 *Feeding*

No difference in feeding behaviour was observed for sooty grunter in different DO treatments (Figure 4.11), possibly because fish did not feed readily whilst being observed in captivity.

An independent samples t-test confirmed that mean food consumption did not differ significantly between the two sets of fish (Table 4.7, $p = 0.076$). Data from the two sets of fish (separately experimented on, as described in Chapter 3), were therefore treated together (Figure 4.12). No difference in food consumption was found between hypoxia treatments by One-Way ANOVA (Table 4.8, $p = 0.112$).

Figure 4.11 Feeding behaviour of juvenile sooty grunter grouped by DO treatment. Data are presented as the percentage of days (total of 28 x 2 = 56 days per treatment) spent by all fish in a treatment at each index of feeding behaviour (except for 100% treatment where one fish died). Black represents days feeding actively, light grey is feeding tentatively and dark grey represents days when feeding was not observed.
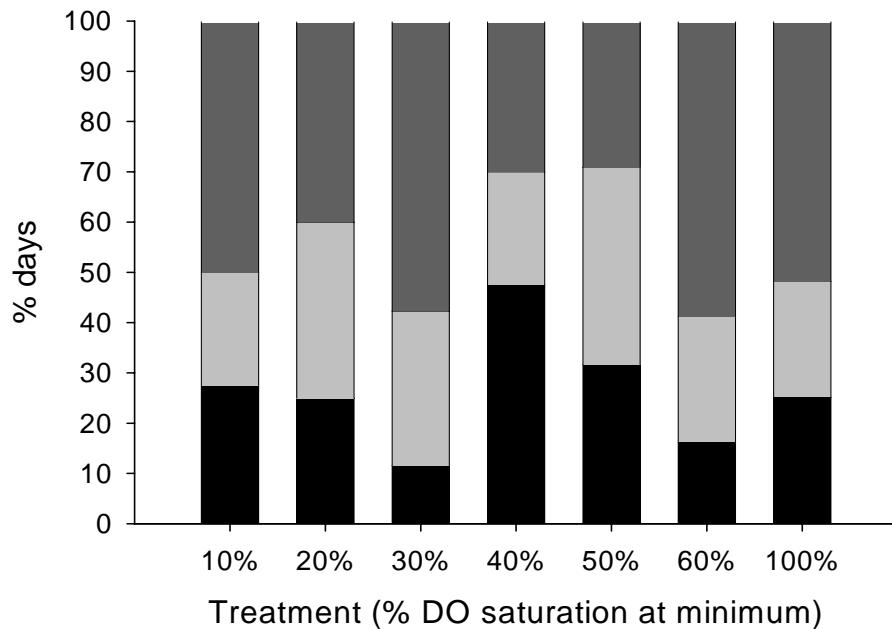
Table 4.7 Independent Samples T-Test showing no significant difference between mean food consumption of the two sets of sooty grunter.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means					95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00012	Equal variances assumed	2.040	.164	1.837	29	.076	6.4263	3.4978	-.7276	13.5802
	Equal variances not assumed			1.853	27.961	.074	6.4263	3.4679	-.6779	13.5304

Table 4.8 One-Way ANOVA comparing food consumption of sooty grunter in all treatments.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1007.291	6	167.882	1.957	.112
Within Groups	2059.354	24	85.806		
Total	3066.645	30			

Figure 4.12 Food consumption (average number of pellets consumed over 28 days) for juvenile sooty grunter.
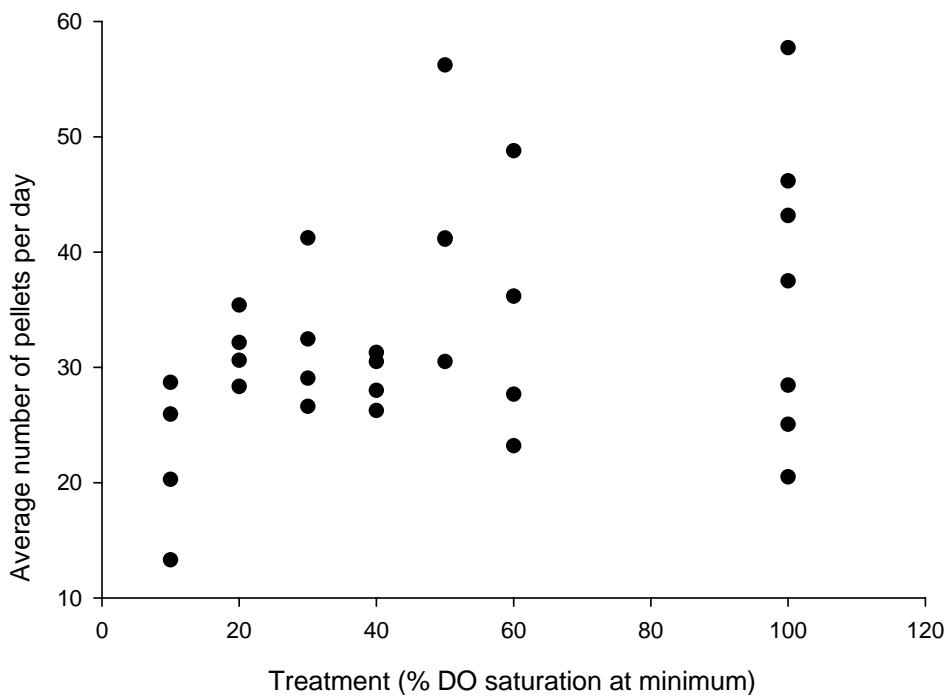
4.5.2 Growth

An independent samples t-test showed that there was no significant difference between means of sooty grunter growth from set 1 and set 2 (Table 4.9, p = 0.622), therefore the two data sets were treated together. Although it was not a statistically significant result at the p < 0.05 level (Table 4.10, p = 0.058), graphical representation of the data suggested an apparent reduction in growth for sooty grunter in the lowest surviving DO treatment. These fish in the treatment reaching 10% saturation each day increased in weight by less than 60% of their initial weight, whereas most fish in other treatments (exception for three individuals) grew more than 60% over 28 days, and in the control treatments the increase in weight was up to 177% of initial weight (Figure 4.14).

Table 4.9 Independent samples T-test showing no significant difference in growth of sooty grunter from sets 1 and 2.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00002	Equal variances assumed	1.749	.196	.498	29	.622	7.5051	15.0775	-23.3318	38.3419
	Equal variances not assumed			.502	27.879	.619	7.5051	14.9447	-23.1137	38.1238

Figure 4.13 Growth of juvenile sooty grunter in each treatment after 28 days of DO cycling, expressed as percentage increase in weight from initial weight.
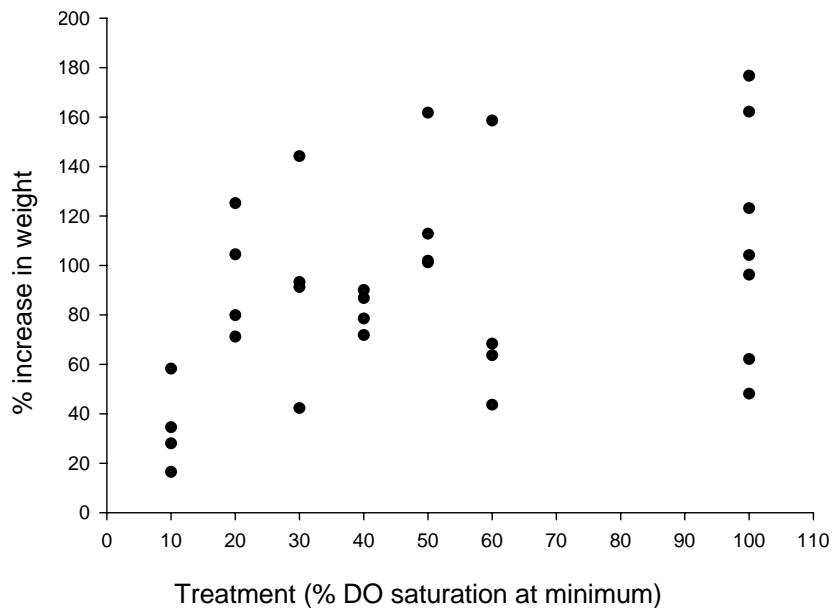
Table 4.10 One-Way ANOVA of growth of sooty grunter from all treatments.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	19333.884	6	3222.314	2.406	.058
Within Groups	32141.409	24	1339.225		
Total	51475.293	30			

4.5.3 Condition

No difference in condition was observed between means of condition data from set 1 and 2 sooty grunter, for either HSI (p = 0.120, Table 4.11) or Fulton's K (p = 0.844, Table 4.12) therefore the two data sets were combined for the following analyses. There was no significant difference in HSI (p = 0.577, Table 4.13, Figure 4.15) or in Fulton's K (p = 0.334, Table 4.14, Figure 4.16) between treatments.

Table 4.11 Independent samples T-test showing no significant difference in HSI between two sets of sooty grunter.

Independent Samples Test

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | 95% Confidence Interval of the Difference | |
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
H	Equal variances assumed	.815	.374	-1.600	29	.120	-.1241	7.753E-02	-.2827	3.449E-02
	Equal variances not assumed			-1.613	28.106	.118	-.1241	7.691E-02	-.2816	3.343E-02

Table 4.12 Independent samples T-test showing no significant difference in Fulton's K between two sets of sooty grunter.

Independent Samples Test

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | 95% Confidence Interval of the Difference | |
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
K	Equal variances assumed	1.597	.216	.199	29	.844	2.019E-02	.1014	-.1872	.2276
	Equal variances not assumed			.196	21.695	.847	2.019E-02	.1032	-.1940	.2344

Table 4.13 One-Way ANOVA of HSI of both sets of sooty grunter from all treatments.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.246	6	4.093E-02	.803	.577
Within Groups	1.223	24	5.097E-02		
Total	1.469	30			

Figure 4.14 HSI of both sets of sooty grunter.
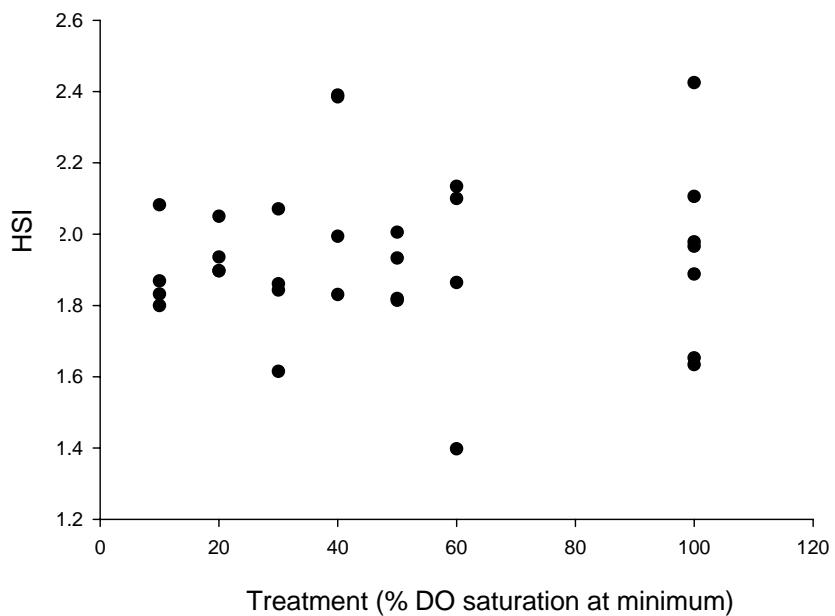
Table 4.14 One-Way ANOVA of Fulton's K for both sets of sooty grunter from all treatments.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.538	6	8.966E-02	1.213	.334
Within Groups	1.775	24	7.394E-02		
Total	2.313	30			

Figure 4.15 Fulton's K of both sets of sooty grunter.
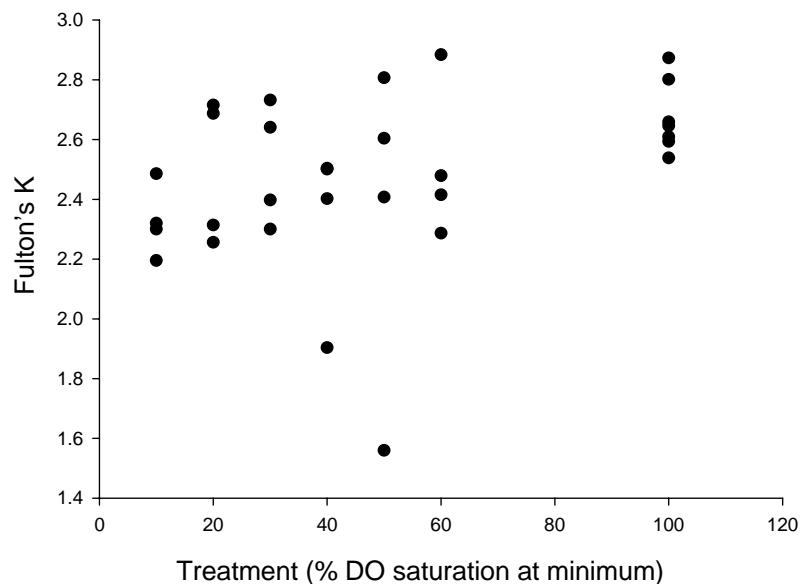
4.6   Discussion

Some slight effects of fluctuating hypoxia on fish growth and condition were identified in the three species studied. No effects on fish condition were identified. Hypoxia treatments affected feeding behaviour of juvenile barramundi, and there was some indication of reduced growth in the low DO treatments for both sooty grunter and barramundi. No effects on eastern rainbowfish were observed.

Many studies have considered the effects of hypoxia on growth of juvenile fish (Table 4.1). Early studies of temperate species including *Micropterus salmoides, Cyprinus carpio* and *Oncorhynchus kisutch* suggested a critical oxygen level of about 4 – 5 ppm (at 26, 22 and 20°C respectively = 50-62%, 46-58%, 44-55% DO saturation respectively) after which growth rate decreases by about 30% for every 1 ppm (11 - 12%) drop in oxygen concentration (Brett, 1979).

Daily fluctuations in DO saturation can result in greater reductions in growth than would occur after chronic exposure to the mean of the fluctuations, as was the case for juvenile coho salmon (*Oncorhynchus kisutch*; Carlson *et al*., 1980) and largemouth bass (*Micropterus salmoides*; Stewart *et al*., 1967). This finding was replicated for channel catfish (*Ictalurus punctatus*) and yellow perch (*Perca flavescens*), whose growth in constant low DO concentrations was higher than in fluctuating DO concentrations with the same mean concentration after 69 and 67 days respectively (Carlson *et al*., 1980).

Subtropical and tropical species tend to be more resistant to hypoxia, and in juvenile southern flounder (*Paralichthys lethostigma*) growth is unaffected by hypoxia down to 2.8 mg/L (at 22°C = 32%) (Taylor & Miller, 2001). Tropical cichlids such as *Oreochromis niloticus* are also remarkably tolerant, and growth is unaffected if oxygen levels are above 10% (Teichert-Coddington & Green, 1993). A progressive reduction in growth was found under 2-3 mg/L (24 - 37% at 24 - 26°C) for tropical guppies (*Poecilia reticulata*) if no surface access was provided; however, if the surface was accessible, growth was unaffected by hypoxia (Weber & Kramer, 1983).

In this study, the tropical fish species considered were found to be similarly resistant to hypoxia in terms of growth. Reductions in growth were identified for juvenile barramundi after 21 days of exposure to severely fluctuating hypoxia (down to 5% and 10% minimum DO saturation each day). Juvenile sooty grunter showed an apparent reduction in weight under a fluctuating DO treatment reaching 10% each day for 28 days (10% DO was the lowest surviving treatment) although the effect was not strongly significant ($p=0.058$). No such effects were identified for eastern rainbowfish, but the experiment period of 28 days may not have been long enough to record changes in growth of this relatively slow-growing fish.

The apparent reduction in growth of juvenile barramundi and sooty grunter under fluctuating hypoxia was likely to be related to decreased food intake during the experimental period. Barramundi showed reduced interest in food (food intake was not directly measured for this species) in severely hypoxic treatments compared to normoxic and intermediate treatments.

In the majority of cases, it appears that the reason for reduced growth under hypoxia is that food intake is also reduced (see Table 4.1). In the case of juvenile northern pike (*Esox lucius*) reductions in food consumption and food conversion efficiency begin at about 3 to 4 ppm of oxygen (at 19°C = 33 - 43% DO saturation). At concentrations near 5 mg/L and higher, fish fed vigorously, at 3.5 mg/L moderately, and at 2 mg/L only a small portion of food was consumed (at 19°C = 54 %, 38% and 22% DO saturation respectively) (Carlson *et al*., 1980).

In this study, the effects of hypoxia on feeding behaviour of juvenile barramundi were marked, with a 30% reduction in active feeding time under the lowest DO treatment (reaching a minimum of 5% each day). Feeding behaviour was used here as a proxy for food intake, which was difficult to measure as barramundi were held in tanks with circulating water currents, and were separated only by mesh grids for individual animals. This meant that uneaten pellets for all four fish per tank were condensed into a single pile after the one-hour feeding period. Feeding behaviour was used to indicate the fish's interest in food, and predict an index of food intake.

In this study, no effect of hypoxia was found on either Fulton's K or HSI for sooty grunter or barramundi (eastern rainbowfish were not tested). The effects of hypoxia on body condition of fish are not extremely well documented, given the comparative ease of measuring these effects. I have been unable to locate any published studies that have attempted to identify the effects of hypoxia on the condition measure Fulton's K, or on HSI. Perhaps other attempts to do this have not been published because results were negative or inconclusive. Nevertheless, the omission of body condition data in studies of hypoxia is unusual, given the regular use of condition indices in ecotoxicology and aquaculture studies (Bolger & Connolly, 1989; Leamon *et al*., 2000).

The relevance of health indices such as HSI and Fulton's K has recently been discussed by Leamon *et al*., (2000) and Bolger & Connolly (1989), respectively. Variability within and between health indices can be strong enough to render comparisons between fish as meaningless, particularly when comparing fish (even of the same species) from different geographical locations (Leamon *et al*., 2000). In light of this variability between individuals it is perhaps not surprising that the study presented in this chapter found no impact of hypoxia on HSI or Fulton's K. Studies of other environmental perturbations have found similar negative or minimal effects on HSI (Steyermark *et al*., 1999; Siligato & Bohmer, 2001) and Fulton's K (Bolger & Connolly, 1989), although different nutritional regimes appear to have measurable impacts on HSI of fish (Foster *et al*., 1993).

The fish studied here displayed few measurable effects of exposure to fluctuating hypoxia. It is important to note that all species were tested in their juvenile phase, and that the effects of hypoxia on adult fish of the same species may not be identical. A study on physiology of adult and juvenile yellow perch (*Perca flavescens*) exposed to hypoxia showed that juveniles were more tolerant than adults (Robb & Abrahams, 2003). The authors postulated that the higher tolerance of younger (and smaller) fish may give them an advantage in seeking refuge from predators by entering hypoxic waters.

The effects of hypoxia on most wild fish are probably worse than is implied by experimental studies (Chabot & Dutil, 1999). Most studies carried out in laboratories

do not replicate natural foraging behaviours necessary in the wild, as food is provided "dead" and ready to consume. In the wild, fish would have greater metabolic costs associated with food capture, predator avoidance, and migration. The increased activity required in natural situations would intensify the effect of hypoxia on food conversion, and result in slower growth.

In addition to the obvious effect of reduced feeding and growth on individual fitness, ecological interactions such as trophodynamics and hence species composition are also affected (Wu, 2002). This is further discussed in Chapter 7.

CHAPTER FIVE: THE EFFECTS OF FLUCTUATING HYPOXIA ON REPRODUCTION OF UTCHEE CREEK RAINBOWFISH

5.1   Introduction

The ability to mate and produce viable offspring is perhaps the most important aspect of fish health. A population or individual's ability to reproduce can be affected by various factors, including environmental stressors such as hypoxia. Reproductive failure and population decline in wild populations of fish and shellfish have been attributed to low oxygen conditions in several instances (e.g., Dombeck *et al*., 1984; Trippel & Harvey, 1989; Breitburg, 1992; Furota, 1996; Fontenot *et al*., 2001).

Despite this correlation, relatively few studies have attempted to identify experimentally the effects of sublethal exposure to hypoxia on reproduction of freshwater fish. Reproduction appears to be one of the most responsive sublethal responses to chemical pollutants (Sprague, 1971), which suggests that hypoxia may also negatively impact reproductive health. Several studies have identified effects of hypoxia on the brood-caring behaviour of freshwater species (Courtenay & Keenleyside, 1983; Jones & Reynolds, 1999a; 1999b; 1999c), and other studies have identified effects on reproductive output of adult fish and viability of resulting embryos. Some of these effects include hormonal disturbance (*Piaractus brachypomus*; Dabrowski *et al*., 2003 & *Cyprinus carpio*; Wu *et al*., 2003); lower gonadosomatic index (*Lepomis macrochirus* and *L. megalotis*; Sabo *et al*., 1998); reduced sperm motility (*Cyprinus carpio*; Wu *et al*., 2003); reduced spawning success (*Pomoxis nigromaculatus*; Carlson & Herman, 1978; and *Cyprinus carpio*; Wu *et al*., 2003); decreased brood size and egg viability (*Oreochromis niloticus*; Bhujel *et al*., 2001; and *Cyprinus carpio*; Wu *et al*., 2003) and diminished larval survival (*Cyprinus carpio*; Wu *et al*., 2003).

None of these studies is from Australia and only one examined the effects of fluctuating rather than chronic hypoxia on fish reproduction (Carlson & Herman, 1978). In this chapter, I investigate reproductive success of Utchee Creek rainbowfish exposed to diel fluctuations in DO saturation, firstly by measuring adult fish health

and reproductive output under hypoxia, and secondly by measuring the effects of parental exposure to hypoxia on embryos.

5.2 Methods

Transportation to Townsville was by air freight. The fish arrived in excellent condition with no mortalities, and males and females were separated for the holding period of four weeks.

5.2.1 Experimental set up and data collecting during the experiment

Mature fish were split into 18 brood groups each consisting of three males and two females, as suggested for maximum egg production in literature from the aquarium trade[7] (since published in Tappin, 2003). Fish were size selected to minimise differences for both males and females. The groups were kept in 30 L aquaria with 25 L of water, with submersible pumps for circulation and additional air bubblers (see Chapter 2). During the holding period and the experiment fish were fed twice during the day – at 3.00 p.m. on Nutrafin flake food, and at 6.00 p.m. on either live *Artemia* nauplii, frozen adult *Artemia* or bloodworms. Live and frozen food supply was alternated day to day to ensure that maximum nutritional value was being provided to fish. This was important to ensure maximum reproductive health. Fifty percent of water was exchanged daily. Spawning substrate was renewed daily, and was made up of 50 strands of green polyester wool tied at the top to form a "mop", as described by Humphrey *et al*. (2003) and attached to a piece of floating Styrofoam to prevent sinking.

The first part of the experiment aimed to identify the effects of fluctuating hypoxia on reproductive output of rainbowfish brood groups, using treatments as detailed in Chapter 2. Each day, the mops were removed from brood stock tanks and eggs were counted. Once a week a sample of five eggs was measured using an Olympus

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[7] http://members.optushome.com.au/chelmon/Breedbow.htm, © Adrian R. Tappin; updated October 1999.
http://www.netpets.org/fish/fishnews/articles/rainbowfishes2.html, article by Adrian R. Tappin, © 1995-2002 NetPets ®, Inc.
http://www.thetropictank.co.uk/Fishindx/mel-pcox.htm, © 2000-2004 The Tropical Tank. Originally added October 2000; last updated 11th February 2004.

objective micrometer under a Nikon stereo dissector. The final batch of eggs produced on day 28 of the experiment (following 28 d of oxygen cycling) was removed from each tank, counted and measured, and placed into nursery tanks for the second part of the experiment (described in Section 5.2.3 below).

5.2.2   Health of adult fish exposed to hypoxia

On the day following the last oxygen cycle, adult fish were euthanased in an icy slurry, weighed and measured. The fish were fixed in FAACC gonad fixative (containing 4% formaldehyde, acetic acid and calcium chloride dihydrate – S. Reilly, pers. comm. 2004[8]) until processing.

Condition of each adult fish was assessed using Fulton's K (Fulton, 1911), which describes the relationship between length and weight (as measured prior to fixation), and assumes isometric growth:

$$\frac{\text{Final somatic weight (g)}}{(\text{Final standard length(cm)})^3} \times 100 = \mathbf{K}$$

Following fixation for several days, fish were processed and gonadosomatic index (GSI) was calculated using somatic and gonad weights (both post-fixation), and the formula:

$$\frac{\text{Gonad weight (g)}}{\text{Somatic weight (g)}} \times 100 = \mathbf{GSI}$$

During processing, the gonads of male and female fish were removed and weighed. Testes were placed into histology cassettes, and ovaries were divided into two sections: approximately 1/3 of the total ovary was dissected immediately, while the remaining 2/3 was placed into a tissue block for histological processing. The piece of ovary to be dissected (approximately 1/3 of the entire ovary) was weighed, dissected and different egg types were identified and counted. These counts could be extrapolated to the whole ovary by calculating the exact fraction of ovary examined (by weight) and multiplying to estimate total egg counts. Gonad tissue blocks were

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[8] Mrs Sue Reilly, Histotechnologist, Biological Sciences Department, James Cook University, Townsville QLD 4811.

then processed and embedded in paraffin using standard histological techniques. Sections were cut using a microtome set to 5 μm, placed on slides, stained with Mayer's haemotoxylin and Young's eosin (H & E) , and mounted in dibutyl-phthalate-polystyrene-xylene (DPX).

All testes and ovaries were checked microscopically for pathological changes. Possible alterations to composition of tissue types within testes were quantified by running five 21-point transects across sections of testes for each fish (at 200x magnification) and identifying tissue type beneath each of the 105 points. To examine quantities of egg types within ovaries five sections of each fish ovary were photographed (at 40x magnification, using a DPI2 microscope digital camera system). From the photographs, eggs of different types were counted within each section using the software Image Tool for Windows Version 2.00.

5.2.3   Health of eggs after parental exposure to hypoxia

The second part of the experiment aimed to identify the effects of parental exposure to hypoxia on resulting embryos. Eggs laid on day 28 of the first part of the experiment (described in Section 5.2.1) were reserved and placed into separate nursery tanks with gentle aeration (DO levels remained > 95% at all times) to hatch. The number of eggs laid on the final day by each brood group is recorded in Table 5.1. Nursery tanks were checked for hatching larvae twice daily until hatching ceased, and for the following two days to make sure no more eggs were viable. The mop was checked visually for dead eggs during and following hatching. On hatching, larvae were removed from the aquarium and euthanased in icy water then checked for abnormalities under a stereo dissector. Five newly hatched larvae from each batch were measured using Kincrome vernier calipers, again under the stereo dissector.

5.2.4   Statistical analyses

Graphs and regression relationships were created and analysed using SigmaPlot. ANOVAs were run using SPSS.

Table 5.1 Number of Utchee Creek rainbowfish eggs used in the egg health section of the reproduction experiment. The number of eggs in each nursery tank was governed by the number of eggs laid by each brood group on the final day of oxygen cycling. Note that the 5% DO treatment was lethal to adult fish and thus no eggs were collected from tanks in that treatment.

3 Tank	Treatment (DO saturation at minimum)	Number of eggs
2A	10%	48
2B	10%	0
3A	20%	47
3B	20%	22
4A	30%	55
4B	30%	43
5A	40%	2
5B	40%	28
6A	50%	7
6B	50%	14
7A	60%	32
7B	60%	38
8A	100%	62
8B	100%	58
8C	100%	45
8D	100%	1

5.3 Results for effect of hypoxia on adult rainbowfish

In this experiment, the immediate lethal level for adult Utchee Creek rainbowfish was found to be about 7% DO saturation. This means that the treatment reaching 5% DO saturation daily (see Chapter 2 for all treatments) was lethal, and the lowest surviving level reached 10% DO saturation daily, for 28 days of cycling.

5.3.1 Egg production

A notable pattern in daily egg production for individual tanks was that, in one of the brood stock tanks reaching 10% minimum DO, egg production virtually ceased after 17 days of oxygen cycling (Figure 5.1). However, the other brood group experiencing the same oxygen saturations continued to reproduce throughout the experiment. All tanks showed variable egg production between days, with some tanks producing no eggs one day, and batches in excess of 100 eggs the next. This variability in daily egg

production by Utchee Creek rainbowfish was not exhibited by eastern rainbowfish in earlier trials, nor were such large egg batches observed for the latter species (unpublished data).

Descriptive statistics considered for the eggs produced each day in individual brood stock tanks included: mean, mode, variance, skewness, kurtosis, interquartile ranges and total egg count for each individual tank. Univariate analyses found the most important descriptor of egg production to be total egg count. 30% of linear variation in total egg count could be explained by DO treatment (Figure 5.2). The relationship was significant at the $p < 0.05$ level ($p = 0.028$).

5.3.2   Egg size

Egg size was measured on days 1, 7, 14, 21 and 28 of the experiment. Regression analyses indicated there was no relationship between egg size and treatment on any of the days, or when data was combined for all measurements (Figure 5.3). A univariate ANOVA with two factors (time and DO treatment) showed a significant variation between means for both factors (Table 5.2). For DO treatment, the results were significant to $p = 0.008$, and for time (day) the difference was significant to $p = 0.002$. The interaction between the two factors was insignificant ($p = 0.396$). However, post hoc multiple comparisons tests identified the difference between DO treatments to be between the 20% and 40% treatments. This result does not suggest that the differences were caused by hypoxia treatment, and are more likely to have been caused by some other 'tank effect'.

Figure 5.1 Daily egg counts of two brood groups of Utchee Creek rainbowfish in treatment 2 (reaching 10% DO saturation each day), the lowest surviving treatment. A) Tank 2A B) Tank 2B.
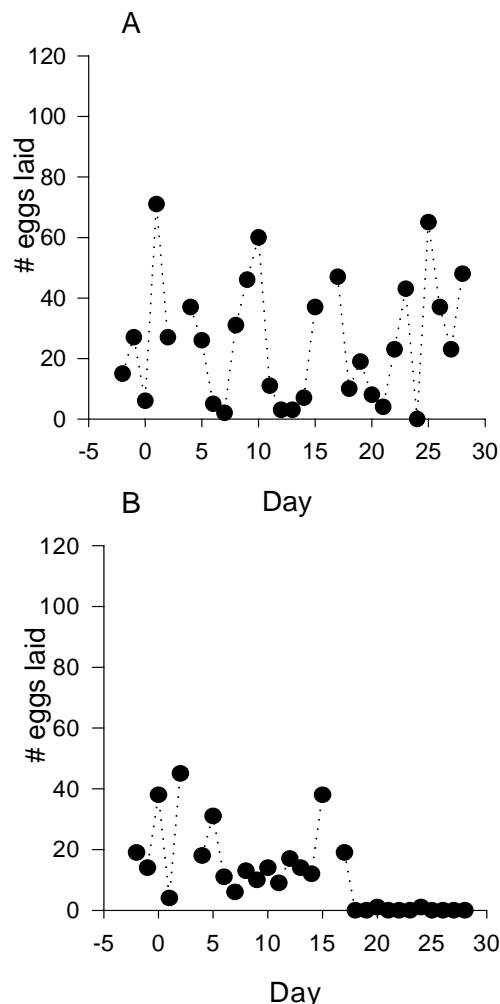
Figure 5.2 Total number of eggs laid by each Utchee Creek rainbowfish brood group in all treatments. $r^2 = 0.300$, $p = 0.028$.
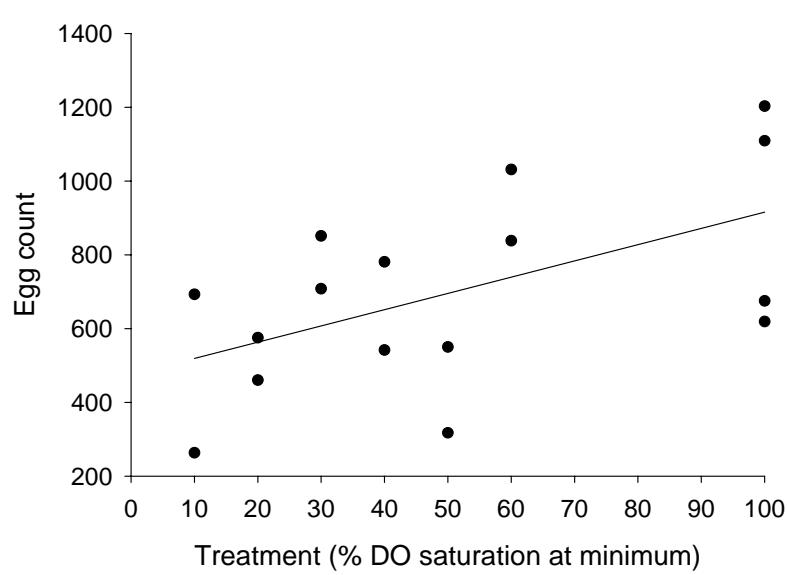
Figure 5.3 Average egg size for each brood group: A) combined for all measures taken; B) measured on day 1 of oxygen cycling, $r^2 = 0.161$, $p = 0.123$; C) day 7 of oxygen cycling, $r^2 = 0.037$, $p = 0.508$; D) day 14 of oxygen cycling, $r^2 = 0.040$, $p = 0.459$; E) day 21 of oxygen cycling, $r^2 = 0.014$, $p = 0.678$; F) day 28 of oxygen cycling, $r^2 = 0.000$, $p = 0.963$.
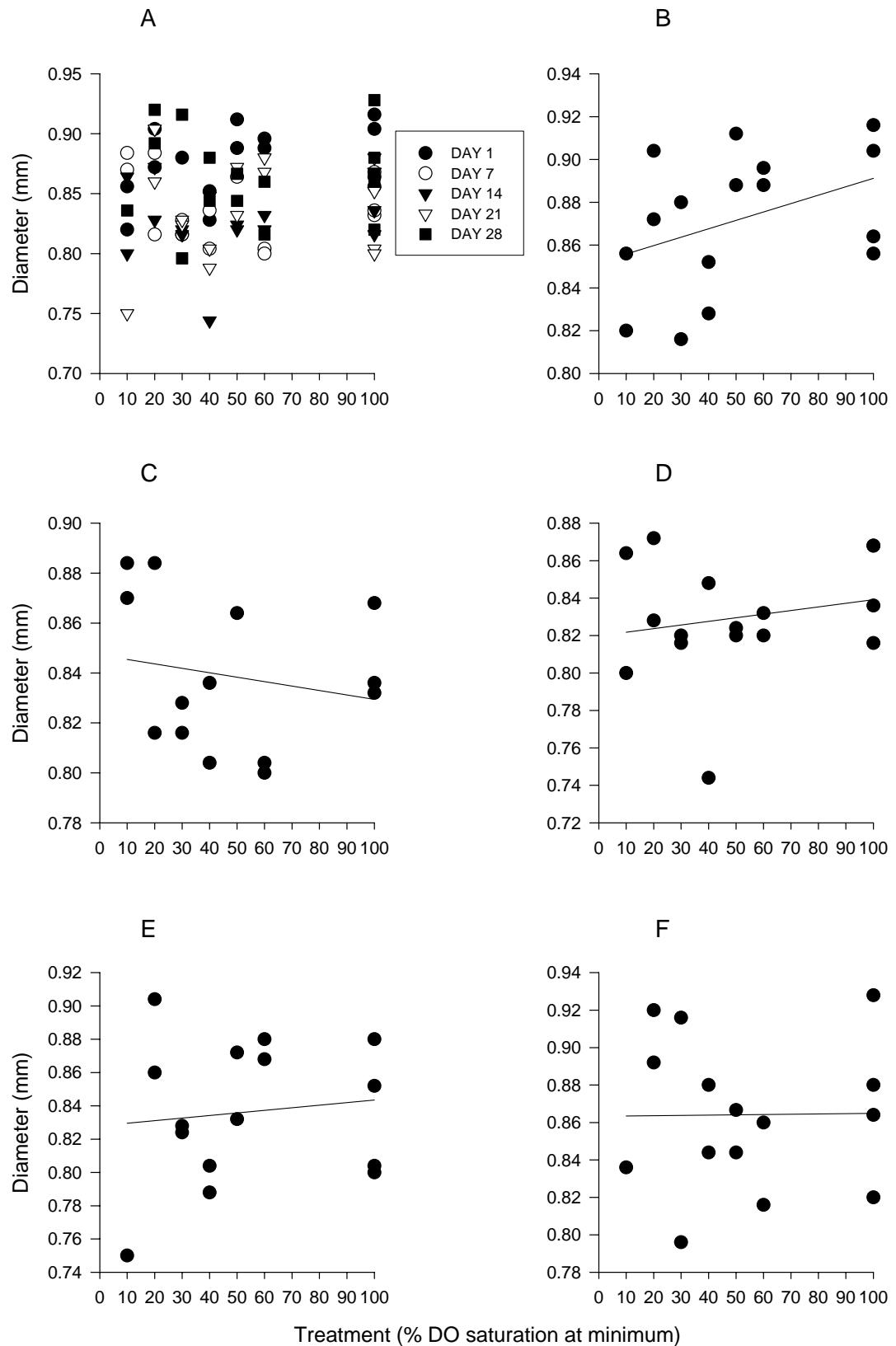
Table 5.2 Two-Way ANOVA of effect of DO treatment (VAR00004) and time (day) (VAR00006) on size of eggs produced by Utchee Creek rainbowfish.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6.810E-02[a]	34	2.003E-03	1.929	.023
Intercept	49.139	1	49.139	47317.855	.000
VAR00004	2.141E-02	6	3.569E-03	3.437	.008
VAR00006	2.054E-02	4	5.134E-03	4.944	.002
VAR00004 * VAR00006	2.713E-02	24	1.131E-03	1.089	.396
Error	4.258E-02	41	1.038E-03		
Total	54.815	76			
Corrected Total	.111	75			

a. R Squared = .615 (Adjusted R Squared = .296)

5.3.3 Adult condition and gonad health

There was no difference between means of Fulton's K (condition) of male and female fish (Table 5.3, p = 0.643), therefore the two data sets were treated together for between treatment analysis. As is clear from the scatter of data in Figure 5.4, no relationship between treatment and Fulton's K was identified by One-Way ANOVA (Table 5.4, p = 0.848) or by linear regression (Figure 5.4, r^2 = 0.000, p = 0.984).

No difference between treatments was found for GSI of either male (Table 5.5, p = 0.945) or female fish (Table 5.6, p = 0.645) (Figure 5.5).

Table 5.3 Independent samples T-test showing no significant difference between means of Fulton's K for male and female rainbowfish.

		Levene's Test for Equality of Variances		t-test for Equality of Means					95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00013	Equal variances assumed	5.588	.021	-.434	78	.665	-1.626E-02	3.742E-02	-9.08E-02	5.825E-02
	Equal variances not assumed			-.466	77.809	.643	-1.626E-02	3.489E-02	-8.57E-02	5.320E-02

Figure 5.4 Fulton's K of adult Utchee Creek rainbowfish following 28 days of oxygen cycling (data from male and female fish combined). $r^2 = 0.000$, $p = 0.984$.
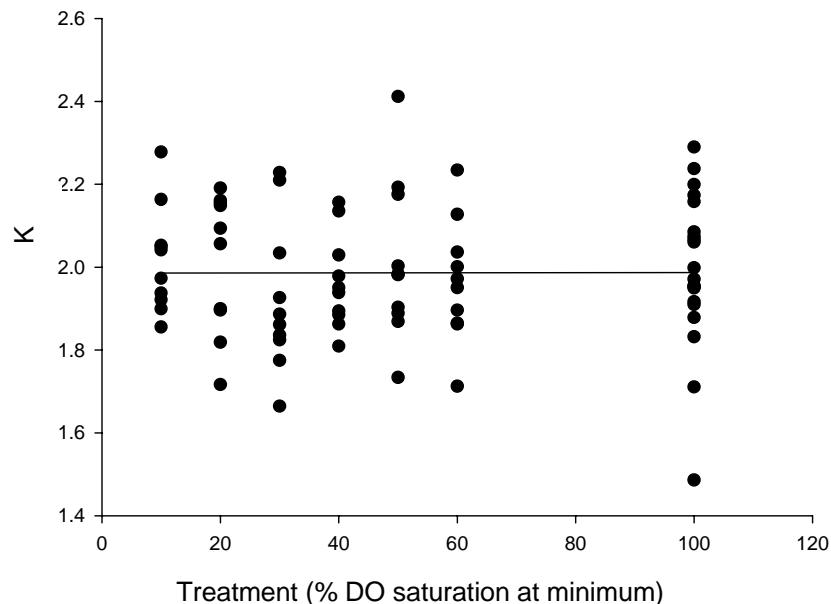
Table 5.4 One-Way ANOVA showing no effect of DO treatment on Fulton's K of adult Utchee Creek rainbowfish.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.371E-02	6	1.229E-02	.442	.848
Within Groups	2.029	73	2.779E-02		
Total	2.102	79			

Table 5.5 One-Way ANOVA showing no effect of DO treatment on gonadosomatic index (GSI) of male Utchee Creek rainbowfish.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.809E-06	6	9.682E-07	.273	.945
Within Groups	8.876E-05	25	3.550E-06		
Total	9.457E-05	31			

Table 5.6 One-Way ANOVA showing no effect of DO treatment on gonadosomatic index (GSI) of female Utchee Creek rainbowfish.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.675E-04	6	1.113E-04	.709	.645
Within Groups	3.922E-03	25	1.569E-04		
Total	4.589E-03	31			

Figure 5.5 GSI of adult Utchee Creek rainbowfish following 28 days of oxygen cycling. A) female fish; B) male fish.
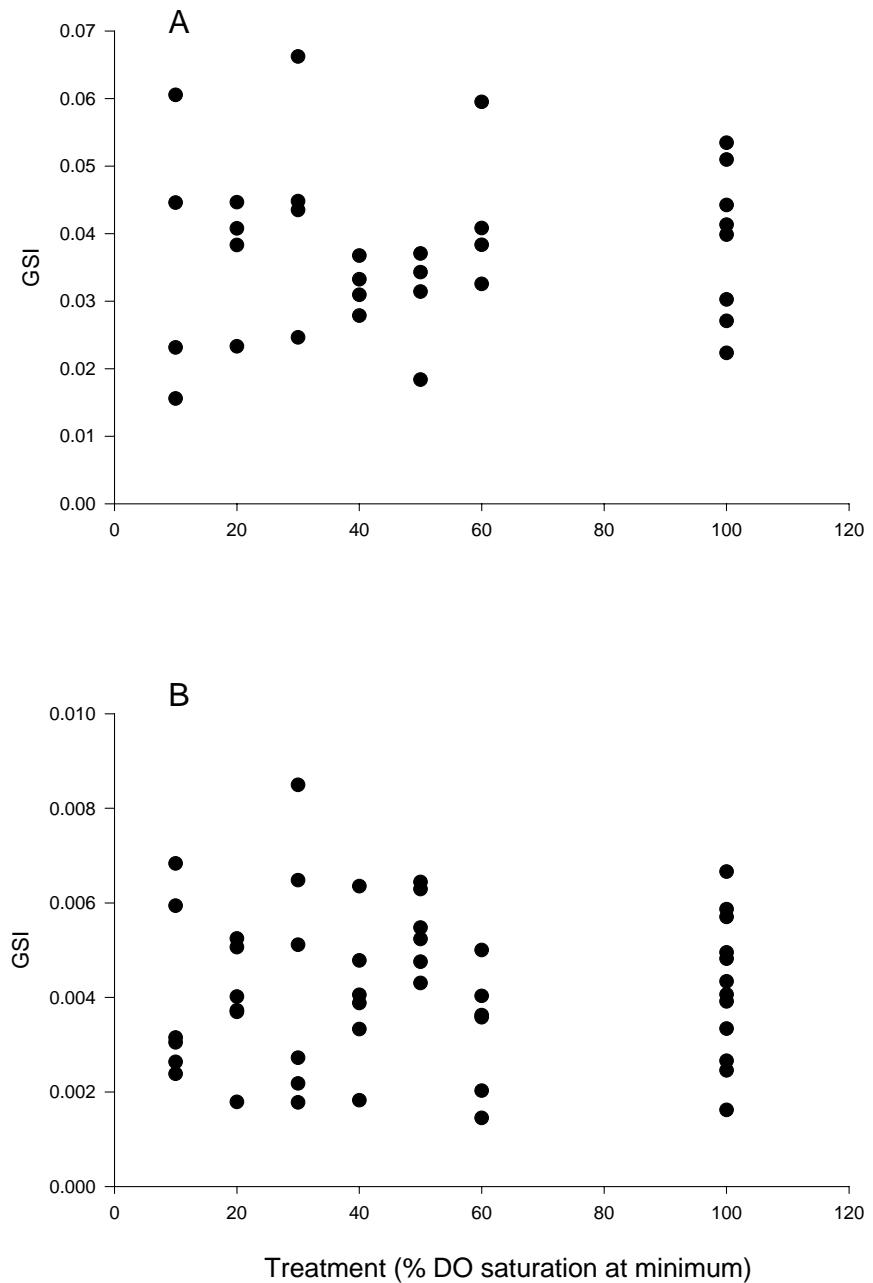
Sections of the ovaries were dissected whole under a stereomicroscope to count egg types, and results were extrapolated to count estimates for the entire ovary. Scatterplots show that the abundance of yolked eggs (an estimate of fecundity), hydrated/ovulated eggs (an estimate of next brood size) and atretic (degenerative) eggs in the ovary, was not affected by DO treatment (Figure 5.6), although ovaries of one female fish from the lowest surviving DO treatment (10% at minimum) contained an abnormally high number of atretic eggs. One-Way AVOVAs confirmed there was

no statistically significant effect of hypoxia on yolked (Table 5.7, p = 0.748), hydrated (Table 5.8, p = 0.244) or atretic (Table 5.9, p = 0.448) eggs.

Figure 5.6 Total ovary count estimates of: A) yolked eggs; B) hydrated eggs; C) atretic eggs for each female Utchee Creek rainbowfish following 28 days of oxygen cycling.
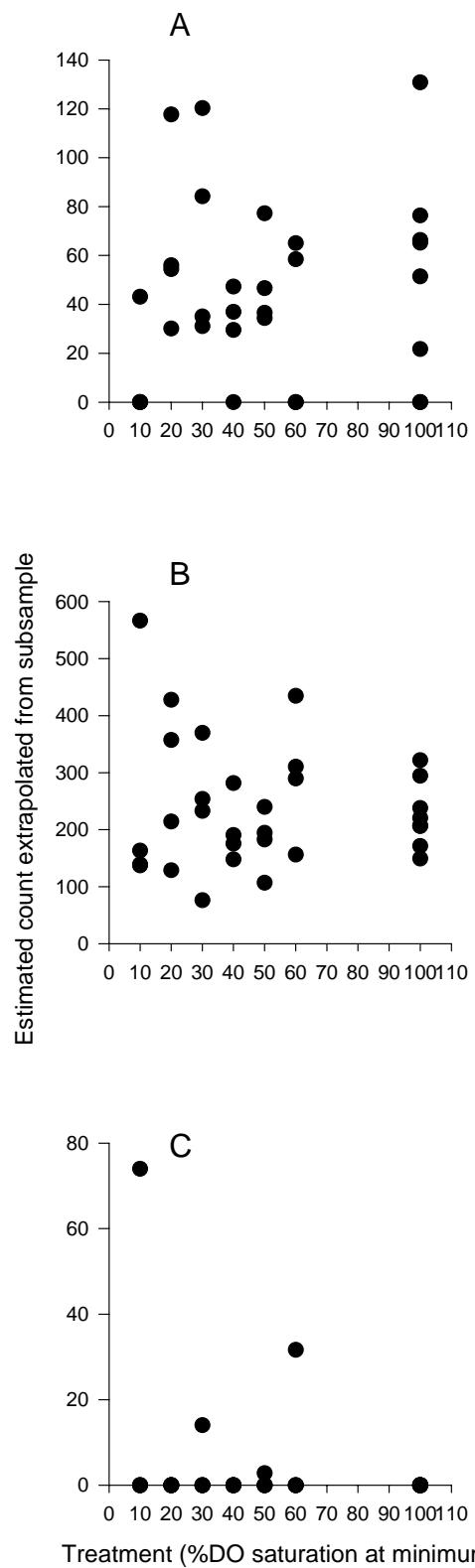
Table 5.7 One-Way ANOVA of effect of hypoxia treatments on abundance of yolked eggs in ovaries of Utchee Creek rainbowfish.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	43099.730	6	7183.288	.573	.748
Within Groups	313310.6	25	12532.423		
Total	356410.3	31			

Table 5.8 One-Way ANOVA of effect of hypoxia treatments on abundance of hydrated/ovulated eggs in ovaries of Utchee Creek rainbowfish.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10537.303	6	1756.217	1.427	.244
Within Groups	30777.039	25	1231.082		
Total	41314.341	31			

Table 5.9 One-Way ANOVA of effect of hypoxia treatments on abundance of atretic eggs in ovaries of Utchee Creek rainbowfish.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1201.884	6	200.314	.999	.448
Within Groups	5012.684	25	200.507		
Total	6214.568	31			

Ovary condition was investigated histologically using photomicrographs of ovaries that had been downloaded onto a computer and counts of egg types taken using a graphics program. Figure 5.7 illustrates the different egg types identified within a typical section. Five sections were analysed for each fish, and several variables were investigated including total egg count, late globule stage (fully yolked) egg count, hydrated (ready to be laid) egg count, atretic (degenerative) egg count and an index of area of scar tissue in the ovary were investigated. One-Way ANOVA with post hoc multiple comparisons (Tukey's) showed a significant difference between the 10% DO treatment and all other treatments for total egg count data (Table 5.10, $p < 0.001$). No statistical relationships between DO treatment and other egg variables existed (Figure 5.8). No pathological changes to ovary tissue were observed.

Figure 5.7 Photomicrograph of ovary section of female Utchee Creek rainbowfish. Types of eggs identified for analysis are indicated.

tissue infiltration

200 µM

fully yolked egg   hydrated egg   atretic egg
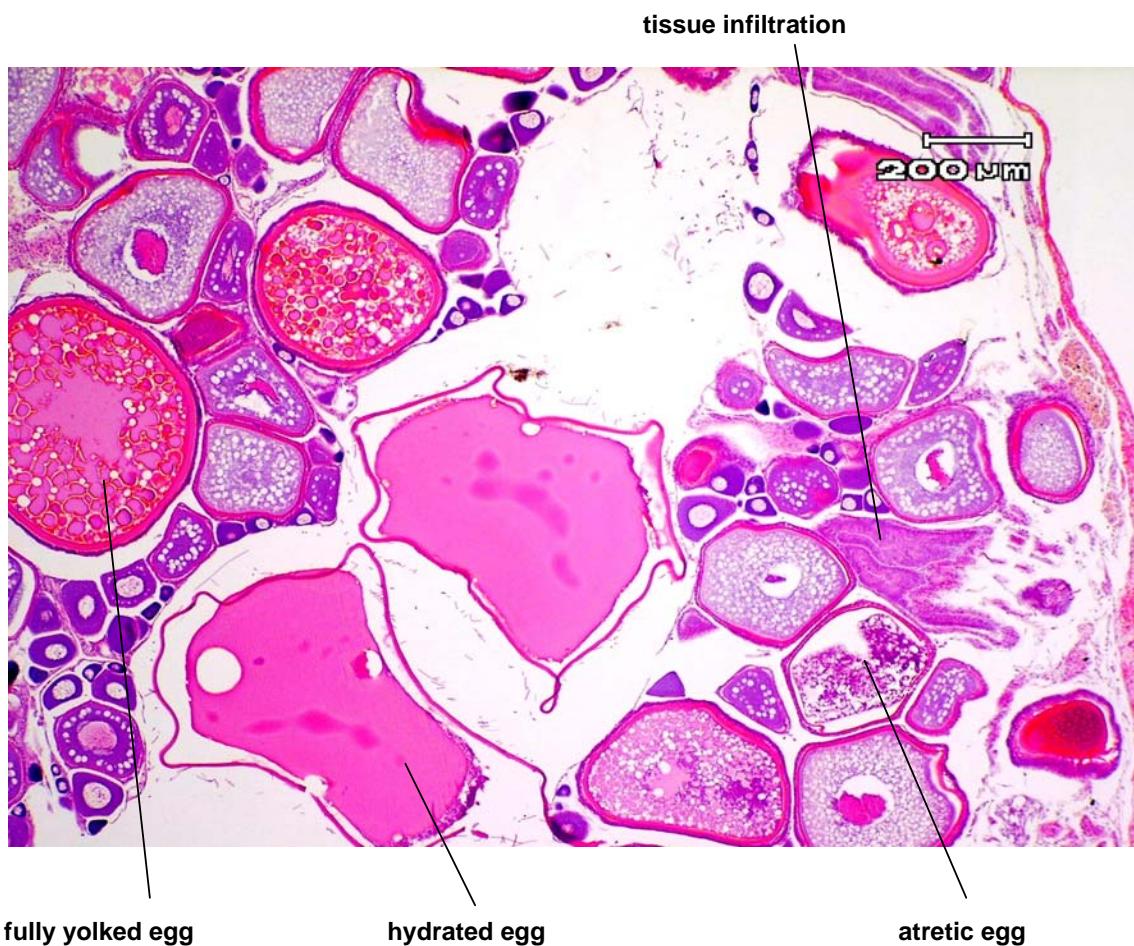
Figure 5.8 Abundance of: total eggs (r^2 = 0.012, p = 0.160), hydrated eggs (r^2 = 0.007, p = 0.303), mature eggs (r^2 = 0.048, p = 0.105), atretic eggs (r^2 = 0.042, p = 0.099) and incidence of scarring – indexed from 1 (least scarring) to 5 (most scarring) (r^2 = 0.000, p = 0.834) within ovaries of female Utchee Creek rainbowfish, counted from five histological sections per fish.
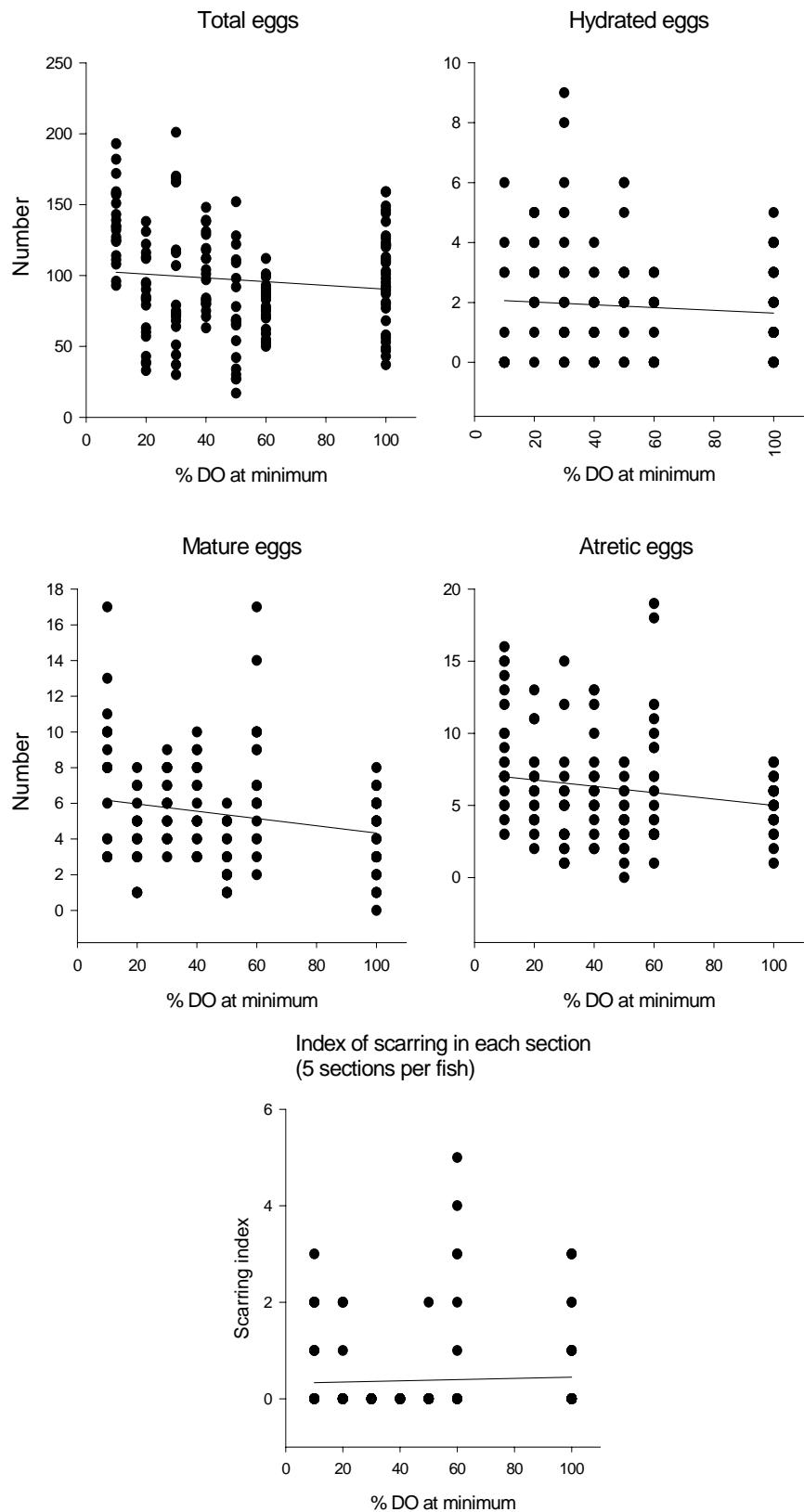
To quantify histological changes to testes, tissue types (Figure 5.9) under 21 points on each of five transects for each male fish were identified. No correlation was found between treatment and abundance of interstitial tissue containing support cells, germ cells and spermatogonia combined, spermatocytes and spermatids combined, or sperm cells (Figure 5.10). The amount of empty space in sections of testes showed a decreasing trend with increasing DO saturation, with a significance of p = 0.010. The linear trend was, however, quite weak, with an r^2 value of only 0.136. This relationship was considered further with a One-Way ANOVA, which was also significant (Table 5.10, p = 0.029). Post hoc multiple comparisons testing (Tukey's test) could not identify the source of difference between treatments. No pathological changes to tissue were observed.

Figure 5.9 Photomicrograph of a section of the testes of a male Utchee Creek rainbowfish indicating tissue types recorded and compared.
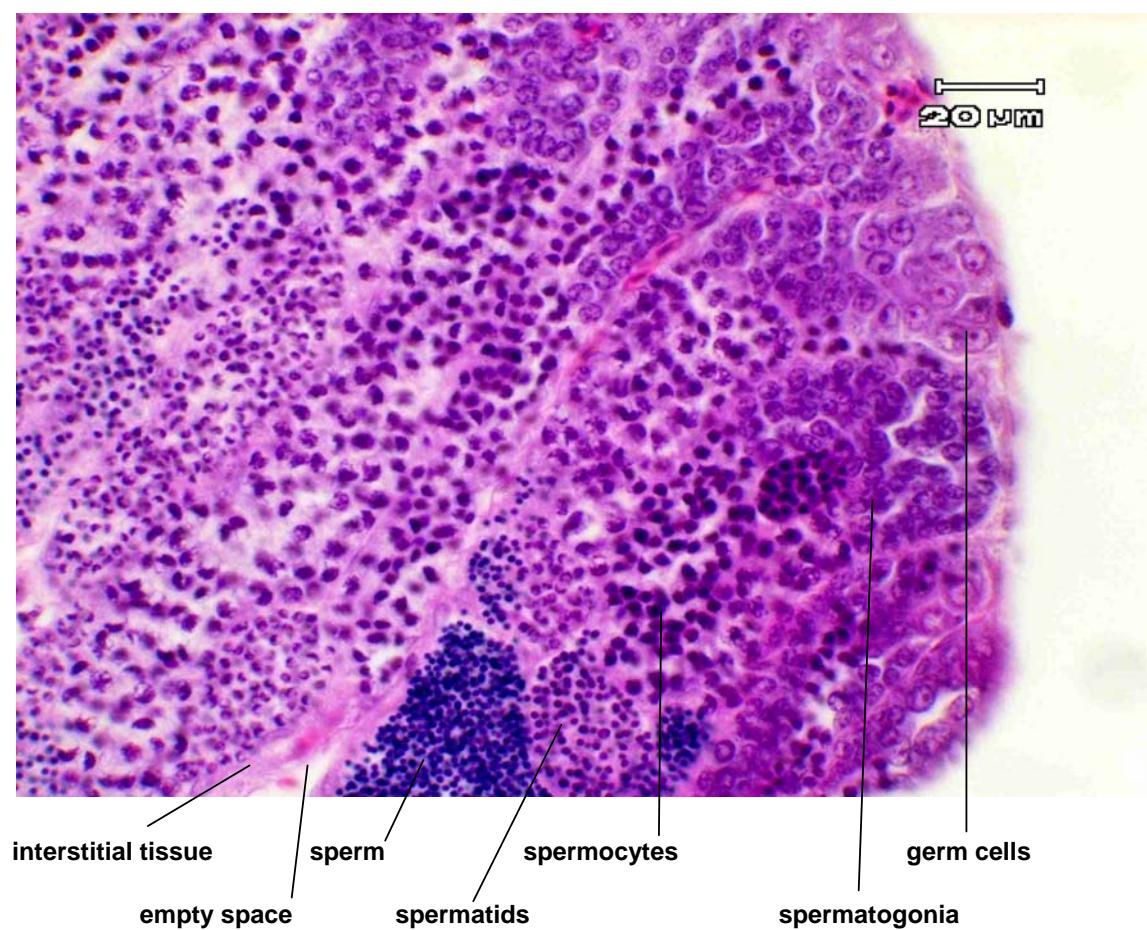
Figure 5.10 Composition of testes as determined by coverage of cell types (number of points on transect) in histological sections. Interstitial tissue, $r^2 = 0.004$, $p = 0.674$; empty lumen/vacuoles, $r^2 = 0.136$, $p = 0.010$; spermatocytes and spermatids, $r^2 = 0.017$, $p = 0.382$; sperm, $r^2 = 0.006$, $p = 0.602$; and germ cells and spermatogonia, $r^2 = 0.034$, $p = 0.208$.
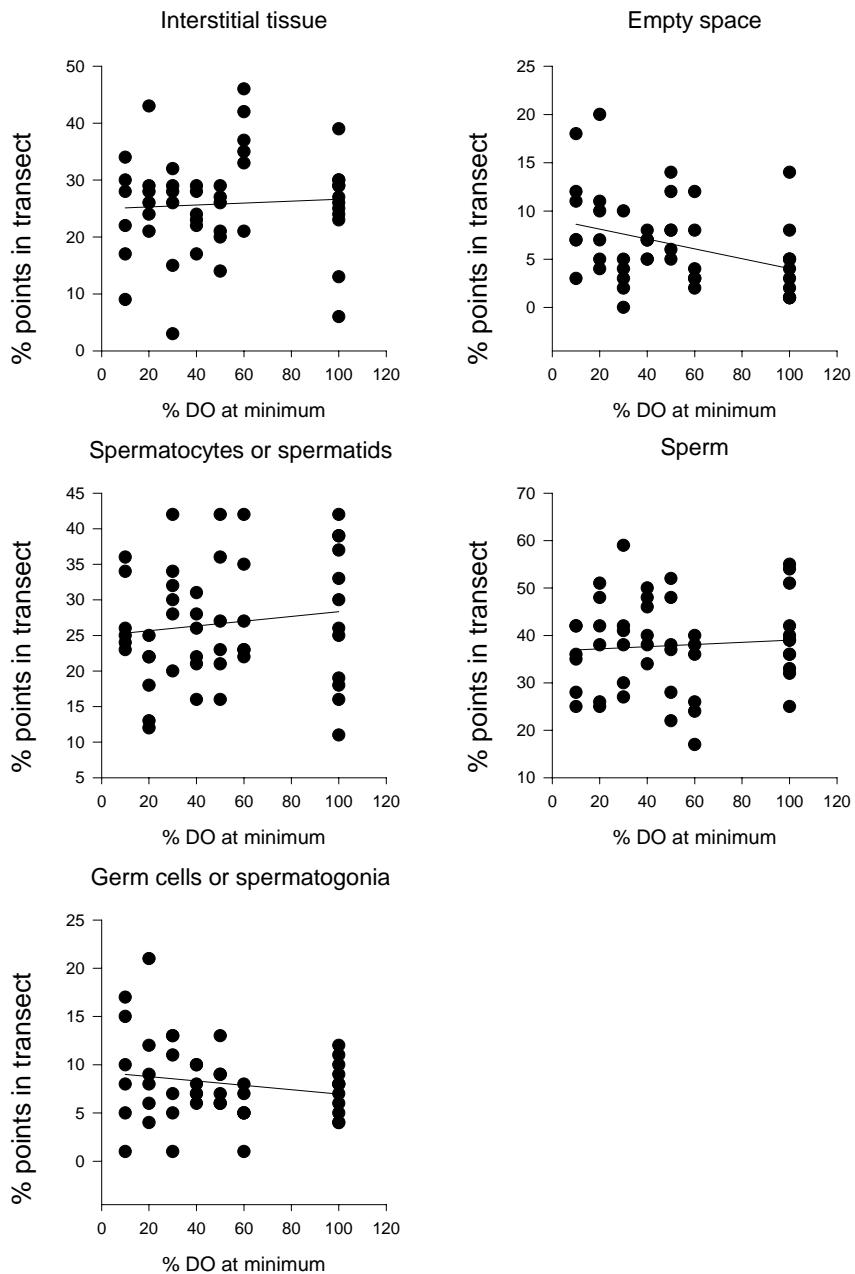
Table 5.10 One-Way ANOVA showing significant relationship between DO treatment and abundance of empty spaces in testes of Utchee Creek rainbowfish.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	257.813	6	42.969	2.645	.029
Within Groups	666.167	41	16.248		
Total	923.979	47			

5.4 Results for effect of parental hypoxia on resulting embryos

Due to air circulation patterns in the aquarium room (which was set on a thermostat to a controlled temperature of 29°C), there was a slight difference in temperature between nursery tanks on different levels of the shelving units used (see Chapter 2). This difference was not as marked in the brood group tanks used in the first part of the experiment, as submersible pumps were used to increase water circulation in those tanks which slightly warmed the water and resulted in more similar water temperatures between tanks on all levels of shelving. In the nursery tanks submersible pumps were not used, because the higher water currents may damage delicate newly hatched larvae. The difference in temperature averaged 1°C between shelves, such that the difference between uppermost and lowest shelves was about 2°C. The average temperatures on the three shelves were: upper – 28°C, middle – 27°C and lower – 26°C. This slight difference in temperature appears to have had a larger effect on viability of embryos than did the parental exposure to hypoxia, as will be illustrated in the following results. Comparison of means analyses are more frequently used to analyse the effects of temperature 'treatments' on data, but is not suitable for analysing effects of hypoxia treatments on the data, as replication of egg batches is low (four control batches, maximum of two batches per treatment).

The number of eggs in the final batch produced in each tank differed (Table 5.1). In some analyses, such as percentage mortality of eggs and larvae, tanks with < 10 eggs have been excluded from the analyses. Because no eggs were laid by the brood group in tank 2B, that tank is not included in the results. The 5% DO treatment (tanks 1A and 1B) was lethal to adult fish, and thus no effect of parental exposure to hypoxia was able to be tested for that treatment.

5.4.1 *Incubation time*

The most informative way of looking at the data from this experiment is graphically. It is clear that larvae showed a trend for longer incubation time (time between spawning and hatching) in tanks on the slightly cooler lower shelf (26°C), and no correlation between incubation time and parental exposure to hypoxia (Figure 5.11).

All tanks on the lower shelf took longer than 7 days to complete hatching, whereas all other tanks had completed hatch by day 7.

To analyse the data statistically, a One-Way ANOVA (Table 5.11) was carried out to determine the effect of temperature on start of hatch (minimum days to incubate) ($p = 0.239$), final day of hatch (maximum days to incubate) ($p < 0.001$), and day of most hatching (mode of days to incubate) ($p = 0.001$). Post hoc multiple comparisons testing (Tukey's test) showed that for both final day of hatch and day of most hatching, the tanks on the lower shelf (with the coolest temperature, at 26°C) were significantly different to the other two temperature 'treatments' (27°C and 28°C).

ANOVA was not a suitable way to determine effects of hypoxia on hatch times, as the replication of each treatment was low, and was more suitable for regression analysis. There was no significant linear relationship between hypoxia treatment and day of first hatch ($r^2 = 0.064$, $p = 0.363$), final day of hatch ($r^2 = 0.002$, $p = 0.861$) or day of most hatching ($r^2 = 0.027$, $p = 0.561$) (Figure 5.12).

Larvae were collected twice daily from the experiment tanks, at 7.00 a.m. and 6.00 p.m., so time to hatch is presented in days and half-days. Time until hatch began in the evening, when eggs were collected from brood stock tanks, such that full days represent evening collections and half days represent morning collections (i.e., eggs hatching on the morning of the sixth day of the experiment took 5.5 d to hatch). Eggs tended to hatch in the morning, and highest numbers of hatching larvae were collected on days 5.5 and 6.5 (i.e. on the mornings of day 6 and day 7) (Figure 5.13).

5.4.2   Hatch success and mortality

When the data are represented graphically, there is an apparent trend for higher viable hatch (% of larvae hatched alive with no deformities) in tanks on the warmer upper shelf (Figure 5.14). This relationship was not statistically significant, however, as shown by One-Way ANOVA in Table 5.12 ($p = 0.402$). There was no evidence of a correlation between parental exposure to hypoxia and viable hatch (Figure 5.15, $r^2 = 0.000$, $p = 0.973$).

Percentage mortality of eggs and larvae combined showed no correlation with parental exposure to fluctuating hypoxia (Figure 5.16, $r^2 = 0.011$, p = 0.748). There was also no significant relationship with temperature (Table 5.13, p = 0.656).

Figure 5.11 Time taken after spawning for larvae from each nursery tank to hatch. Note the tanks on the lower shelf with a slightly cooler temperature took longer to hatch, as marked on the graph by thicker lines.
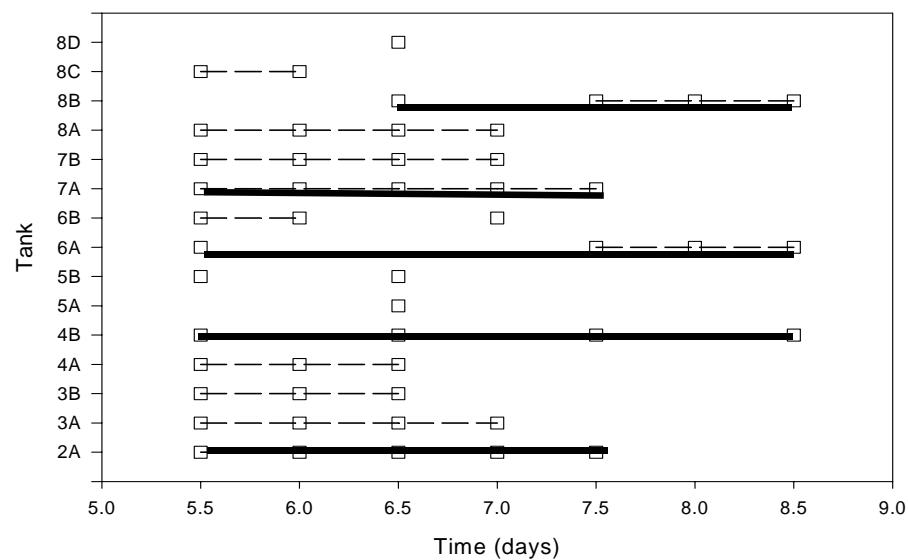
Table 5.11 One-Way ANOVA showing significant differences between temperature 'treatments' for last day of hatching (HL), and day of most hatching activity (HG). No significant difference for day of first hatch (H1) between temperatures.

		Sum of Squares	df	Mean Square	F	Sig.
H1	Between Groups	1.133	2	.567	1.619	.239
	Within Groups	4.200	12	.350		
	Total	5.333	14			
HL	Between Groups	7.158	2	3.579	20.699	.000
	Within Groups	2.075	12	.173		
	Total	9.233	14			
HG	Between Groups	5.400	2	2.700	14.727	.001
	Within Groups	2.200	12	.183		
	Total	7.600	14			

Figure 5.12 Hypoxia treatment versus day of first hatch (A, $r^2 = 0.064$, p = 0.363), final day of hatch (B, $r^2 = 0.002$, p = 0.861) or day of most hatching (C, $r^2 = 0.027$, p = 0.561) of Utchee Creek rainbowfish eggs.
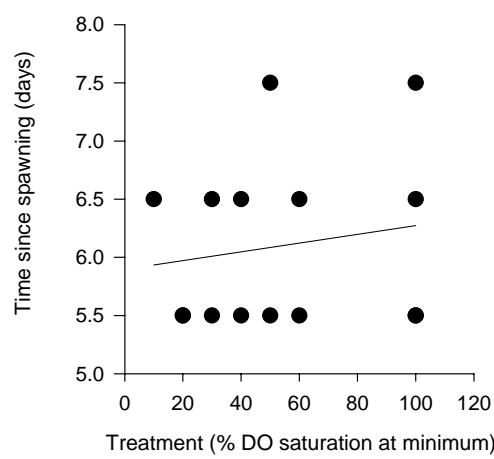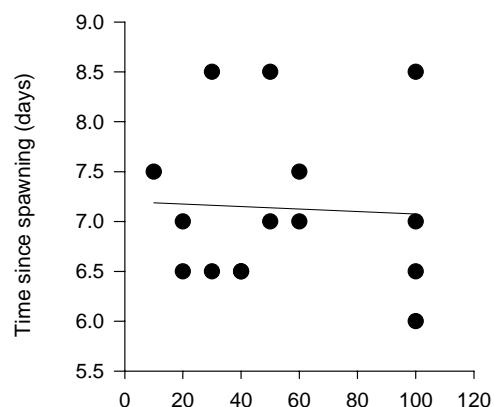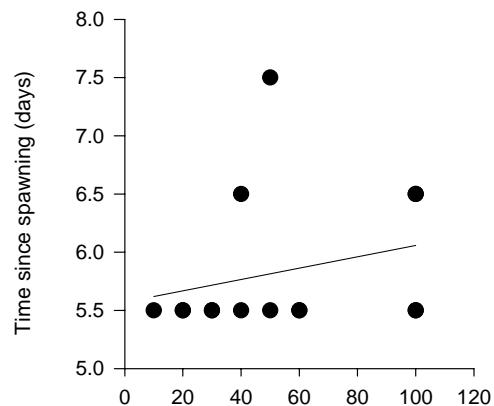
Figure 5.13 Number of days taken for Utchee Creek rainbowfish eggs to hatch. Includes only larvae that were collected alive and without deformities. Morning collections were recorded as 0.5 days and evening collections as 0.0 days.
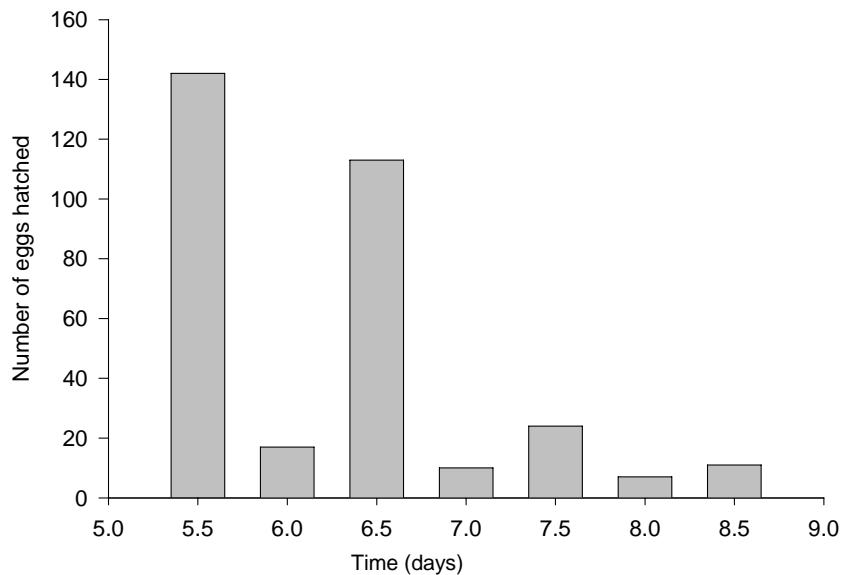
Figure 5.14 Viable hatch (% of eggs that hatched alive with no deformities) for each tank. The data excludes tanks with less than 10 eggs. Arrows indicate tanks on the top shelf, which experienced slightly higher temperatures and a trend for increased viability.
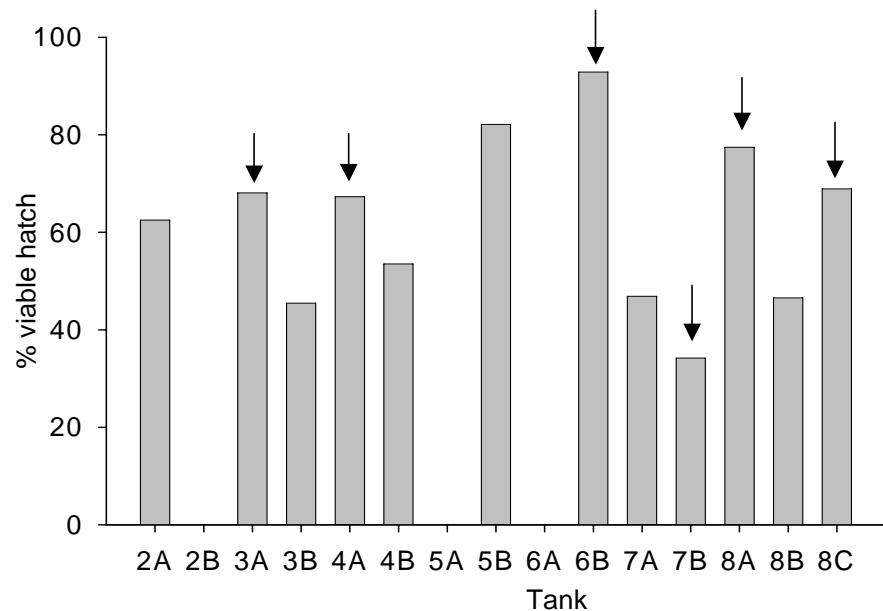
Table 5.12 One-Way ANOVA showing no significant difference in viable hatch between temperature treatments.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	603.310	2	301.655	1.009	.402
Within Groups	2690.497	9	298.944		
Total	3293.807	11			

Figure 5.15 Viable hatch (% of eggs that hatched alive with no deformities) for each hypoxia treatment. The data excludes tanks with less than 10 eggs. $r^2 = 0.000$, $p = 0.973$.
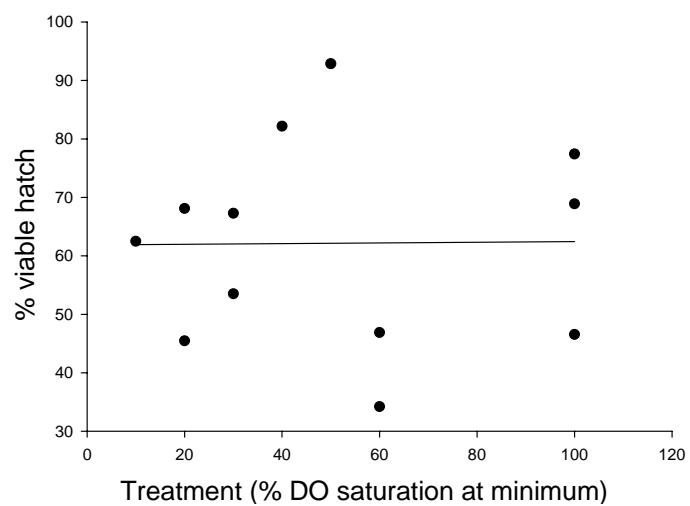
Figure 5.16 Mortality of eggs and larvae in each tank, expressed as a percentage of the total number of eggs. Data excludes tanks with less than 10 eggs. $r^2 = 0.011$, $p = 0.748$.
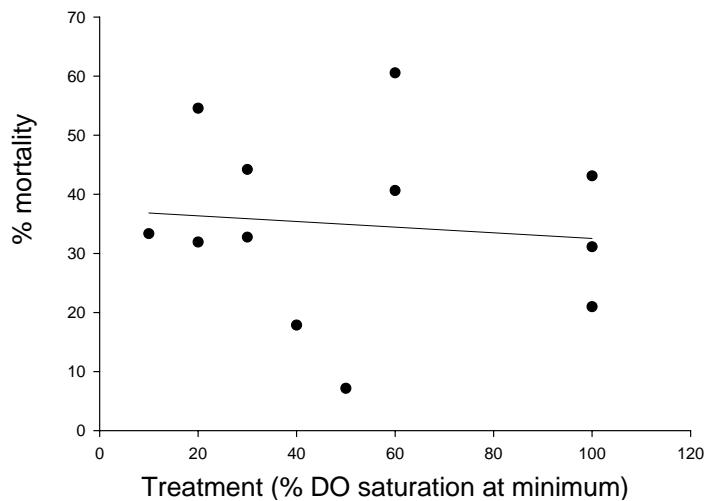
Table 5.13 One-Way ANOVA showing no significant effect of temperature 'treatment' on mortality of eggs and larvae.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	224.744	2	112.372	.442	.656
Within Groups	2289.630	9	254.403		
Total	2514.374	11			

5.4.3   Size of hatching larvae and incidence of deformity

One-Way ANOVA showed no significant difference in larval size between temperature 'treatments' (Table 5.14, p = 0.785). There was no difference in larval size between parental DO treatments when all hatch days were considered (Figure 5.17 A, r^2 = 0.013, p = 0.198). To attempt to remove a time until hatch effect on larval size, individual days were analysed separately. The days analysed were those when most larvae hatched, days 5.5 (142 larvae hatched) and 6.5 (114 larvae hatched). Larval size on day 5.5 was unaffected by DO treatment (Figure 5.17 B, r^2 = 0.002, p = 0.799) or temperature (Table 5.15, p = 0.173). For larvae hatched on day 6.5, there was a significant linear regression relationship between larval size and DO treatment (Figure 5.17 C, r^2 = 0.244, p = 0.001), and no relationship between size and temperature (Table 5.16, p = 0.698).

The percentage of larvae that hatched alive but with deformities was very low, precluding quantitative analysis (Figure 5.18). When the total number of deformed larvae that were collected both dead and alive was considered, there appeared to be a trend for higher rate of deformities in tanks on the lower, cooler shelf (Figure 5.19). This trend was significant according to a One-Way ANOVA (Table 5.17, p = 0.006), and post hoc multiple comparisons showed the difference to be between the lower (coldest) shelf of tanks, and all other tanks. There was no relationship between prevalence of deformities and DO treatment (Figure 5.20, r^2 = 0.014, p = 0.717).

Figure 5.17 Total length of newly hatched larvae (mm). A) all days combined, r^2 = 0.013, p = 0.198; B) Day 5.5 (when 142 larvae hatched), r^2 = 0.002, p = 0.799; C) Day 6.5 (when 113 larvae hatched), r^2 = 0.244, p = 0.001.

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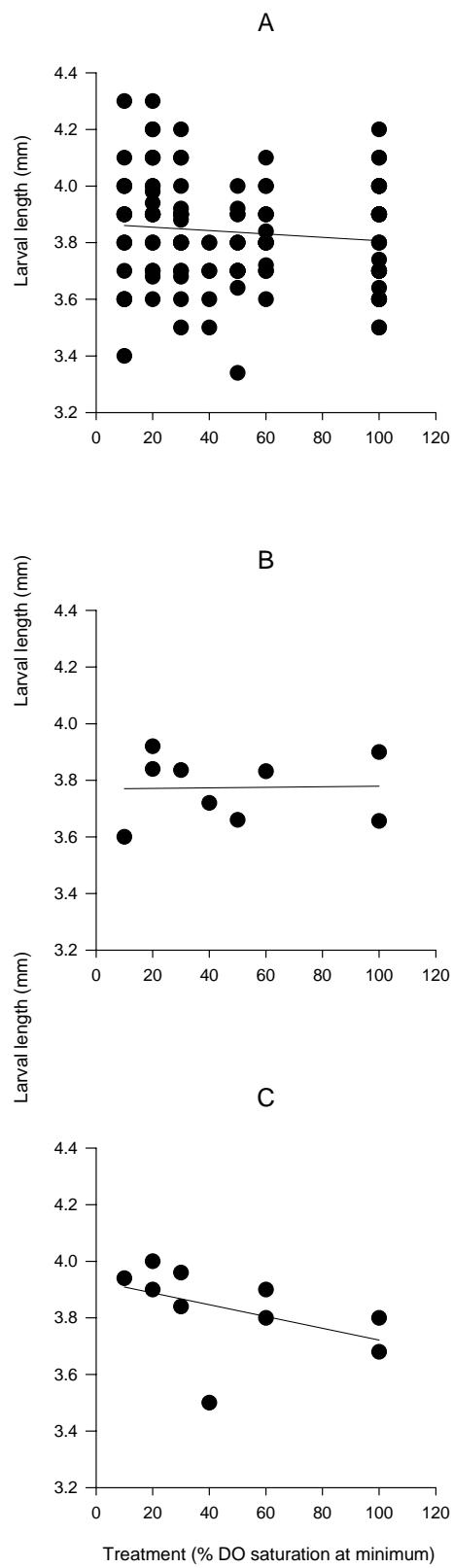
Table 5.14 One-Way ANOVA showing no significant difference in larval size between temperature 'treatments'.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.585E-02	2	7.925E-03	.242	.785
Within Groups	4.226	129	3.276E-02		
Total	4.242	131			

Table 5.15 One-Way ANOVA showing larval size on day 5.5 was unaffected by temperature.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.110	2	5.493E-02	1.837	.173
Within Groups	1.196	40	2.991E-02		
Total	1.306	42			

Table 5.16 One-Way ANOVA showing larval size on day 6.5 was unaffected by temperature.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.930E-02	2	9.649E-03	.364	.698
Within Groups	.955	36	2.653E-02		
Total	.974	38			

Figure 5.18 Larvae that hatched alive with deformities (% hatch in each nursery tank).
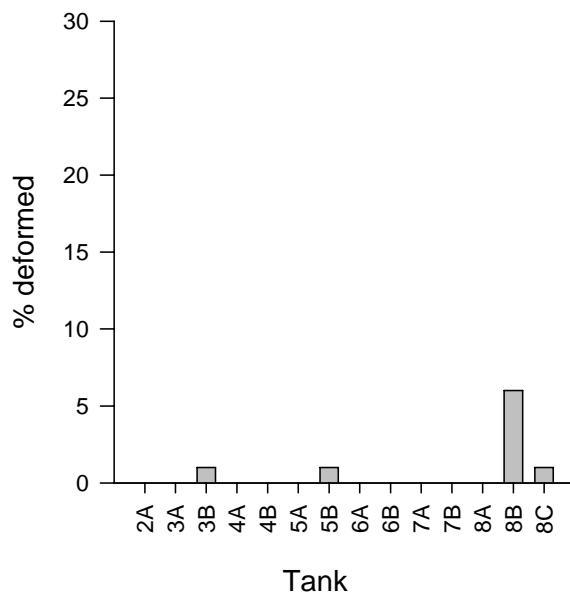
Figure 5.19 Larvae that hatched with deformities including both live and dead larvae (% hatch in each nursery tank). Black arrows indicate tanks that were on the lower shelf, which had slightly cooler temperatures, and showed a trend for a higher incidence of larval deformity.
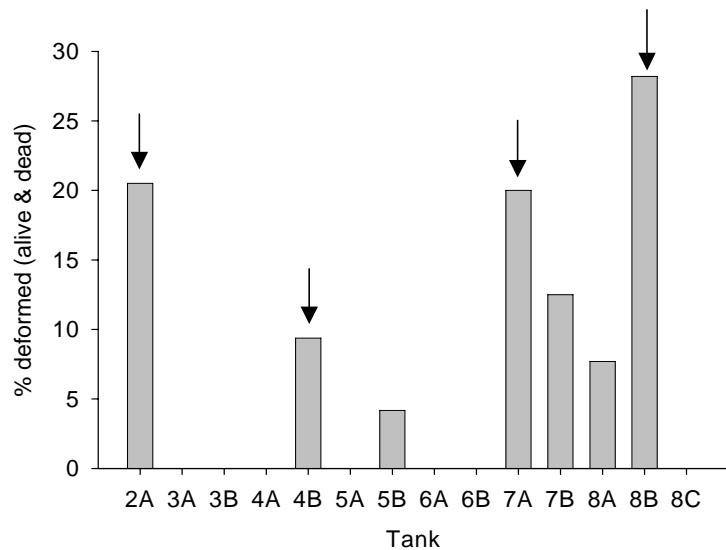
Table 5.17 One-Way ANOVA showing a significant difference in the rate of larval deformity (live and dead larvae) between temperature 'treatments'.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	726.563	2	363.281	9.739	.006
Within Groups	335.716	9	37.302		
Total	1062.279	11			

Figure 5.20 Rate of larval deformity (live and dead larvae). $r^2 = 0.014$, $p = 0.717$.
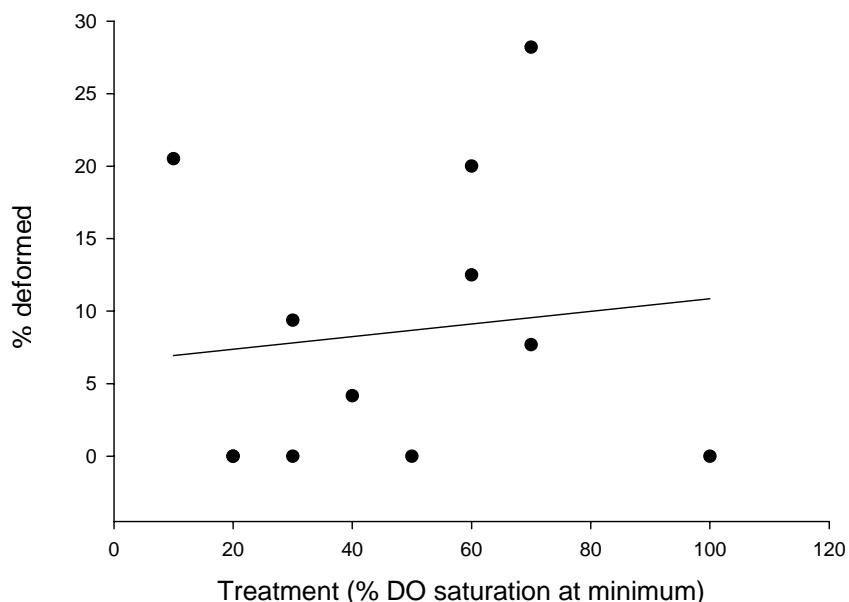
5.5 Discussion

Utchee Creek rainbowfish were surprisingly tolerant to what might be considered to be extremely stressful hypoxia regimes. The immediate lethal level for adults of the species was found to be about 7% DO saturation, when levels were gradually lowered over five hours. This lethal level may be even lower in the field where animals have access to the less hypoxic surface layer and are able to successfully carry out aquatic surface respiration (ASR), which they can readily perform (pers. obs.). In these

experiments although fish could access the surface of the water the nitrogen atmosphere above the surface prevented successful ASR.

The most severe sublethal hypoxia regime tested here reached minimum daily levels of 10% DO saturation, only 3% above the immediate lethal limit. Even under these very strenuous conditions, some fish continued to produce large numbers of viable eggs, and showed only minor signs of an effect on reproductive health. However, one brood group in the lowest treatment did cease to lay eggs on day 18 of cycling to 10% DO, which suggests there may be a negative impact of the treatment on parts of a fish population. In the same tank as egg laying ceased, dissection of gonads showed that one female fish had a very high count of atretic eggs.

Histological analysis of sections of ovaries showed there was a significant difference in total egg count (all types and stages of eggs within the ovary) between the 10% DO treatment and all other treatments. Interestingly, this was a result of the 10% treatment having higher egg counts than other treatments, rather than vice versa.

Thirty percent of the variation in total number of eggs laid over 28 days of the experiment could be explained by hypoxic treatments when fitted with a linear regression, with the lower egg counts found in the more severely hypoxic treatments. The remaining variation represents differences between individuals and brood groups.

Although the number of eggs laid by female rainbowfish appeared to be affected by hypoxia treatments, no such pattern was identified for the number of ovulated eggs (ready to be laid) within dissected ovaries following the experiment. It is possible that the reason for this is that egg-laying behaviour was inhibited, rather than egg production. Although there are no specific studies on the effects of hypoxia on both egg-laying behaviour and ovary condition with which to compare this result, previous studies have identified an effect of hypoxia on various reproductive behaviours (e.g. Jones & Reynolds, 1999c; Reynolds & Jones, 1999; Wu, 2002).

A reduction in courtship behaviour and reproductive success under hypoxia has been documented in a small number of studies. Black crappies (*Pomoxis nigromaculatus*) exposed to two months of fluctuating hypoxia showed later spawning times, although

success of spawning was otherwise unaffected (Siefert & Herman, 1977). In another study, fluctuating oxygen conditions prevented spawning in black crappies in a study by Carlson & Herman (1978). At the end of the test gonads of both male and female fish were similar to control fish, with testes of males containing motile sperm. It was noted, however, that a parasitic infection affecting experimental fish was treated with, among other things, malachite green. Malachite green is a copper derivative, and although it is successful in curing parasitic and fungal infections, copper is known to increase susceptibility to hypoxic conditions (Heath, 1991). It may be for this reason that the experimental fish were slower to spawn than the controls.

In the study presented here, many other parameters of reproductive health, including size of eggs produced, gonadosomatic index, fecundity, egg-type composition of ovaries or cell-type composition of testes, were found to be unaffected by the hypoxia regimes imposed.

The results are contrary to results from field studies do not suggest tolerance to hypoxic stress. In a study of female bluegill (*Lepomis macrochirus*) and longear sunfish (*L. megalotis*) Sabo *et al.* (1998) found dramatic differences between wild-caught fish from normoxic and hypoxic habitats. Fish from normoxic habitats had gonadosomatic indices (ratio of gonad weight to somatic weight) twice as high as fish caught in hypoxic habitats. They also collected spotted sunfish (*L. punctatus*) from both habitats but found no difference in gonadosomatic indices between habitats for this species. Females with yolked eggs were more abundant in normoxic habitats for all three species, but mean number of yolked eggs did not differ between habitats for any species, although smaller eggs were found in fish from hypoxic habitats for all species. The results from this field study suggest that although bluegill and longear sunfish are able to survive under hypoxic conditions, their metabolism is affected enough to impair egg production.

Histopathological effects of hypoxia on fish organs have seldom been reported, and never to my knowledge for gonads. The effects of various pollutants on fish gonads are frequently considered in studies of sublethal exposure – PCB's for example, have been shown to cause fragmentation of developing oocytes in rainbowtrout (*Salmo gairdneri*) and carp (*Cyprinus carpio*) (Sivarajah *et al.*, 1978) and reduced number of

mature eggs in ovaries of zebrafish (*Danio rerio*; Örn *et al*., 1998). The organophosphate, malathion, causes clumping of cytoplasm, degeneration of follicular cells, increased numbers of nucleoli, shrunken nuclear materials and adhesion of oocytes with rupture of follicular epithelium in catfish (*Heteropneustes fossilis*; Dutta *et al*., 1994). Pollutants with the ability to mimic the action of endogenous oestrogen, such as non-ionic surfactants used in cleaning products, paints, herbicides and cosmetics, have the ability to alter the number and size of nutrient cells and germ cells within the testes of male fish (Miles-Richardson *et al*., 1999).

Hussainsagar Lake is a heavily polluted water body in India which is a sink for sewage and untreated industrial effluents and experiences hyper-eutrophication, frequent fish kills, low DO, high nitrates, high mercury levels, poisonous gases and heavy algal blooms. Female *Channa punctatus* collected from the lake showed scattering and vacuolation of oocytes, and cytoplasmic and nucleolar degeneration (Kumari & Kumar, 1997). Textile mill effluents running into the River Tambarparani (India) have high levels of copper, chromium, zinc, sodium, sulphate, chlorides, suspended solids and biological oxygen demand, and *Heteropneustes fossilis* exposed to such effluents experience pathological damage to ovaries in the form of vacuolation of the nucleus and nuclear atrophy (Murugesan & Hannita, 1992). Although in both these examples hypoxia is clearly a problem, the combined effects of various heavy metals and biological toxins are more likely to have caused the observed histopathological changes.

No histopathological changes to reproductive tissues were identified for Utchee Creek rainbowfish exposed to hypoxia in this study. Other studies of environmental stress have had similar negative results in terms of histopathology, including a study on rocky mountain whitefish (*Prosopium williamsoni*) and longnose sucker (*Catostomus catostomus*) from waters with high levels of biologically treated bleached-kraft effluent (Kloepper-Sams *et al*., 1994). Because of the lack of previous research on the subject, it is unclear at this stage whether the lack of histopathological response to hypoxia in this study indicates hypoxia has no effect on gonads of fish, or whether the negative results were simply due to a high tolerance of this particular species to low DO saturations.

The second part of the experiment aimed to determine the effects of parental exposure to fluctuating hypoxia on resulting embryos. No effect of DO treatments on adults was found to carry through to the next generation, and variables including incubation time, size and viability of hatching larvae and incidence of deformities were more affected by slight temperature differences in the nursery tanks. These results are discussed below.

Carp (*Cyprinus carpio*) exposed to chronically low DO saturations of about 12% (1.0 mg/L at 22°C) for 12 weeks during maturation produced eggs with lower viability than fish kept in constantly normoxic (81%; 7 mg/L at 22°C) conditions (Wu *et al*., 2003). Ninety-nine percent of fertilized eggs laid by fish from the normoxic treatment hatched, while only 56% hatched in the hypoxic group. Additionally, larvae from parents kept under normoxia showed 94% survival, while only 46% of larvae from parents exposed to hypoxia survived to 24 hrs post-hatch (Wu *et al*., 2003). Clearly, the results found by Wu *et al*. are not directly comparable to the study presented here, as carp were exposed to chronic hypoxia, while rainbowfish were exposed to diel fluctuations in hypoxia, and carp were exposed for a much longer time period. The ability of Utchee Creek rainbowfish to tolerate fluctuating hypoxia may not extrapolate to chronic hypoxia – only further research on the topic can determine the full extent of the tolerance of the species to hypoxia.

Because of the air-circulation patterns in the aquarium room, and the inability to provide stablising water pumps in tanks containing the delicate rainbowfish larvae, slight differences in temperature were experienced by tanks on shelves at three different heights. Tanks on the bottom set of shelves were coolest at about 26° C. Tanks on the middle shelf averaged approximately 27° C and tanks on the top shelf (about 1.5 metres higher than the bottom shelf) experienced temperatures of about 28° C. These differences in temperature only existed for the second part of this experiment, when water pumps were removed from the tanks to prevent damage to the tiny larvae. During the first half of the experiment, where adults were in the tanks along with water pumps, all tanks averaged 28° C.

The difference in temperature experienced by embryos had a more significant effect on than did the fluctuating hypoxia treatments imposed upon their parents. Eggs in the coolest tanks (26° C 'treatment') were significantly slower to hatch than eggs in the other tanks. There was a trend for increased viable hatch in the warmer tanks, although this was not proven to be statistically significant. There were also increased percentages of deformed larvae in tanks on the cooler shelf.

For other fish species, temperature is known to affect egg health. For striped trumpeter (*Latris lineate*) for example, the optimal temperature during incubation is about 10.5° – 12.3° C. At temperatures higher or lower than this, egg mortality, time to hatch, length of hatched larvae and yolk volume are affected (Bermudes & Ritar, 1999). Rohu (*Labeo rohita*) show decreased hatch survival, increased hatching duration and increased malformation of embryos at temperatures higher than 31° C (Das *et al*., 2006). I have been unable to locate any published studies on the effects of temperature on Australian freshwater fish.

It was very interesting to find that the effect of a couple of degrees temperature had more detrimental effects to the hatching larvae than did the severe hypoxia treatments their parents were exposed to. Apparently there was no impact of the stress caused to parents in this manner on resulting eggs.

Perhaps the reproductive guild each fish species belongs to may hold some answers to their ability to withstand hypoxia. Rainbowfish (family Melanotaeniidae) can be described as non-guarding open substratum spawners, probably most accurately described by the reproductive guild known as phyto-lithophils. Phyto-lithophils deposit their eggs on submergent vegetation or other materials such as rocks or gravel, and hatch with moderately developed embryonic respiratory organs (Balon, 1975).

Unlike phyto-lithophilic larvae, however, newly hatched rainbowfish larvae are phototrophic, and swim at the surface during the day, and on the benthos when they are in darkness (pers. obs.). This trait is more like larvae of fish from the reproductive guild of psammophils, and the phototrophic behaviour is thought to prevent larvae from wandering into crevices where oxygen content may be lower (Balon, 1975).

Phytophils are a more specifically adapted guild of fishes that will spawn only on submergent vegetation. Rainbowfish do preferentially spawn on vegetation, but in the absence of vegetation will spawn on other complex substrates that look nothing like plants, for example plastic mesh placed in an aquarium (pers. obs.). Additionally, and unlike larvae of phytophils, rainbowfish larvae are not known to possess cement glands on their heads as a tool to attach to plants prior to active swimming. Psammophils are thought to be less adapted to hypoxic conditions, whereas phytophils and phyto-lithophils are adapted to survive in habitats with dense plant growth and hence periodically low oxygen concentrations (Balon, 1975).

The fact that rainbowfish lay their eggs on submerged macrophytes does suggest a certain tolerance to hypoxia is necessary, and this has certainly been established through this study, and will be further considered in Chapter 6. Future research may help to classify the group more accurately into a known reproductive guild or as an intermediate group, and help us to understand the specific characteristics of rainbowfish that make them hypoxia-tolerant.

The vital function of reproduction in maintaining populations has been largely ignored in studies to determine the sublethal effects of hypoxia, and it is impossible to gain a true picture of the tolerance of fish populations to this environmental stressor without such knowledge. Whether the capability of Utchee Creek rainbowfish to withstand hypoxia reflects that of other tropical fish, or even of other rainbowfish is unknown. Further research in the field is vital to accurately determine sublethal levels of hypoxia for freshwater fish.

Because this experiment was somewhat confounded by the incidental temperature 'treatments' imposed upon embryos, it would be useful to repeat the experiment to ensure the effects of hypoxia were not missed as a result. What can be gleaned from this experiment is that the temperature is a more important factor to rainbowfish embryos than whether or not their parents were exposed to the stress of fluctuating hypoxia.

CHAPTER SIX: THE EFFECTS OF FLUCTUATING HYPOXIA ON EGG VIABILITY AND LARVAL HEALTH

6.1   Introduction

In recent decades great interest has developed in the conditions required for successful hatching and development of fish embryos and larvae. This research is largely based on commercial interests, as the importance of recruitment to fisheries has been realized, and through the development of aquaculture techniques to achieve maximum survival of cultured species (Blaxter, 1988). Much research has been carried out on salmonid embryos as they are very sensitive to environmental change and are important to commercial and recreational fisheries in the northern hemisphere.

The developing embryo and newly hatched larva are considered the most sensitive stages of the teleost life-cycle with particular sensitivity to environmental change (Rosenthal & Alderdice, 1976; von Westernhagen, 1988). The effects of hypoxia are no exception, with embryos and larvae generally considered to be more vulnerable than older fish to low DO saturations. Mortality due to oxygen deficiency during the early life history of fish has been considered as a possible limiting factor to population size for a number of species, including largemouth bass (*Micropterus salmoides*; Spoor, 1977), naked gobies (*Gobiosoma bosc*; Breitburg, 1992), Baltic cod (*Gadus morhua*; Wieland *et al.*, 1994) and dragonet (*Repomucenus valenciennei*; Ikejima & Shimizu, 1999).

Sublethal exposure to hypoxia during the egg or larval stage can also be detrimental. Hypoxia can retard embryonic development (McDonald & McMahon, 1977; Keckeis *et al.*, 1996), causing delayed hatching (Oseid & Smith, 1971a; Rosenthal & Alderdice, 1976; Giorgi & Congleton, 1984), and smaller size of hatching larvae (Brooke & Colby, 1980; Giorgi & Congleton, 1984); or it can cause premature hatching (Czerkies *et al.*, 2001). Increased occurrence of twinning (polyembryonic fish) has been observed in some species following embryonic exposure to hypoxia (Stockard, 1921 - cited in Stephens, 1973); and other morphological variations and deformities of hatching larvae have been frequently reported (Rombough, 1988). One of the most common embryonic effects of sublethal exposure to environmental stress,

including hypoxia, is injury to the developing vertebral column (Rosenthal & Alderdice, 1976).

Rombough (1988) provides a review of the sublethal effects of hypoxia on fish eggs, and Rosenthal & Adlerdice (1976) and von Westernhagen (1988) reviewed the sublethal effects of various environmental stressors on fish eggs and larvae. Many studies have found that as development proceeds, sensitivity to hypoxia and other stressors increases. The period from fertilization to gastrulation appears to be the most resistant stage of the egg to hypoxia, while hatching is a transitional period where optimal conditions are most critical (Keckeis *et al.*, 1996).

Sensitivity of eggs to hypoxic stress differs not just between development stages, but also between species, even if these species are closely related. For example whitefish (*Coregonus lavaretus*) are less tolerant to hypoxia than the closely related vendace (*C. albula*) (Czerkies *et al*., 2001). Hypoxia tolerance of fish embryos appears to be related to the environmental characteristics of their natural habitat, rather than phylogeny (Rombough, 1988).

There is little published data describing the effects of hypoxia on adult and juvenile Australian freshwater fishes, and there is no published data describing tolerance of their embryos. Since embryos and larval fish are the most sensitive life stage to hypoxia in many temperate species, this type of information may prove imperative in quantifying water quality standards for Australian wetlands. For this reason, I investigated the effects of fluctuating hypoxia on eggs of three species of tropical Australian freshwater fish: Utchee Creek rainbowfish (*Melanotaenia utcheensis*), eastern rainbowfish (*Melanotaenia splendida splendida*) and sooty grunter (*Hephaestus fuliginosus*). I examined effects on incubation time (time from fertilization until hatch), egg and larval mortality, viability of hatching larvae, incidence of deformities and size of larvae upon hatching.

6.2   Methods

6.2.1   *Utchee Creek rainbowfish*

Two groups of mature Utchee Creek rainbowfish were used to produce eggs for this experiment. The groups each consisted of three male and two female rainbowfish, as suggested for maximum egg production in the literature from the aquarium trade (see Chapter 5). These groups were kept in 30 L aquaria with 25 L of water, with submersible pumps for circulation and additional air bubblers (for detailed description of experimental setup see Chapter 2). The fish were fed twice daily, first with Nutrafin commercial flake food as soon as the lights in the aquarium room were switched on, and several hours later with newly hatched live *Artemia* nauplii. Fifty percent of water was exchanged daily. Spawning substrate was renewed daily, and was made up of 50 strands of green polyester wool tied at the top to form a "mop", as described by Humphrey *et al*. (2003) and attached to a piece of floating Styrofoam to prevent sinking.

Each day, the mops were removed from brood stock tanks, eggs were counted, and if there were more than 10 eggs on each single mop they were used for the experiment. If either mop contained less than 10 eggs, neither mop was used in the experiment that day. If more than 10 eggs were counted on both mops, 5 eggs from each mop were then measured (quickly and carefully to prevent damage, and without removal from the mop) using an Olympus objective micrometer under a Nikon stereo dissector. The egged mops were then placed in experimental aquaria, and water quality measures were recorded from both the breeding tanks and the experimental aquaria. Breeding groups were labeled A and B and each breeding group contributed a batch of eggs to each treatment in the experiment, and two batches of eggs to the control aquaria. In this way, there were 14 experimental tanks (within seven treatments) and four control aquaria, allowing differences in egg health due to breeding groups to be identified if present.

Once eggs were placed in the experimental aquaria, diel cycling of DO was started, and continued daily until two days after all eggs had hatched from that mop. The mop was then checked for dead eggs. On hatching, larvae were removed from the

aquarium and euthanased using benzocaine, then checked for abnormalities under the stereo dissector. Five individuals from each batch of hatching larvae were measured using Kincrome vernier calipers, again under the stereo dissector.

6.2.2 Eastern rainbowfish

Adult eastern rainbowfish were wild caught from Leichhardt Creek north of Townsville. The methods for this experiment were as described for Utchee Creek rainbowfish eggs in section 6.2.1 above.

6.2.3 Sooty grunter

This experiment was carried out at QDPI Walkamin Research Station near Atherton, North Queensland. The change of location was necessary as eggs develop quickly and I was unable to transport them back to Townsville post-spawning. Sooty grunter eggs were donated by the Tablelands Fish Stocking Society, and were sourced from one adult female fish collected in Tinaroo Dam and induced by hormone injection 24 hrs prior to spawn. I collected eggs 3.5 hrs post-spawn and transported them in an aerated container to the laboratory. Eggs were subsampled and counts estimated, to allow volumetric measurement of approximately 800 eggs into each experimental tank. A sample of eggs was taken and diameters measured using a stereo dissector with calibrated eyepiece. Tanks for this experiment were made from 2 L plastic softdrink bottles, from which the bottom had been removed, and an airstone placed in the lid, with a tube leading to the manifold containing a mix of industrial nitrogen and compressed air. Bottles were held upside down (open end upwards) in PVC stands. Eggs were subjected to one round of oxygen depletion (treatments are described in Chapter 2), beginning at 4 hrs post-spawn (at the blastodisc stage), and continuing for 5 hrs as in other experiments. Due to the use of smaller containers, it was necessary to use a mixture of nitrogen and air for oxygen depletion, rather than nitrogen gas alone. After the 5 hr dissolved oxygen depletion cycle, tanks were switched to compressed air for the remainder of the experiment. Larvae were removed as they hatched, and counted, checked for deformities and total lengths measured of five fish per hatching episode. Hatched larvae were euthanased in benzocaine before measuring.

6.3   Results for Utchee Creek rainbowfish

6.3.1   Number and size of eggs

The number of eggs placed in each tank varied from 20 to 95, depending on the eggs laid by brood stock groups each day (Table 6.1). Therefore, much of the following data are presented as percentages, rather than numbers of eggs. Initial size of eggs averaged 0.81 mm (range: 0.70 – 0.90, standard deviation: 0.05).

Table 6.1 Number of eggs placed in each experiment tank from two brood groups to determine the effects of fluctuating hypoxia on Utchee Creek rainbowfish and eastern rainbowfish.

Treatment (DO saturation at minimum)	Utchee Creek rainbowfish		Eastern rainbowfish	
	Brood group A	Brood group B	Brood group A	Brood group B
5%	23 (1A)	21 (1B)	21 (1A)	34 (1B)
10%	20 (2A)	60 (2B)	14 (2A)	36 (2B)
20%	30 (3A)	95 (3B)	35 (3A)	24 (3B)
30%	79 (4A)	45 (4B)	20 (4A)	39 (4B)
40%	21 (5A)	25 (5B)	35 (5A)	42 (5B)
50%	38 (6A)	20 (6B)	48 (6A)	20 (6B)
60%	31 (7A)	36 (7B)	27 (7A)	38 (7B)
100%	47 (8A)	29 (8B)	34 (8A)	24 (8B)
100%	20 (8C)	24 (8D)	21 (8C)	57 (8D)

6.3.2   Time to hatch

Larvae were collected twice daily from the experiment tanks, at 7.00 a.m. and 6.00 p.m., so time to hatch is presented in days and half-days. Time until hatch began in the evening, when eggs were collected from brood stock tanks, such that full days represent evening collections and half days represent morning collections (i.e., eggs hatching on the morning of the sixth day of the experiment took 5.5 d to hatch). No effect of hypoxia treatments on time to hatch was identified. Time until hatch of all Utchee Creek rainbowfish eggs is shown in Figure 6.1. There was no relationship between hypoxia treatment and day of first hatch, day of last hatch, or day of greatest hatch (Figure 6.2).

The highest numbers of hatching larvae were collected on mornings 5.5 (184 eggs over all tanks) and 6.5 (141 eggs) (Figure 6.3).

Figure 6.1 Time to hatch of Utchee Creek rainbowfish eggs. Days when hatching occurred in each tank are represented by squares. Lines connect consecutive days of hatching. Treatments of each tank were: 1A & 1B = 5% minimum DO saturation; 2A & 2B = 10%; 3A & 3B = 20%; 4A & 4B = 30%; 5A & 5B = 40%; 6A & 6B = 50%; 7A & 7B = 60%; 8A − 8D = 100% (controls).
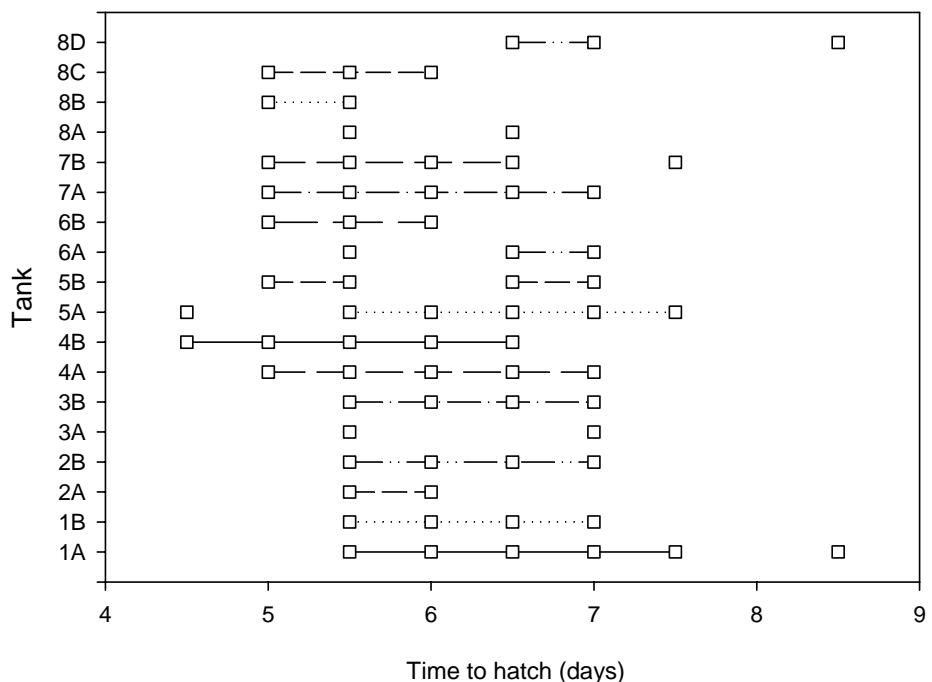
6.3.3   Hatch success and mortality

Hatch success was variable, but success did not appear to be related to hypoxia treatments. Viable hatch rate (% of larvae hatched alive with no deformities) was unaffected by hypoxia (Figure 6.4, r^2 = 0.109, p = 0.181).   The lowest viable hatch rate was exhibited by tank 7A, with 9.7% hatch success, while the highest viable hatch was recorded in tank 4B at 94% (mean = 59%, standard deviation = 25%). Likewise, mortality rates of eggs and hatching larvae was unaffected by hypoxia (Figure 6.5, r^2 = 0.103, p = 0.195). Dead eggs were identified by their milky colouration. Mortality rates were high, and ranged from 7% to 84%.

Figure 6.2 Relationship between hypoxia treatment and day of first hatch (A, r^2 = 0.004, p = 0.798), final day of hatch (B, r^2 = 0.046, p = 0.395) and day of most hatching (C, r^2 = 0.153, p = 0.109) for Utchee Creek rainbowfish eggs.
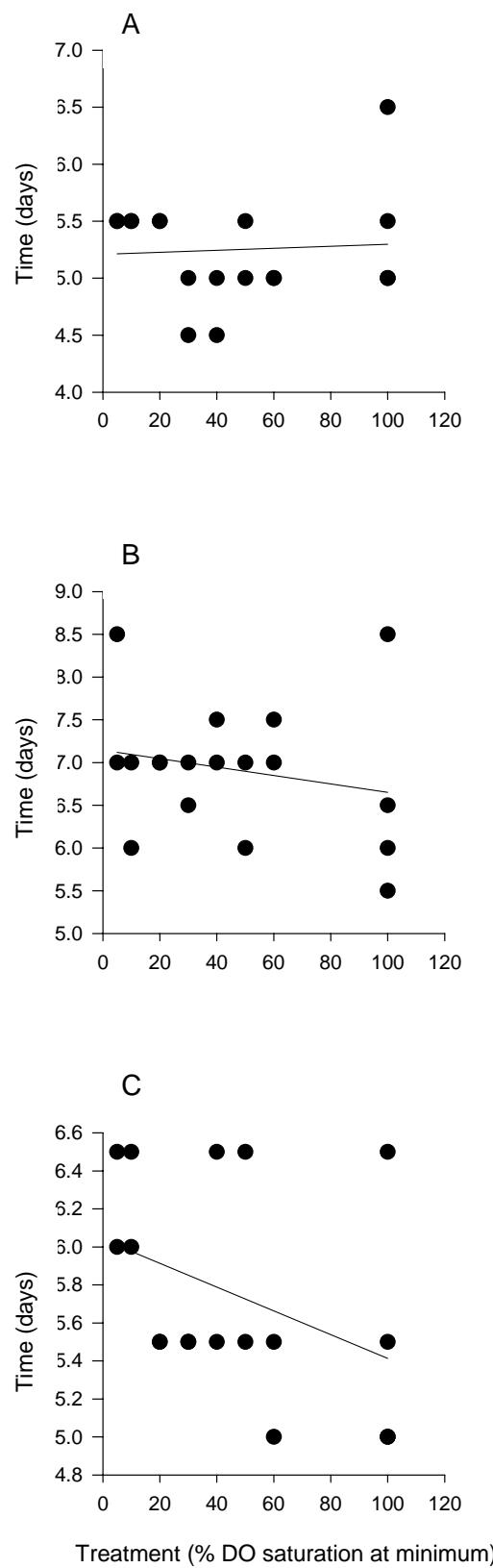
Figure 6.3 Number of Utchee Creek rainbowfish larvae that hatched on each day post-spawn in all tanks. Eggs were collected from spawning adults in the evening, and thus half days represent morning collections of larvae and whole days represent evening collections.
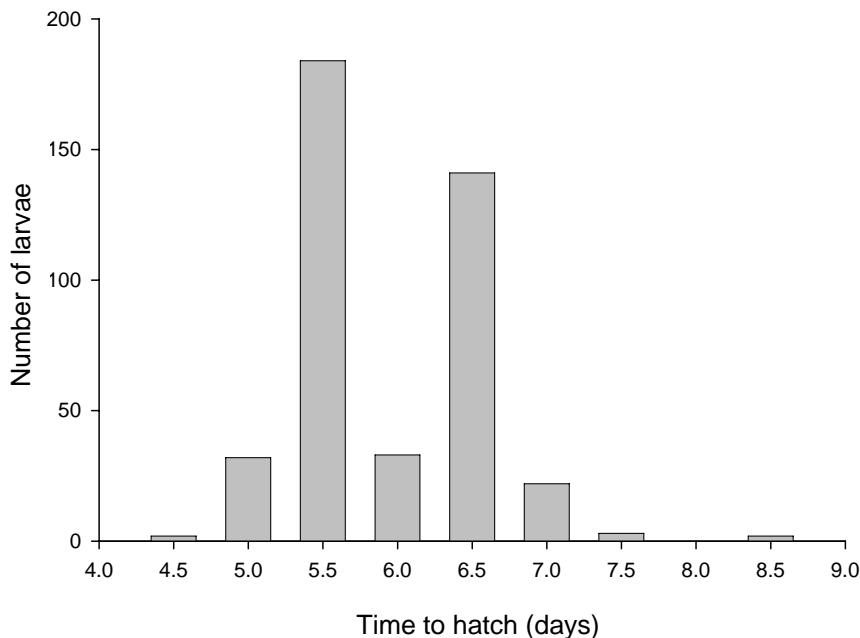
Figure 6.4 Percentage of Utchee Creek rainbowfish larvae that hatched from eggs alive and without deformity (viable hatch). $r^2 = 0.109$, p = 0.181.
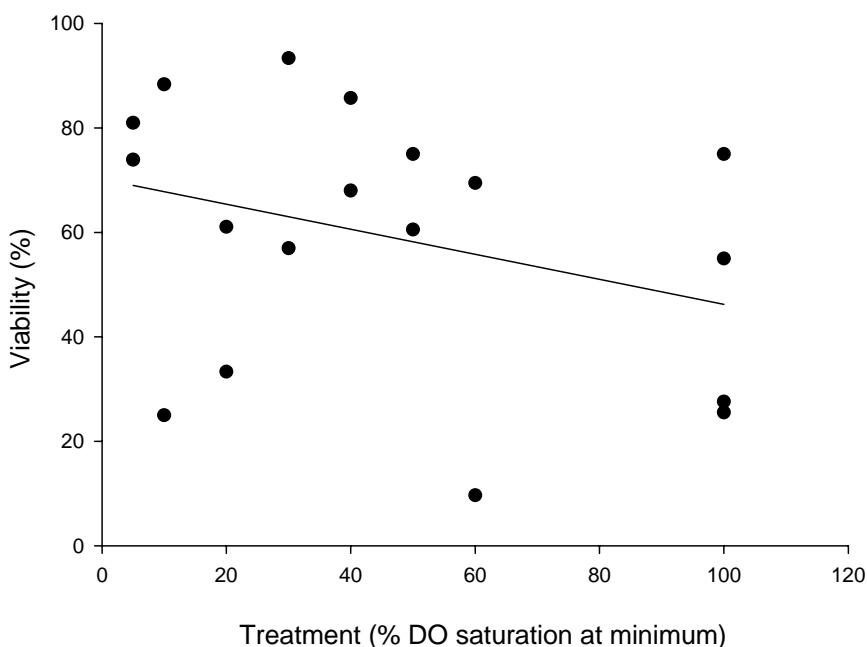
Figure 6.5 Percent mortality of Utchee Creek rainbowfish eggs and larvae and each tank. $r^2 = 0.103$, p = 0.195.
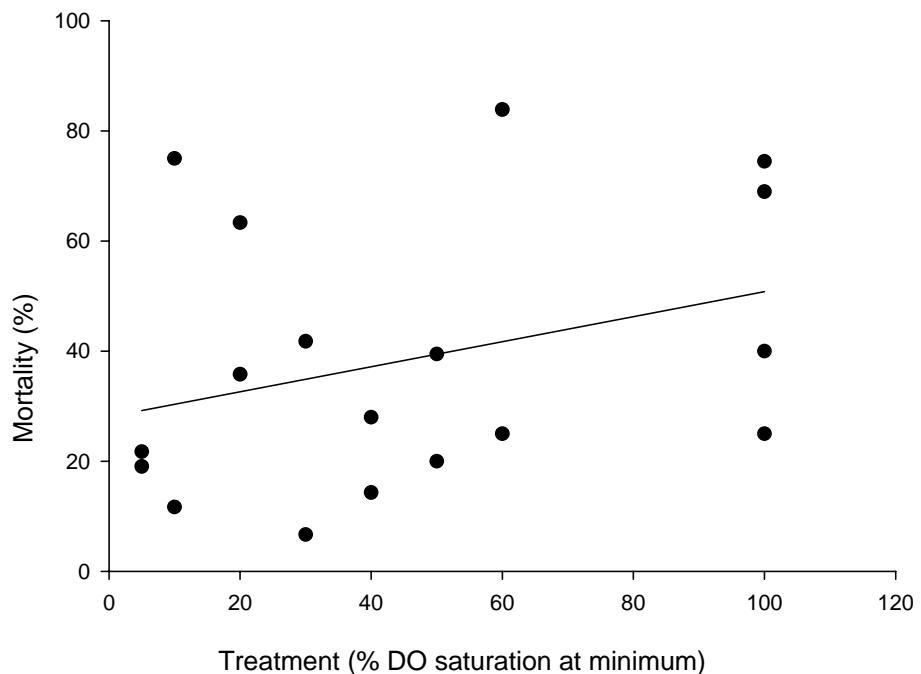
6.3.4   Size of hatching larvae and incidence of deformity

There was no difference in larval size between DO treatments when all hatch days were considered (Figure 6.6 A, $r^2 = 0.000$, p = 0.912). To attempt to remove a time until hatch effect on larval size, individual days were analysed separately. The days analysed were those when most larvae hatched, days 5.5 (184 larvae hatched) and 6.5 (141 larvae hatched). Larval size on day 5.5 was unaffected by DO treatment (Figure 6.6 B, $r^2 = 0.003$, p = 0.690), as was larval size on day 6.5 (Figure 6.6 C, $r^2 = 0.005$, p = 0.622).

Few live deformed larvae were recorded for any tanks in this experiment, and there was no effect of hypoxia treatment on deformity rates (Figure 6.7, $r^2 = 0.032$, p = 0.476). Although larvae in 10 of the 18 tanks showed some level of deformity, all had less than 7% deformity rates. Deformities included fish with spinal curves that were generally alive on collection, and under-developed larvae that died during or immediately after hatching. There was no trend for increased deformity rates with decreasing DO saturation at minimum.

Figure 6.6 Total length (mm) of Utchee Creek rainbowfish larvae at hatch. A) all days combined, r^2 = 0.000, p = 0.912; B) Day 5.5 (when 184 larvae hatched), r^2 = 0.003, p = 0.690; C) Day 6.5 (when 141 larvae hatched), r^2 = 0.005, p = 0.622.
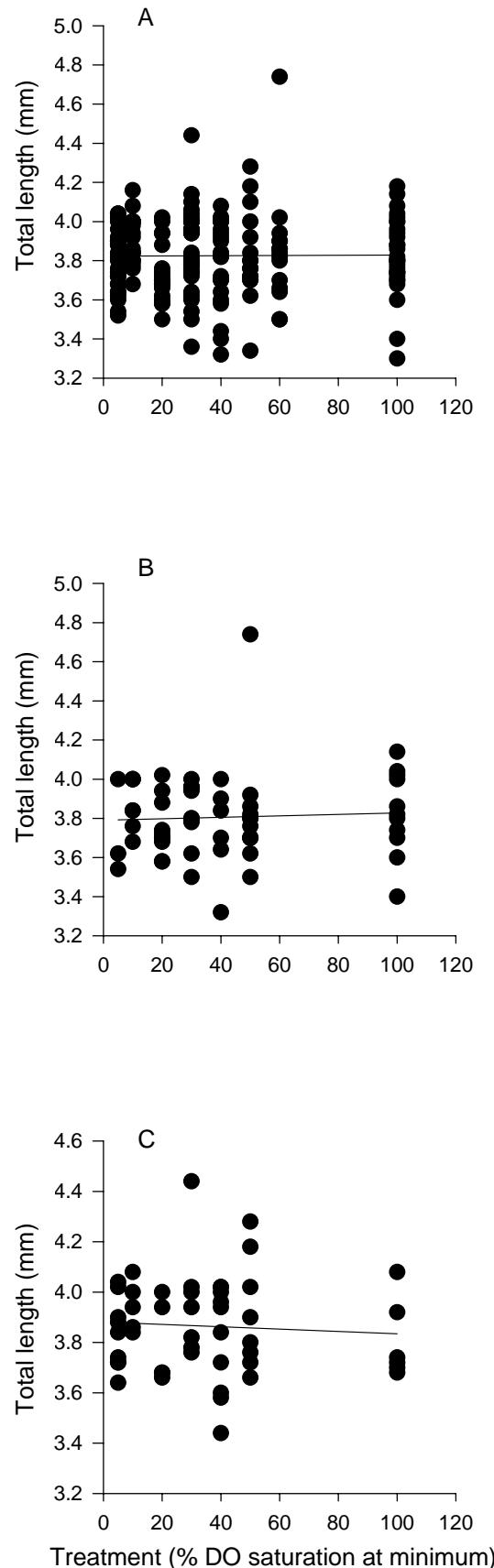
Figure 6.7 Percentage of Utchee Creek rainbowfish larvae that hatched with deformities (found either alive or dead). $r^2 = 0.032$, p = 0.476.
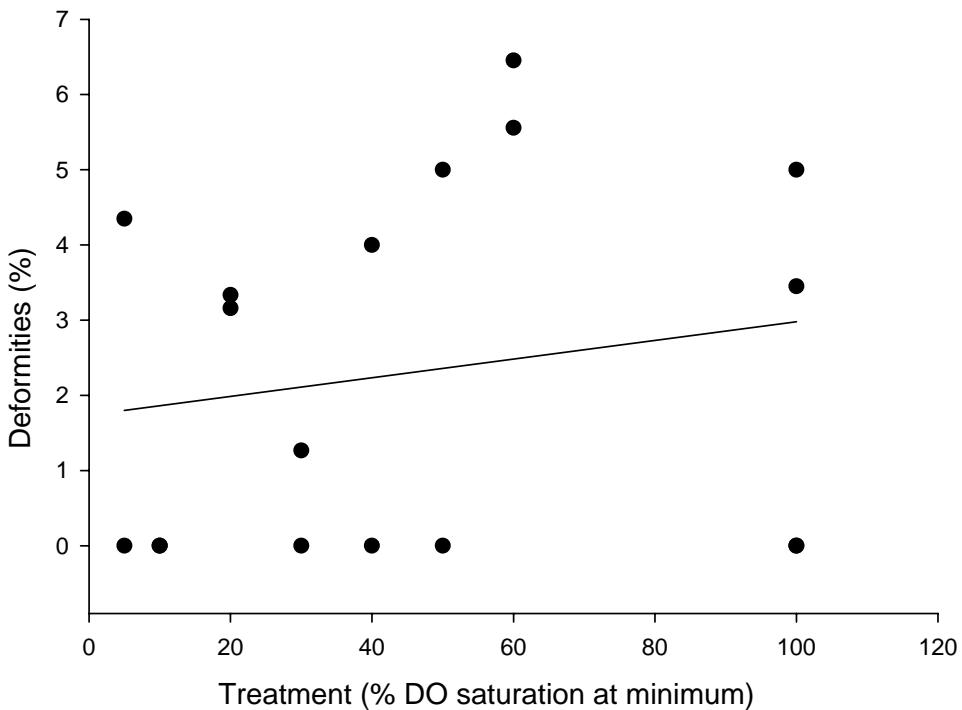
6.4   Results for eastern rainbowfish

6.4.1   Number and size of eggs

For the eastern rainbowfish egg experiment, number of eggs placed in each tank varied from 14 to 47, depending on the eggs laid by brood stock groups each day (Table 6.1). Thus, much of the following data are presented as percentages, rather than numbers of eggs. Initial size of eggs averaged 0.9 mm (range: 0.75 – 1.05, standard deviation: 0.06).

6.4.2   Time to hatch

Eastern rainbowfish larvae were collected twice daily from the experiment tanks at 7.00 a.m. and 6.00 p.m., so time to hatch is presented in days and half-days as for the Utchee Creek rainbowfish. Time until hatch began in the evening, when eggs were

collected from brood stock tanks, such that full days represent evening collections and half days represent morning collections (i.e., eggs hatching on the morning of the sixth day of the experiment took 5.5 d to hatch). No effect of hypoxia treatments on time until hatch was identified. Time until hatch of eastern rainbowfish eggs is shown in Figure 6.8. There was no relationship between hypoxia treatment and day of first hatch, day of last hatch, or day of greatest hatch (Figure 6.9). The greatest hatch in all tanks occurred on day 5.5.

Most hatching occurred at 5.5 d post-spawn (430 eggs), followed by 6 d post-spawn (95 eggs) (Figure 6.10).

Figure 6.8 Time to hatch of eastern rainbowfish eggs. Days when hatching occurred in each tank are represented by squares. Lines connect consecutive days of hatching. Treatments of each tank were: 1A & 1B = 5% minimum DO saturation; 2A & 2B = 10%; 3A & 3B = 20%; 4A & 4B = 30%; 5A & 5B = 40%; 6A & 6B = 50%; 7A & 7B = 60%; 8A − 8D = 100% (controls).
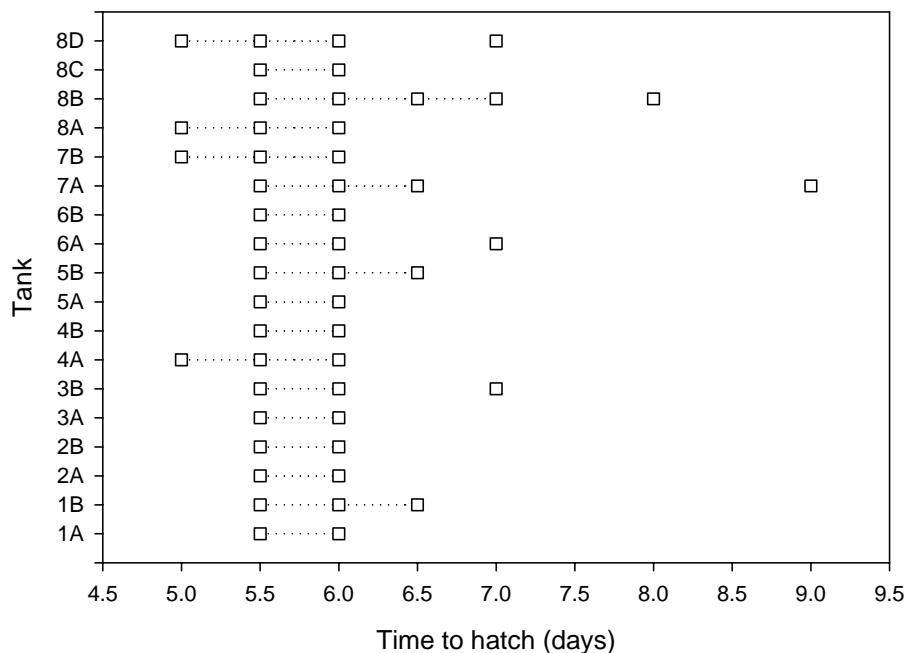
Figure 6.9 Relationship between hypoxia treatment and day of first hatch (A, $r^2 =$ 0.179, p = 0.080), final day of hatch (B, r^2 = 0.098, p = 0.207) or day of most hatching (C – same for all tanks – statistics cannot be computed) for eastern rainbowfish eggs.
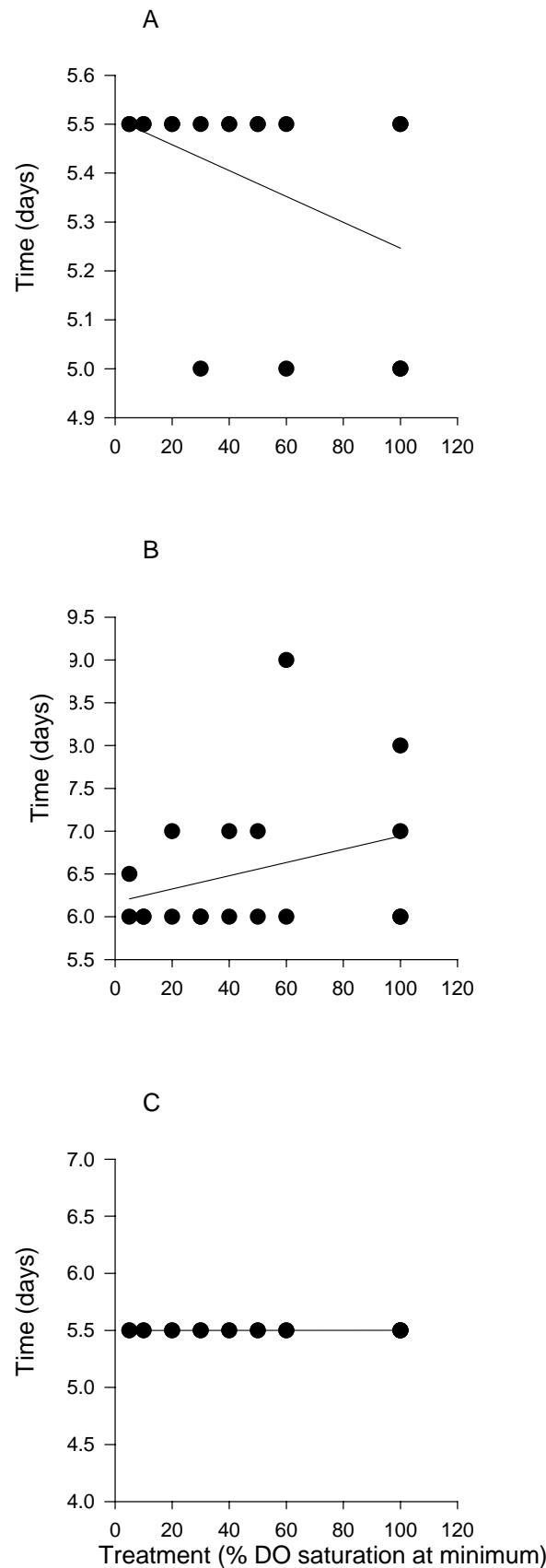
Figure 6.10 Number of eastern rainbowfish larvae that hatched on each day post-spawn. Eggs were collected from spawning adults in the evening, and thus half days represent morning collections of larvae and whole days represent evening collections.
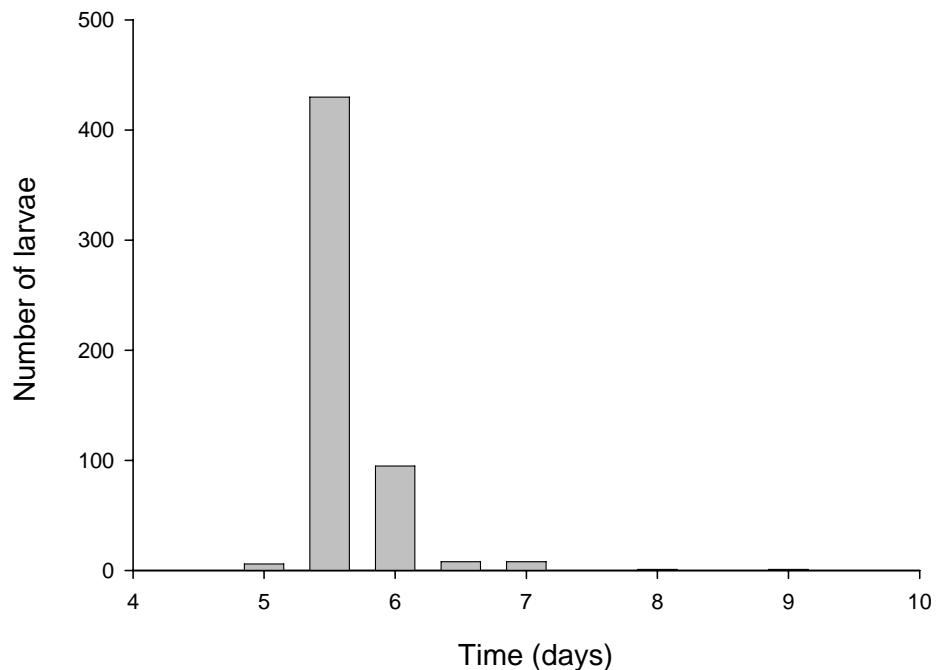
6.4.3   Hatch success and mortality

Eastern rainbowfish eggs in all treatments had high hatch success. The proportion of eggs resulting in a viable hatch, or in live, undeformed larvae was not decreased by increasingly severe hypoxia treatments, and viability was high in all treatments. There was a significant relationship between increasing viability and decreasing oxygen levels, although this relationship was not supported by the coefficient of determination (r^2) which was low, indicating a limited ability of the independent variable (treatment) to predict the dependent variable (viability) (Figure 6.11, $r^2 = 0.245$, $p = 0.021$). The lowest viable hatch rate was exhibited by tank 8B, one of the control tanks, with 79% hatch success. 100% hatch success was recorded for tanks 1A (5% DO minimum) and 2A (10% DO minimum). Dead eggs were identified by their milky colouration. The percentage mortality of eggs and hatching larvae was unaffected by hypoxia at the $p < 0.05$ level, and the fit of the relationship was poor as described by the coefficient of determination (Figure 6.12, $r^2 = 0.157$, $p = 0.058$). The

highest mortality rates were exhibited by two control tanks, 8B and 8C, at 21% and 19% mortality respectively.

Figure 6.11 Percentage of eastern rainbowfish larvae that hatched alive and without deformity (viable hatch). $r^2 = 0.245$, $p = 0.021$.
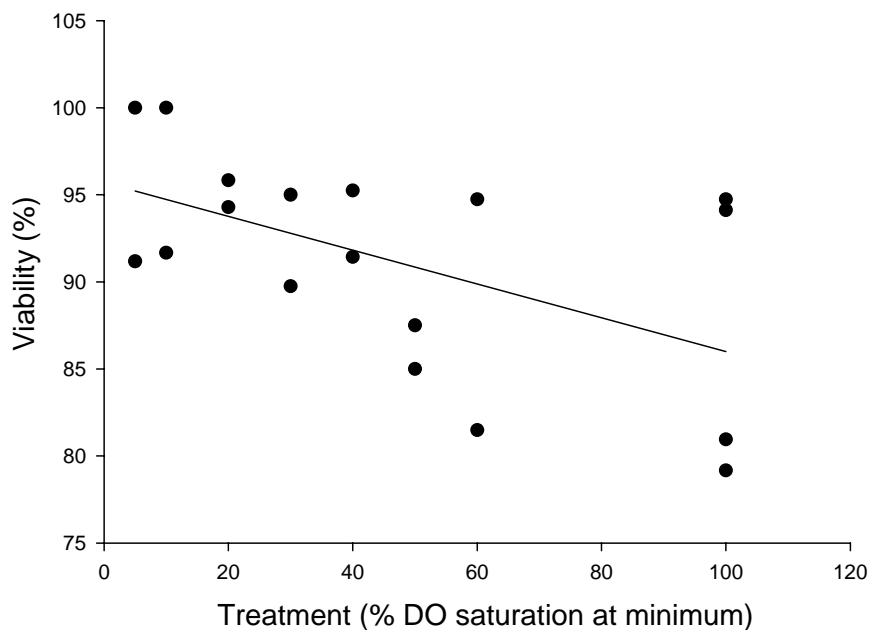
Figure 6.12 Percent mortality of eastern rainbowfish eggs and larvae in each tank. Treatments are as described in Figure 6.7. $r^2 = 0.157$, $p = 0.058$.
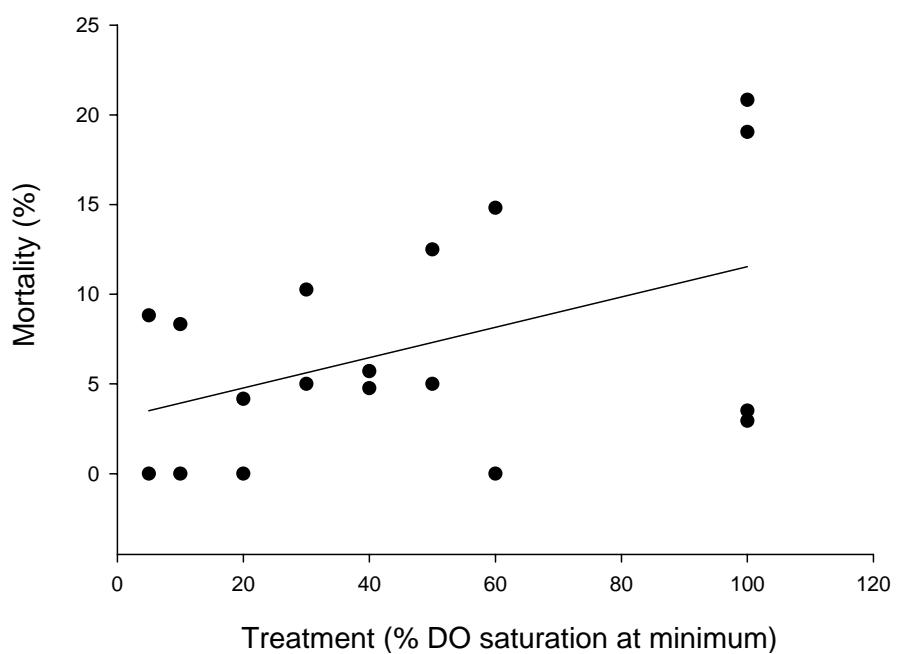
6.4.4   Size of hatching larvae and incidence of deformities

There was no difference in larval size between DO treatments when all hatch days were considered (Figure 6.13 A, $r^2 = 0.000$, p = 0.809). To attempt to remove a time until hatch effect on larval size, individual days were analysed separately. The days analysed were those when most larvae hatched, days 5.5 (430 larvae hatched) and 6.0 (95 larvae hatched). Larval size on day 5.5 was unaffected by DO treatment (Figure 6.13 B, $r^2 = 0.001$, p = 0.761), as was larval size on day 6.0 (Figure 6.13 C, $r^2 = 0.012$, p = 0.417).

Few live deformed larvae were recorded for any tanks in this experiment, and there was no effect of hypoxia treatment on deformity rates (Figure 6.14, $r^2 = 0.015$, p = 0.628). Larvae in 7 of the 18 tanks showed some level of deformity, all had less than 6% deformity rates. Deformities included fish with spinal curves that were generally alive on collection, and under-developed larvae that frequently died during or immediately after hatching. There was no trend for increased deformity rates with decreasing DO saturation at minimum.

Figure 6.13 Total length (mm) of eastern rainbowfish larvae at hatching. A) all days combined, $r^2 = 0.000$, p = 0.809.; B) Day 5.5 (when 430 larvae hatched), $r^2 = 0.001$, p = 0.761; C) Day 6 (when 95 larvae hatched) , $r^2 = 0.012$, p = 0.417.
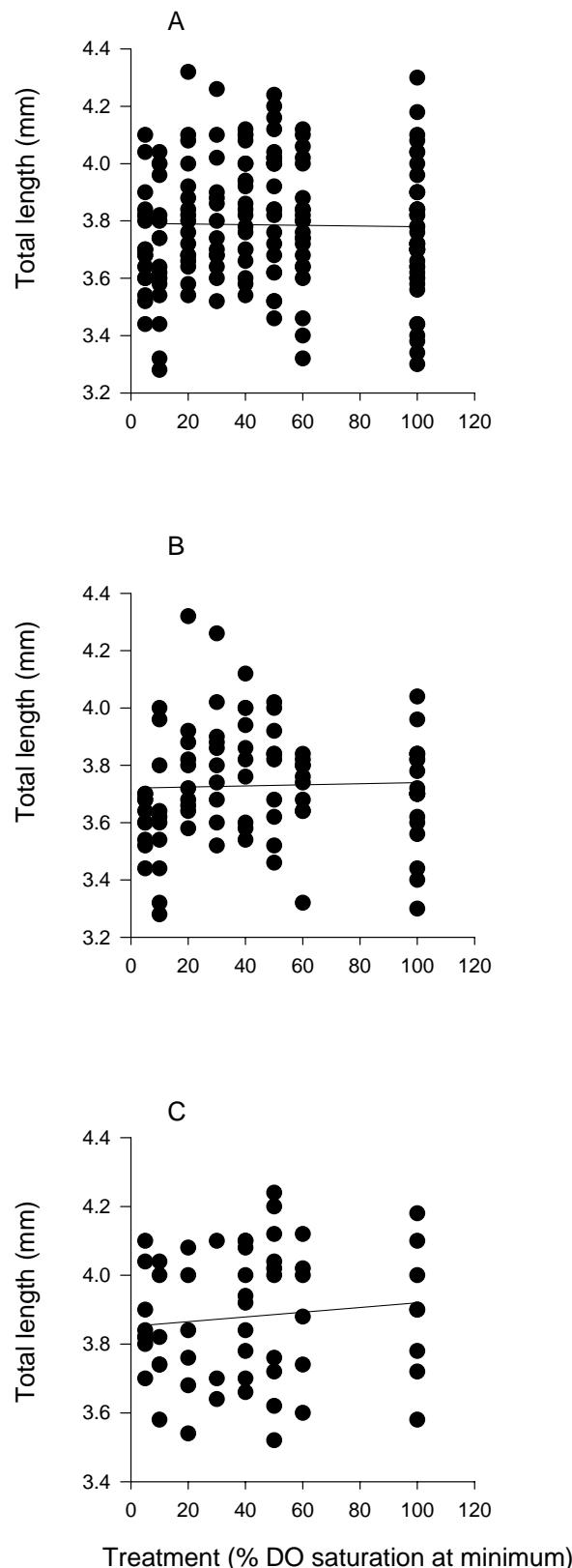
Figure 6.14 Percentage of eastern rainbowfish larvae that hatched alive, with deformities. $r^2 = 0.015$, p = 0.628.
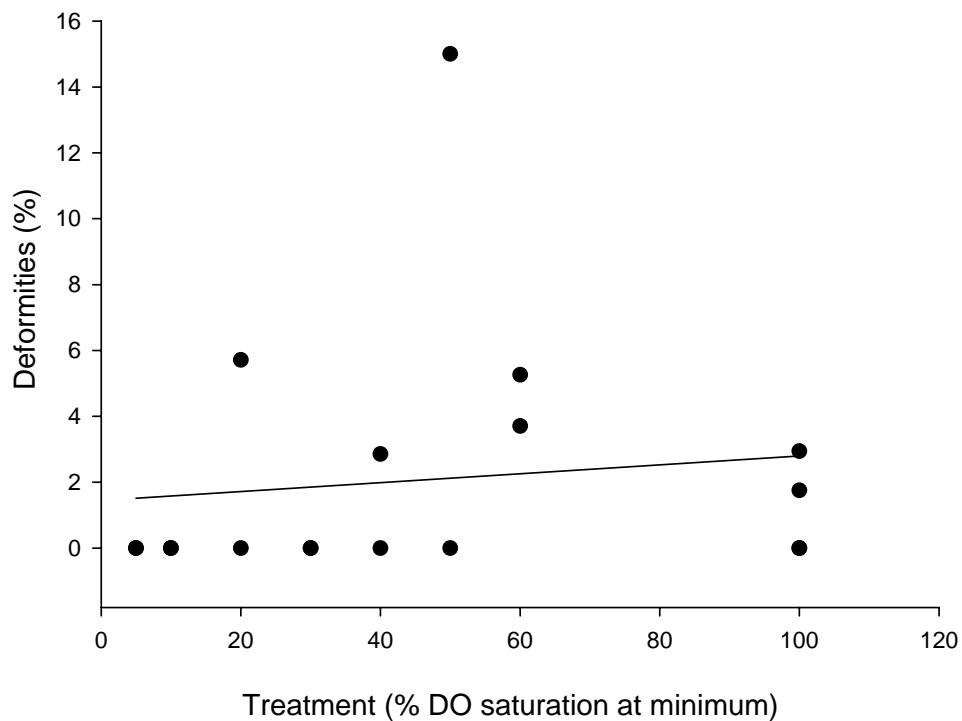
6.5   Sooty grunter

6.5.1   Number and size of eggs.

Sooty grunter eggs used in this experiment were collected from one spawning of a single wild caught female fish that had been chemically induced 24 hrs before. The number of eggs placed in each experimental tank was determined volumetrically, with subsample counts. Approximately 800 eggs were placed in each tank. Initial average diameter of eggs was 2.24 mm (n = 10, range = 1.92 - 2.4, standard deviation = 0.16).

6.5.2   Time to hatch

Hatching began in experimental tanks at 40 hrs and continued until 152.5 hrs after spawning. Figure 6.15 displays all times that larvae were collected from tanks. No difference in hatch times is visible for different treatments. In Figure 6.16 major hatch

times of all tanks are shown, when >20% of total hatching occurred for each tank. There was no relationship between treatment and time of first hatch (Figure 6.17 A, r^2 = 0.012, p = 0.667), or the day that most hatching occurred (Figure 6.17 C, r^2 = 0.174, p = 0.085). There was a linear relationship between treatment and final day of hatch (i.e. time until hatch completion) at the p < 0.05 level, although the coefficient of determination (r^2) was not very high, indicating the relationship was not very strong (Figure 6.17 B, r^2 = 0.280, p =0.024).

Most hatching occurred between 80 and 110 hrs post-spawn (Figure 6.18). The collection time when most hatched larvae were collected was at 107 hrs post-spawn (546 eggs). These eggs had hatched in the 11.5 hours since the previous collection time of 95.5 hrs post-spawn.

Figure 6.15 Time to hatch of sooty grunter eggs. Times when hatching occurred in each tank are represented by squares. Treatments of each tank were: 1A & 1B = 5% minimum DO saturation; 2A & 2B = 10%; 3A & 3B = 20%; 4A & 4B = 30%; 5A & 5B = 40%; 6A & 6B = 50%; 7A & 7B = 60&; 8A − 8D = 100% (controls).
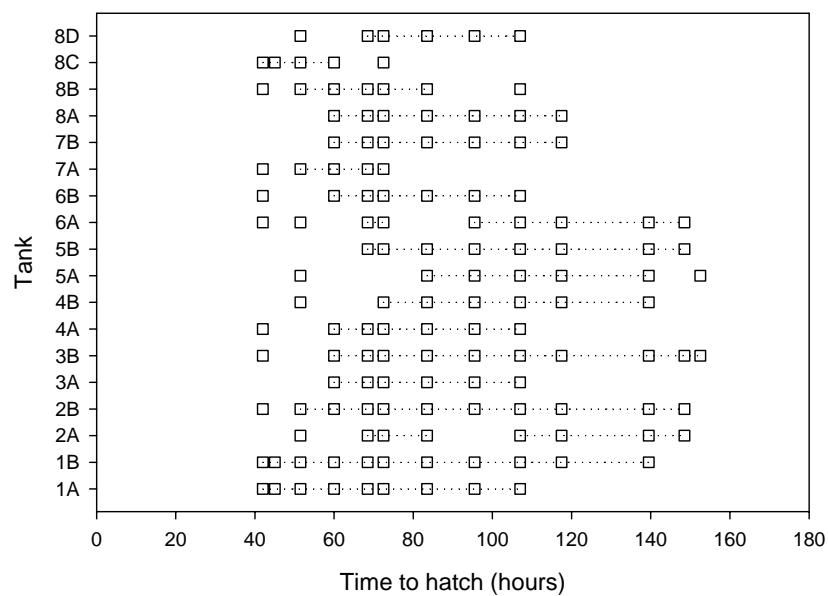
Figure 6.16 Major hatching periods of sooty grunter eggs. Points indicate >20% of total hatching occurred at that time. Treatments in each tank are as for Figure 6.15 above.
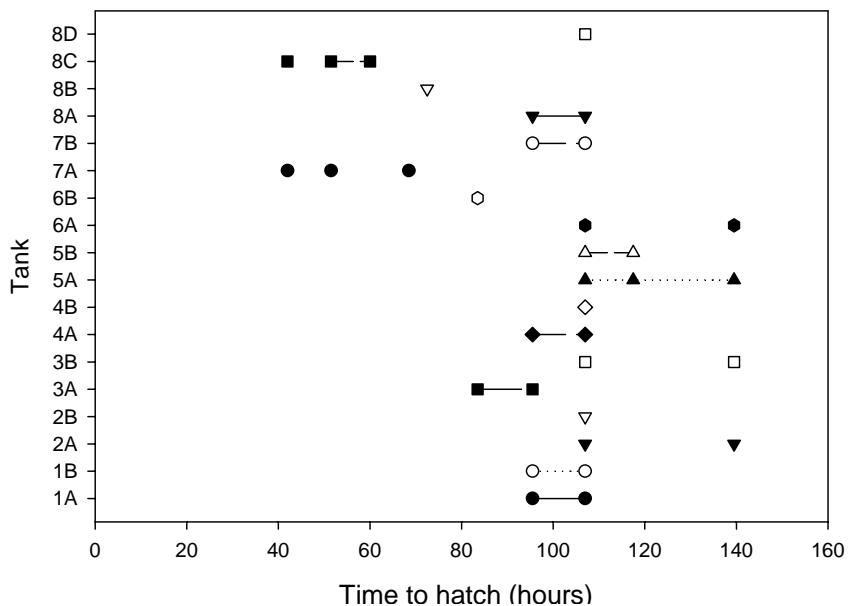
6.5.3 Hatch success and mortality

Percent of viable hatch (larvae that were collected alive and with no deformities) was low for all tanks (Figure 6.19, $r^2 = 0.004$, $p = 0.812$). Less than 20% egg survival was recorded for all tanks. The minimum number of viable larvae was 9 (1.125% hatch success, tank 7A), and the maximum was 152 (19% hatch success, tank 7B). Average number of eggs to hatch to live larvae for all tanks was 83 (10.3% hatch success), with a standard deviation of 5.3%. There was no apparent pattern in hatch success compared to DO treatment. Correspondingly, mortality of eggs and larvae was high in all tanks (Figure 6.20, $r^2 = 0.010$, $p = 0.690$).

6.5.4 Size of hatching larvae and incidence of deformity

There was no difference in larval size between DO treatments when all hatch days were considered (Figure 6.21 A, $r^2 = 0.005$, $p = 0.171$). To attempt to remove a time until hatch effect on larval size, individual collection times were analysed separately. The times analysed were those when most hatching occurred, at 107 hrs post-hatch (546 larvae hatched) and 95.5 hrs post-hatch (353 larvae hatched). Larval size was

unaffected by DO treatment for both collection times (Figure 6.21 B, $r^2 = 0.008$, p = 0.475 and Figure 6.21 C, $r^2 = 0.000$, p = 0.906).

When hatch size is considered in relation to incubation time, every individual tank shows a graphical trend of increasing larval size with increasing time to hatch (Figure 6.22). Many, but not all of these relationships are statistically significant linear regressions (Table 6.2). When lengths of all larvae measured are graphed against incubation time (Figure 6.23) there is evidence of a convincing trend for exponential increase in size with increased incubation time (f=a*(1-exp(-*x)) where a = 5.858 and b = 0.026; $r^2 = 0.587$, p < 0.001). Similar trends were not identified for either rainbowfish species.

The percentage of live larvae hatching with deformities (including underdeveloped embryos hatching prematurely and larvae with spinal cord deformation) was low, and showed no pattern that related to treatments (Figure 6.24, $r^2 = 0.203$, p = 0.061).

Figure 6.17 Relationship between hypoxia treatment and time of first hatch (A, r^2 = 0.012, p = 0.667), final day of hatch (B, r^2 = 0.280, p = 0.024) or day of most hatching (C, r^2 = 0.174, p = 0.085) for sooty grunter eggs.

Treatment (% DO saturation at minimum)
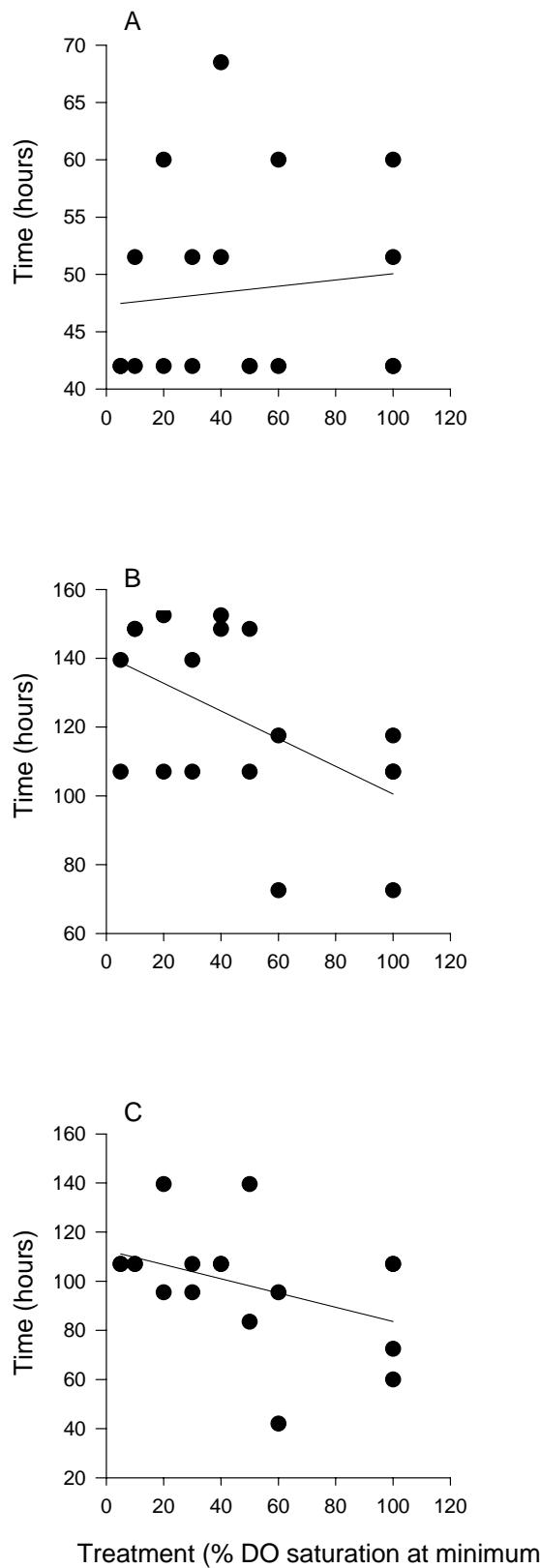
Figure 6.18 Number of sooty grunter larvae that were found to have hatched at each collection time. Larvae were collected at 10-12 hour intervals.
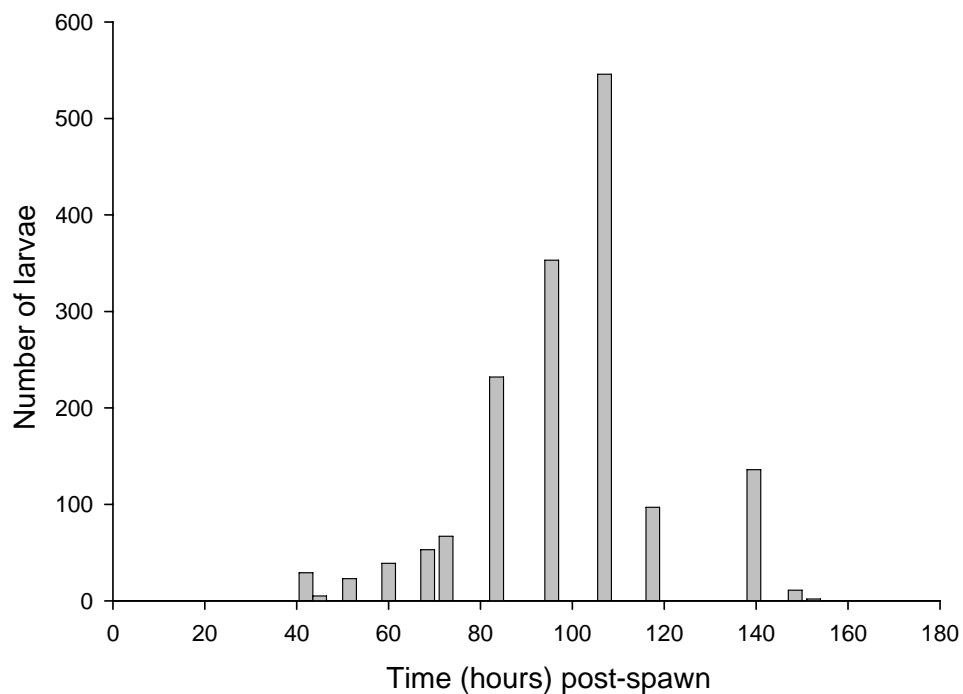
Figure 6.19 Percentage of sooty grunter larvae that hatched alive and without deformity (viable hatch). $r^2 = 0.004$, $p = 0.812$.
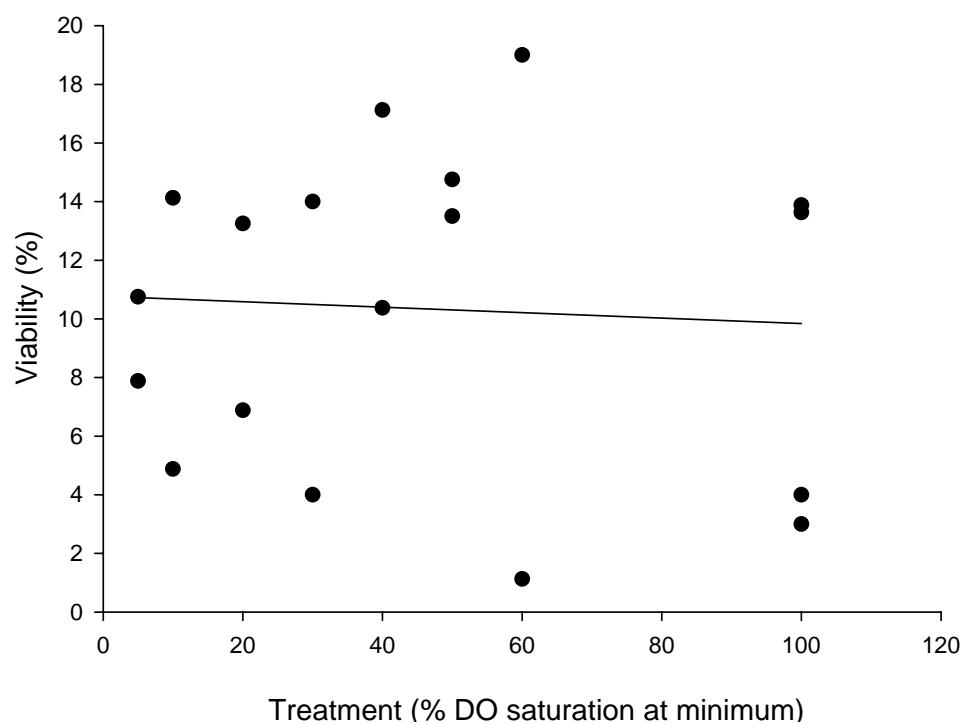
Figure 6.20 Percent mortality of sooty grunter eggs and larvae in each tank. $r^2 =$ 0.010, p = 0.690.
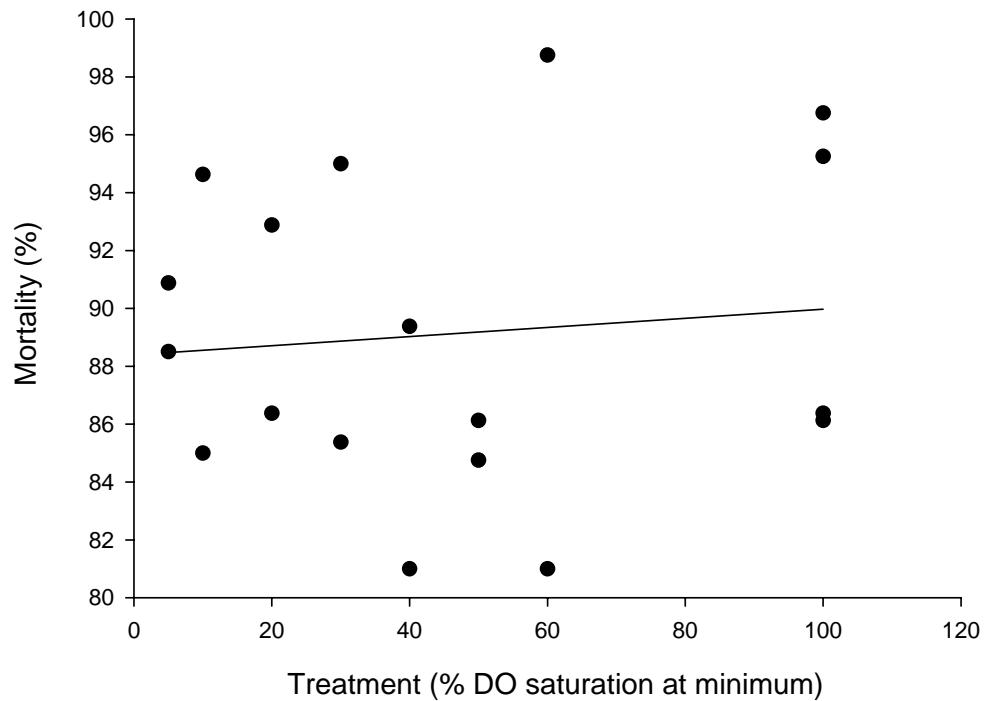
Figure 6.21 Total length (mm) of sooty grunter larvae at hatching. A) all collection times combined, r^2 = 0.005, p = 0.171; B) collection at 107 hr post-spawn (when 546 eggs hatched) r^2 = 0.008, p = 0.475; C) collection at 95.5 hr post-spawn (when 353 eggs hatched) , r^2 = 0.000, p = 0.906.
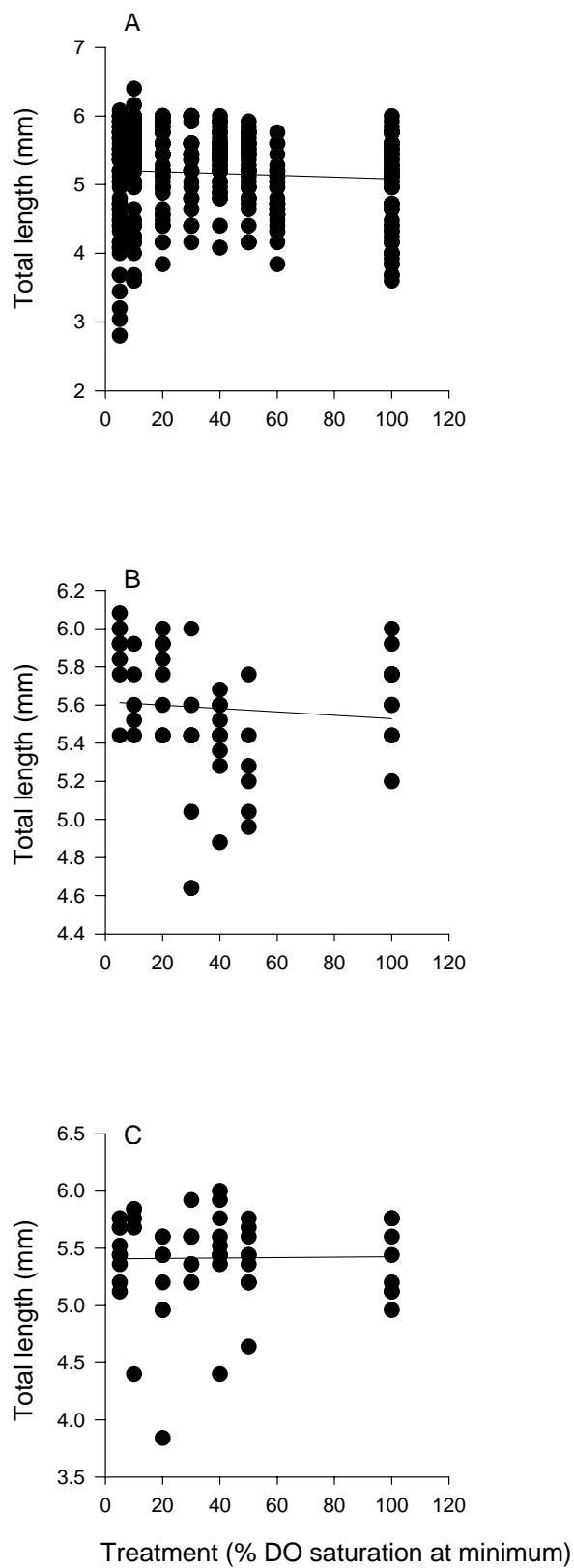
Figure 6.22 Relationship between incubation time and size of hatching sooty grunter level for each tank. Results of linear regression analyses are given in Table 6.2 below.
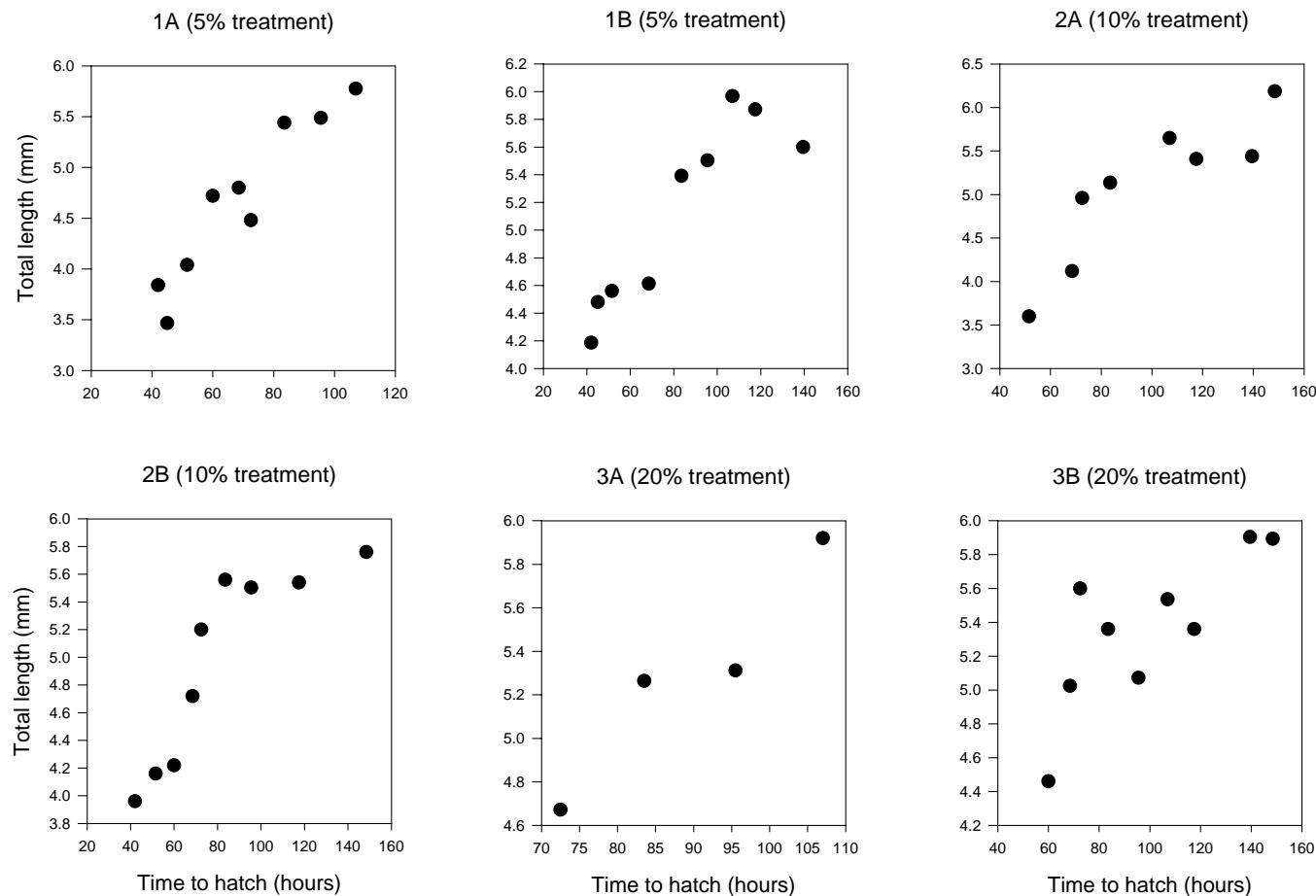
Figure 6.22 Relationship between incubation time and size of hatching sooty grunter level for each tank (continued).
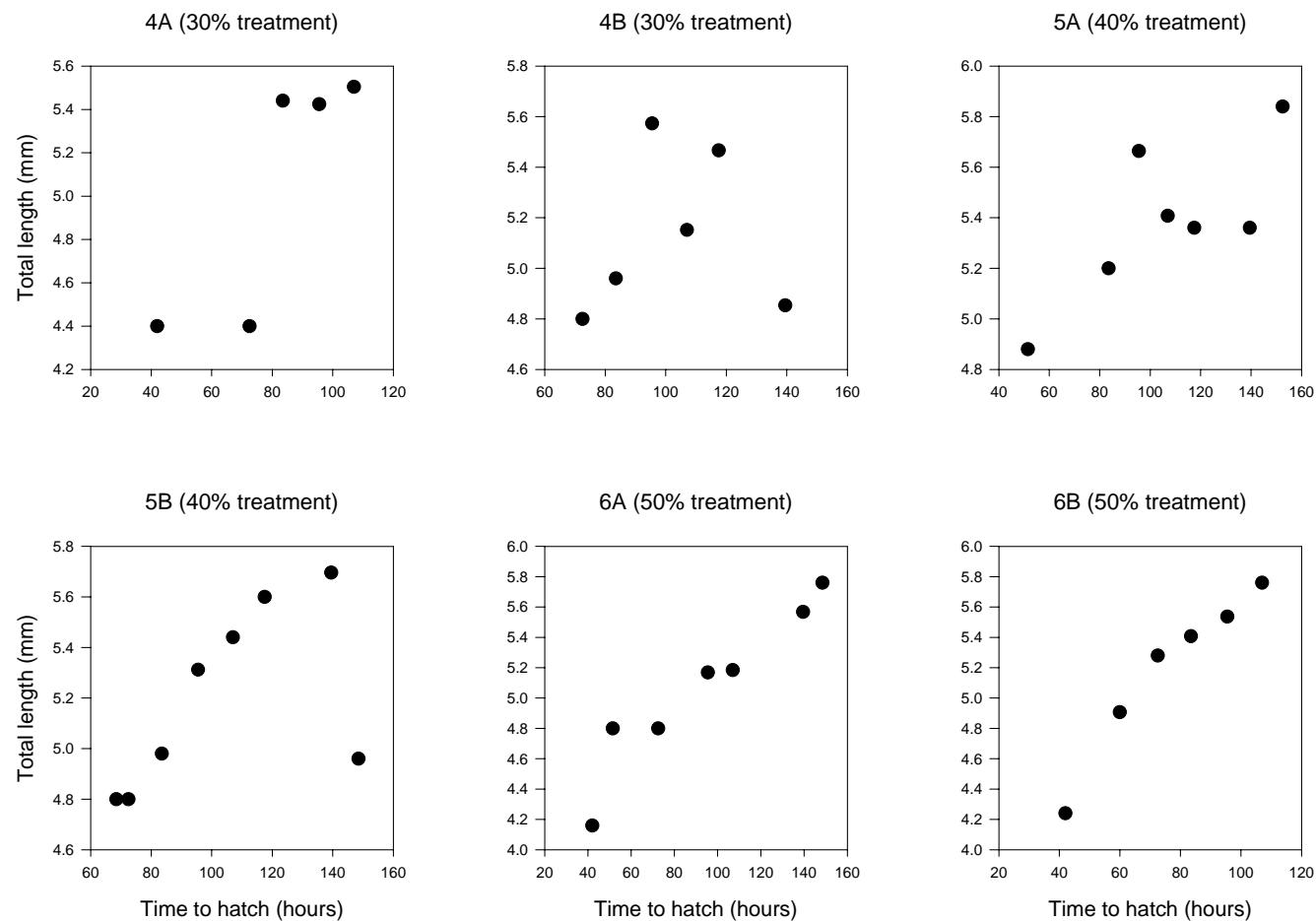
Figure 6.22 Relationship between incubation time and size of hatching sooty grunter level for each tank (continued).
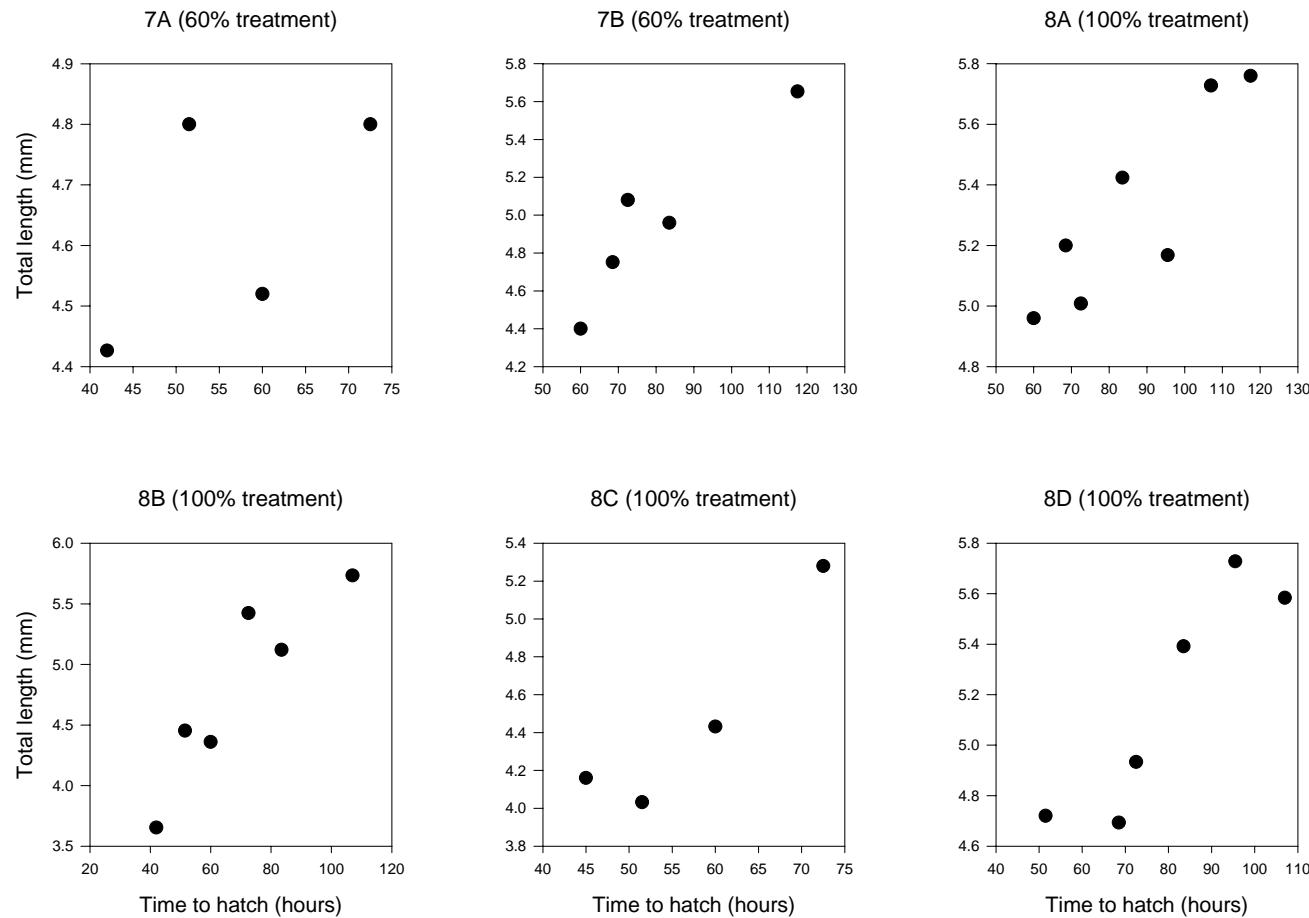
Table 6.2 Results of linear regression analyses to test for relationship between treatment and incubation time. Graphical results are shown in Figure 6.22 above.

	Treatment (% DO saturation at minimum)	Degrees of Freedom	r^2 value	p value (* = significant at p < 0.05 level)
Tank 1A	5%	8	0.898	0.000*
Tank 1B	5%	8	0.813	0.001*
Tank 2A	10%	7	0.780	0.004*
Tank 2B	10%	8	0.744	0.003*
Tank 3A	20%	3	0.915	0.044*
Tank 3B	20%	8	0.610	0.013*
Tank 4A	30%	4	0.721	0.069
Tank 4B	30%	5	0.016	0.812
Tank 5A	40%	6	0.395	0.181
Tank 5B	40%	7	0.338	0.131
Tank 6A	50%	6	0.913	0.001*
Tank 6B	50%	5	0.937	0.002*
Tank 7A	60%	3	0.351	0.407
Tank 7B	60%	4	0.875	0.019*
Tank 8A	100%	6	0.774	0.009*
Tank 8B	100%	5	0.834	0.011*
Tank 8C	100%	3	0.861	0.072
Tank 8D	100%	5	0.821	0.013*

Figure 6.23 Relationship between incubation time and total length of sooty grunter larvae. $r^2 = 0.587$, $p < 0.001$; $f = a*(1-exp(-b*x))$ where $a = 5.858$ and $b = 0.026$.
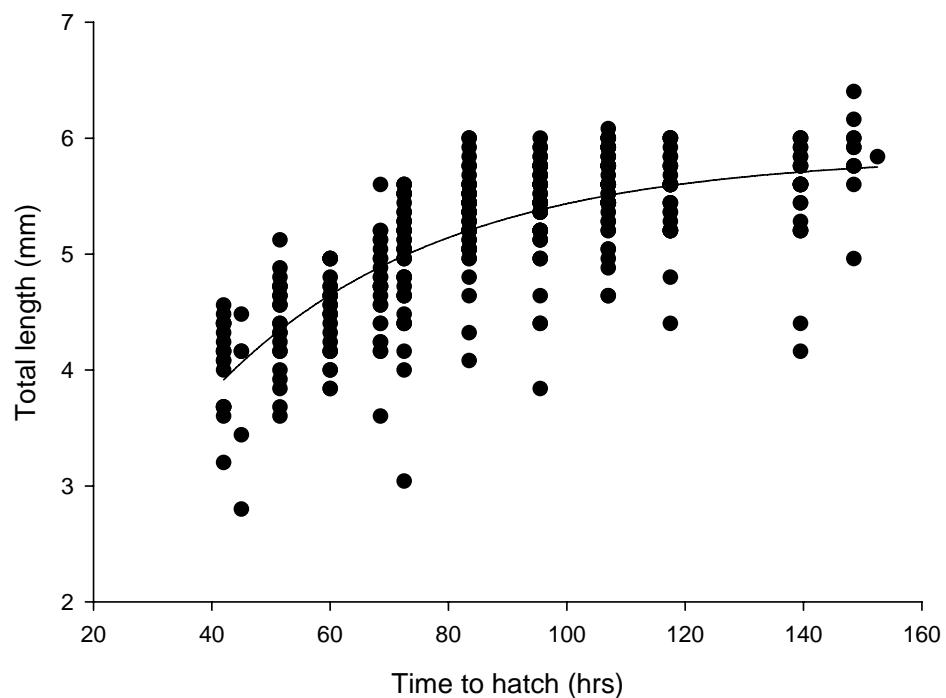
Figure 6.24 Percentage of sooty grunter larvae that hatched alive, with deformities. r^2 = 0.203, p = 0.061.
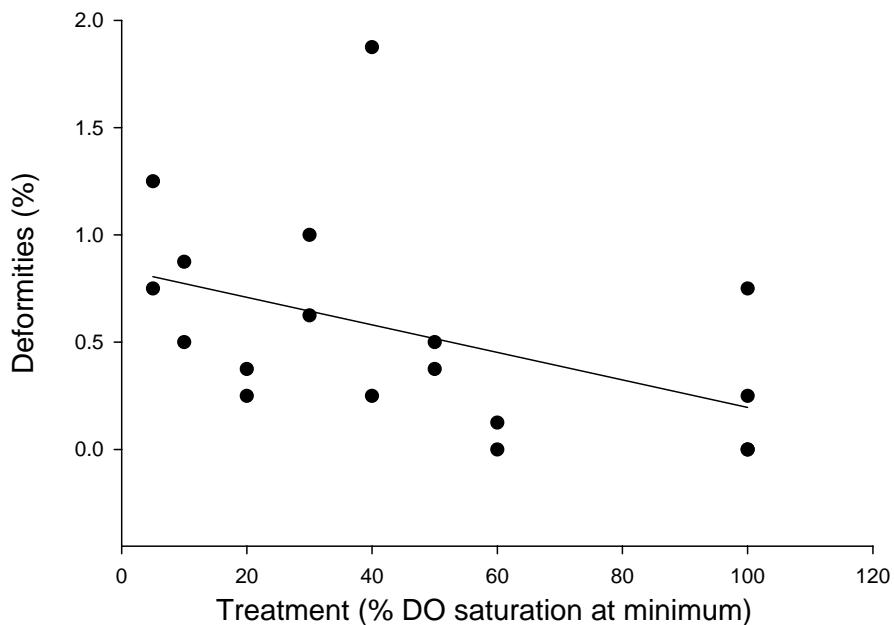
6.6 Discussion

After considering factors such as incubation time, egg and larval mortality, viability of hatching larvae, incidence of deformities and size of hatching larvae, I was unable to identify any strong changes in these parameters under different oxygen conditions for any of the three species tested.

The experiment on sooty grunter eggs showed very high egg mortality (>80% in all treatments) and quite a high rate of larval deformities (up to 20% of live hatched larvae), with no suggestion of higher mortality or deformities in treatments with lower dissolved oxygen minima. The fact that there was no difference between treatments in this experiment cannot be considered a significant result due to the mass mortality in eggs that was experienced. Usually when spawned artificially, sooty grunter eggs show a high survival to hatch, and eggs are discarded by hatchery personnel if pre-hatch mortality is greater than fifty percent (Hogan, 1990). The reason for the poor survival rate in this case is unknown, but may have been caused by insufficient fertilization by male sooty grunters, poor health of the female sooty grunter, or

external factors such as water quality during spawning. The eggs were all sourced from a single female sooty grunter, which would have reduced variation, but may also have detrimentally affected the experiment. It should be mentioned that remaining eggs in the batch from which I collected my sample also showed very high mortality, and were discarded by the stocking group prior to hatch. To determine the effects of fluctuating hypoxia on sooty grunter eggs, this experiment would need to be repeated. Unfortunately I was unable to repeat the experiment in the time available, as I could not source another batch of sooty grunter eggs.

There was an indication that increased incubation time of sooty grunter eggs resulted in larger larvae. Once again, these results need to be treated with caution due to the high mortality rates experienced. No relationship between incubation time and length at hatch was not found in the rainbowfish experiments.

The experiments on eastern rainbowfish and Utchee Creek rainbowfish eggs resulted in the conclusion that embryos of this species are very tolerant to fluctuating hypoxia to a minimum of 5% DO saturation. No examples of the effects of fluctuating hypoxia could be found in the literature. However, in chronic exposure experiments lethal levels for fish embryos a number of studies on cold water or temperate fish species have been carried out. For example, the lethal level for Baltic cod (*Gadus morhua*) embryos is 16% DO saturation (2 mg/L at 7°C) (Wieland *et al*., 1994); for sea trout (*Salmo trutta*) embryos 53 – 57% (6.9 mg/L at 4 – 7°C) (Ingendahl, 2001); 30% (4 mg/L at 4°C) for lake herring (*Coregonus artedii*; Brooke & Colby, 1980); for steelhead trout (*Salmo gairdneri gairdneri*) 14% (1.6 mg/L at 9.5°C) (Silver *et al*., 1963); for chinook salmon (*Oncorhynchus tshawytscha*) 15% (1.6 mg/L at 11°C) (Silver *et al*., 1963); and for nase (*Chondrostoma nasus*) embryos (after gastrulation) 10% DO saturation (1 mg/L at 16°C) (Keckeis *et al*., 1996).

It is surprising that eggs of the two rainbowfish species were able to survive unaffected to such low DO concentrations, particularly as in other experiments juveniles of eastern rainbowfish and adults of Utchee Creek rainbowfish were found to die when exposed to <7% dissolved oxygen (Chapters 3 and 5). Early life stages, including embryos and larvae of most fish species are considered less tolerant to poor

conditions than juvenile and adult stages (Rosenthal & Alderdice, 1976; von Westernhagen, 1988). This is apparently not the case for rainbowfish eggs under hypoxia, which suggests there may be some remarkable physiological adaptations of the embryos to withstand hypoxic stress.

Oxygen requirements of fish eggs generally increase as embryos develop (Spoor, 1977; Levels *et al*., 1986; Rombough, 1988). The oxygen consumption of individual embryonic stages of rainbowfish was not tested during this experiment, but it does not appear that there is a single developmental stage that is particularly sensitive to hypoxia, as daily fluctuations did not cause developmental retardation or higher mortality. It is possible that rainbowfish embryos are adapted to undergo periods of higher metabolism during the day, when oxygen levels in their natural environment would increase, and the maximum amount of dissolved oxygen would be available. Further studies on this topic would be interesting, particularly deliberately exposing fish to hypoxia during critical stages such as blastodisc formation, gastrulation, early organogenesis and hatching. It would also be interesting to determine whether embryos were similarly tolerant to chronic hypoxia.

Deformities were not recorded in high numbers in either rainbowfish experiment, but did occur in the form of spinal curvature and underdeveloped larvae. Spinal curves were usually towards the tail end of the fish, and did not appear to cause immediate death of larvae. Spinal curves have been previously reported as a result of embryonic exposure to hypoxia (Rosenthal & Alderdice, 1976), but in the experiments reported here there was no correlation between treatments and incidence of deformities. Underdeveloped larvae were more likely to be dead or very weak on collection than larvae with spinal curves. Underdeveloped larvae were very small (about 2/3 of length of normal larvae) and had larger heads and shorter tails, with less pigmentation on the body. Deformed hatchlings are not expected to survive through the larval period (Keckeis *et al*., 1996). Twinning (polyembryony) has been previously recorded following embryonic exposure to hypoxia for killifish (*Fundulus heteroclitus*; Stockard, 1921 - cited in Stephens, 1973). There were no recorded polyembryonic events from the experiments on sooty grunter or either species of rainbowfish; however, polyembryony has never been reported for the test species and may not be a possibility.

Larval size on hatching is reduced by low dissolved oxygen concentration in several fish species including steelhead trout (*Salmo gairdneri gairdneri*; Silver *et al.*, 1963), Chinook salmon (*Oncorhynchus tshawytscha*; Silver *et al.*, 1963), walleye (*Stizostedion vitreum vitreum*; Oseid & Smith, 1971a), white sucker (*Catostomus commersonnii*; Oseid & Smith, 1971b), lake herring (*Coregonus artedii*; Brooke & Colby, 1980) and lingcod (*Ophiodon elongatus*; Giorgi & Congleton, 1984). Reduced larval size is thought to be a response to increased metabolic costs of embryonic development under hypoxia (Rosenthal & Alderdice, 1976). It may have deleterious consequences for resulting larvae, making them less able to compete with conspecifics (Mason, 1969 - cited in Giorgi & Congleton, 1984) and subject to greater predation over a longer period of time (Ware, 1975 - cited in Giorgi & Congleton, 1984), although these consequences of smaller hatch size have been queried (von Westernhagen, 1988). No effect of hypoxia on larval hatch size was found for the rainbowfish species examined during this study.

Hypoxia causes premature hatching in fish, depending on the developmental phase at which they are exposed (Keickeis *et al.*, 1996; Czerkies *et al.*, 2001). Hypoxia can be advantageous as a hatch stimulant, and is used to induce synchronous hatching in aquaculture hatcheries for Atlantic salmon (*Salmo salar*) suggesting hypoxia may be involved in cuing hatch of eggs of the species in natural situations (Oppen-Berntsen *et al.*, 1990). Hypoxia is also a cue to hatch for intertidal spawners such as killifish (*Fundulus heteroclitus*) that rely on their natural respiratory stress response to release the hatching enzyme, chorionase, and facilitate hatching on the high tide (reviewed by Taylor, 1999). Conversely, reduced oxygen has also been shown to retard development and increase incubation time of early stage eggs of some fish species (Oseid & Smith, 1971a; Rosenthal & Alderdice, 1976; Giorgi & Congleton, 1984; Keckeis *et al.*, 1996). Delayed hatch times increase vulnerability of eggs to predation (Oyen *et al.*, 1991). Neither species of rainbowfish showed reduced or increased incubation time in response to hypoxia. During the experiments on the two species of rainbowfish, it was observed in all tanks that most hatching occurred in the 12 hours prior to beginning the DO cycling regime each day, seldom during the five-hour cycling period, and in small numbers for several hours following cycling. This may indicate an inhibitory role of hypoxia to hatching of rainbowfish on a diel time scale,

which might allow larvae to complete hatch and locate a high oxygen environment to occupy before onset of the next hypoxic episode. Further studies may investigate this hypothesis.

It is perhaps not surprising that rainbowfish embryos are well adapted to dealing with diel fluctuations in hypoxia, given that eggs are spawned on plant material and reside there until hatching. The natural rhythm of plants respiring and consuming oxygen during the night, and producing oxygen throughout the day via the process of photosynthesis likely causes huge diel shifts of oxygen content in the still water surrounding eggs. It is likely that the eggs have adapted to the variable environment their parents leave them in, and are hence resistant to fluctuating hypoxia, provided the timing is similar to that in their natural environment.

Eggs of some molluscs also show unique adaptations to hypoxia caused by the spawning habits of their parents. The sand snail (*Polinices sordidus*) lays gelatinous egg masses with three layers of eggs on the outer surfaces. Eggs on the inner layer are exposed to extremely hypoxic conditions, which slows and even arrests their development. However, following hatching of their siblings on the surface layer of the egg mass (which have not been exposed to hypoxia) the oxygen environment of the inner egg layers improves, and eggs continue to develop and hatch viable veligers, with no apparent ill effects from their hypoxic experience (Booth, 1995).

The eggs of some amphibians, including salamanders (*Ambystoma* spp.) inhabit ephemeral environments with high biological oxygen demand and low water currents, similar to that which rainbowfish eggs may experience. Salamander eggs are able to adjust their oxygen conductance and oxygen transport capacities in response to oxygen availability, which may increase their survival in low oxygen environments (Mills *et al*., 2001). It is conceivable that rainbowfish eggs have a similar mechanism for tolerating hypoxia.

It is possible that there were effects on the embryos or newly hatched rainbowfish larvae that would have developed over time. For example, larval growth may have been slower, or larval mortality higher as larval development progressed. A study on the effects of PCBs (polychlorinated biphenyls) on carp (*Cyprinus carpio*) eggs

showed no effect until resorption of the yolk sac occurred at 72 hrs post-hatch. At this point larvae began to exhibit yolk sac and periocardial oedema and craniofacial deformities (Stouthart *et al*., 1998). The authors postulated that PCB was stored in the yolk sac and was not available for toxic action until resorption occurred. Yolk sac oedema resembles a condition known as blue-sac disease, which is prevalent in hatchery-reared salmonids that have been exposed to hypoxia (Stouthart *et al*., 1998) but to my knowledge has not been reported as a result of hypoxic exposure for any other species.

CHAPTER SEVEN: GENERAL DISCUSSION

The effects of diel fluctuations in DO saturation (fluctuating hypoxia) on Australian native freshwater fish have not previously been studied in experimental situations. As poor water quality conditions such as hypoxia are becoming more commonplace in catchments of tropical north Queensland (Pearson *et al.,* 2003) it is important to appreciate their effects on the unique fish fauna of the region. Fluctuating hypoxia occurs naturally in water bodies when dissolved oxygen increases during the day, provided by the photosynthetic processes of aquatic plants and algae, and decreases during the night when no photosynthesis occurs and all organisms in the system are respiring (Hunt & Christiansen, 2000). However, fluctuating hypoxia is more likely to occur, and with greater severity, in enclosed lentic water bodies or slow-flowing streams with high nutrient loads and high plant biomass, conditions that often result from anthropogenic practices such as cropping and grazing (Oliveira & Kjerfve, 1993; Bonsdorff *et al*., 1997; Martin & Saiki, 1999; Tucker & Burton, 1999; Collins *et al*., 2000; Pearson *et al.,* 2003); aquaculture (Hargrave *et al.*, 1993; Bonsdorff *et al.*, 1997); industrial effluents (Winn & Knott, 1992) and urban runoff (Tucker & Burton, 1999).

This study tested the tolerance of various life history stages of several native fish species to fluctuating hypoxia, and found that the fish tested were very resistant to it at both lethal and sublethal levels. A summary of the results of the seven experiments carried out during this study is provided in Table 7.1. The rank order of resistance to fluctuating hypoxia of each species/life history stage from highest to lowest was: eggs of eastern rainbowfish (*Melanotaenia splendida splendida*) and Utchee Creek rainbowfish (*M. utcheensis*) (no immediate lethal level identified), juvenile barramundi (*Lates calcarifer*) (lethal level ~2% DO saturation), juvenile eastern rainbowfish and adult Utchee Creek rainbowfish (lethal level 6-7% for both), and juvenile sooty grunter (*Hephaestus fuliginosus*) (lethal level ~7%).

Table 7.1 Experiments carried out during this study.

4 Species	Life history stage	Parameters studied	Length of experiment	4.1 Effects	Thesis chapter
Barramundi (***Lates calcarifer***)	Juvenile	• ventilation rates • ventilation behaviour • gill histopathology • growth • feeding behaviour • condition	21 days, terminated 7 days early due to rapid growth of fish	• Ventilation rate described by moving average regression • ASR commenced at ~7% DO saturation • No effect of treatment on gill health • Some suggestion of reduced growth in treatments reaching 10% & 5% minimum DO saturation • 30% reduction in "active" feeding under low DO treatments • No effect on condition	3 and 4
Sooty grunter (***Hephaestus fuliginosus***)	Juvenile	• ventilation rates • ventilation behaviour • gill histopathology • growth • feeding behaviour • food consumption • condition	28 days	• Ventilation rate described by linear regression • Incapable of performing ASR • No effect on gill health • Some suggestion of reduced growth in treatment reaching 10% DO saturation • Feeding behaviour erratic in all treatments • Food consumption reduced with decreasing DO saturation in set 1 fish	3 and 4
Sooty grunter (***Hephaestus fuliginosus***)	Embryos	• time to hatch • egg & larval mortality • larval deformity • size of larvae	Until completion of hatch	Very high mortality rates in all tanks making any results skeptical.	6
Eastern rainbowfish (***Melanotaenia splendida splendida***)	Juvenile	• ventilation rates • ventilation behaviour • growth • feeding behaviour • condition	28 days	• Ventilation rate described by moving average regression • ASR commenced at ~7% DO saturation • Little growth recorded for all fish • Feeding behaviour erratic in all treatments • No effect on condition	3 and 4
Eastern rainbowfish (***Melanotaenia splendida splendida***)	Embryos	• time to hatch • egg & larval mortality • larval deformity • size of larvae	Until completion of hatch	No effect of treatments on eggs or resulting larvae.	6

Species	Life history stage	Parameters studied	Length of experiment	Effects	Thesis chapter
Utchee Creek rainbowfish (*Melanotaenia utcheensis*)	Embryos	• time to hatch • egg & larval mortality • larval deformity • size of larvae	Until completion of hatch	No effect of treatments on eggs or resulting larvae.	6
Utchee Creek rainbowfish (*Melanotaenia utcheensis*)	Breeding adults and resulting embryos	• egg production • egg size • gonad histology • embryo effects as for other egg experiments	28 days for adults, plus until completion of hatch	On adults: One tank in lowest surviving treatment (10% DO) stopped laying eggs, the other did not. Reduced egg production in lower DO treatments. High number of atretic eggs in one female fish from 10% treatment. No effect on gonad histology or egg size. On resulting embryos: None. Slight variations in temperature had greater effect.	5

The resistance of rainbowfish embryos (eggs – Chapter 6) to daily exposure to fluctuating hypoxia reaching a minimum of 5% DO saturation (~ 0.05 mg/L at 28°C) was remarkable. No ill effects at all were found on eggs placed in these extremely stressful hypoxic situations in which juveniles or adults of the same species would be dead. The natural situation for rainbowfish eggs being spawned onto plant material and not tended by parents (Allen *et al.,* 2003; Pusey *et al.,* 2004) undoubtedly results in natural exposure to diel cycles in DO saturation in the immediate vicinity of the embryo, especially if there is little water movement.

Natural exposure to hypoxia is probably also the mechanism that has prepared juvenile barramundi, juvenile eastern rainbowfish (Chapters 3 and 4) and adult Utchee Creek rainbowfish (Chapter 5) for greater survival and tolerance rates in experiments. Barramundi could survive to ~2% DO saturation following a gradual reduction over several hours. Both species of rainbowfish survived to 6-7% DO saturation under the same circumstances. Previous studies show that if oxygen levels drop suddenly the DO saturation that is lethal to rainbowfish and barramundi is a much higher threshold, stressing the importance of rate of reduction of DO in determining lethality (Pearson *et al*., 2003). Sooty grunter also survived to ~7% DO saturation but unlike the other species tested were unable to perform aquatic surface respiration (ASR – Chapter 3) which might increase survival rates at much lower DO saturations if surface access were available. Rainbowfish and juvenile barramundi are more likely to experience hypoxia in the field, inhabiting slow running, highly vegetated water bodies, whereas sooty grunter prefer running streams or large open water bodies over smaller tributaries (Pusey *et al.,* 2004).

Note that in this study, ASR although attempted, was unsuccessful. The reason for this is that the aquarium set up, and use of nitrogen as an oxygen-removal agent, precluded access to additional oxygen at the air:water interface. Detail on aquarium set up is provided in Chapter 2. The experiments thus examined the ability of fish to survive fluctuating hypoxia to the minimum levels tested without gaining additional oxygen via ASR.

The ability of different species of tropical Australian freshwater fish to survive and prosper under notionally challenging DO regimes is probably due to their long-term experience with oxygen in natural situations. This is also the case for fish from the Amazon, where hypoxia is commonplace. Even closely related species of Amazonian fish such as fish within the genus *Hoplias* can have different tolerances to hypoxia depending on the oxygen regimes experienced in their natural habitat (Rantin *et al.,* 1992). It appears quite likely that, with some exceptions, tropical and subtropical fish species are more tolerant of hypoxia than fish from temperate and cold-water climates, as is the case for macroinvertebrates (Connolly *et al.,* 2004). This is a hypothesis that warrants further investigation.

Sublethal effects on fish were found to be slight during the 3-4 week experiment periods in this study. Ventilation rates and behaviour changed during oxygen depletion for all three species tested, representing a mechanism for living with hypoxia rather than a negative reaction to it. Growth was reduced in barramundi exposed to hypoxia cycles reaching 5% and 10% minimum DO saturation each day, and in sooty grunter exposed to cycles reaching 10% minimum DO saturation (5% was a lethal treatment for this species). Feeding behaviour of barramundi was also negatively affected by these treatments, suggesting that it is the amount of food consumed, rather than any alteration in metabolism or food conversion rates that caused the reduction in growth for this species. This has been the case for many studies on other species, for example: *Ictalurus punctatus* and *Perca flavescens* (Carlson *et al.,* 1980); *Pseudopleuronectes americanus* (Bejda *et al.,* 1992); *Gadus morhua* (Chabot & Dutil, 1999); and *Scophthalmus maximum* and *Dicentrarchus labrax* (Pichavant *et al.,* 2001). However, the theory of greater importance of food consumption over food conversion under hypoxia is contested by Buentello *et al.* (2000) in a study on *Ictalurus punctatus*.

Condition of juvenile eastern rainbowfish, barramundi and sooty grunter was unaffected by hypoxia treatments. No published information was found that reported effects or lack of effects of hypoxia on these condition indices for other species of fish.

Reproduction of surviving adult Utchee Creek rainbowfish remained largely unaffected by exposure to diel fluctuations in DO saturation, although one of the two tanks treated with a minimum DO saturation of 10% daily ceased egg production after 18 days of oxygen cycling and in this same aquarium one of the two female fish was found to have a very high percentage of atretic (degenerative) eggs in her ovary (Chapter 5). Aside from that fish, no adverse effects on ovaries of female fish or testes of male fish were identified to correlate with exposure to hypoxia. Studies on other fish species have also identified reduced spawning success (*Pomoxis nigromaculatus*; Carlson & Herman, 1978) and decreased brood size and egg viability (*Oreochromis niloticus*; Bhujel *et al.,* 2001) under hypoxia.

Very little research has been carried out to identify the effects of diel fluctuations in DO saturation on fish, which means there is little to be compared with this study. Of the research that has been carried out, Carlson *et al.* (1980) found that growth of *Perca flavescens* was affected by chronic hypoxia, but not by fluctuating hypoxia where the mean of fluctuations was the same as the level of chronic hypoxia. Bejda *et al.* (1992) found that *Pseudopleuronectes americanus* grew more slowly in chronic hypoxia than in fluctuating hypoxia, but that recovery times for fish that had been exposed to chronic hypoxia were faster than for fish that had been exposed to fluctuating hypoxia. Conversely, *Paralichthys lethostigma* showed lower growth under fluctuating hypoxia than under a chronic treatment of approximately the same mean DO saturation (Taylor & Miller, 2001). However, fish in the fluctuating treatment grew more than fish in a chronic treatment that was equal to the minimum DO saturation reached each day in the fluctuating treatment. It would be valuable to carry out more research to further examine the effects of fluctuating versus chronic hypoxia on fish, and particularly for effects other than growth, which has been quite widely reported in the literature.

One concerning result identified by this study was the reduction in appetite of barramundi under fluctuating hypoxia treatments reaching a daily DO saturation of 10% and under. Fish were fed after oxygen levels had returned to normal (i.e. several hours after the completion of the depletion stage of the oxygen cycle) and still were too weak to feed normally. Eventually, if the experiments had continued for longer, this reduction in food intake may have led to wasting and death. In a field situation

the impacts of hypoxia on feeding are likely to be much greater, especially for predatory species like barramundi which have to chase and capture food before consuming it. In a laboratory study on juvenile *Paralichthys flesus*, significant reductions in predation efficiency were identified under 20% and 30% DO saturation when compared with fish exposed to 40% and 100% DO saturation (Tallqvist *et al.,* 1999). Fish unable to capture sufficient food would eventually die, weakening every day as both continuing hypoxia and lack of food reduce the likelihood of recuperation following cessation of hypoxic conditions.

The implications of reduced feeding by barramundi are of concern not just for the species, but also for the trophodynamics of the ecosystems they occupy. Barramundi are key predators and their removal from the food chain would be expected to result in substantial alterations to the fish communities, as reported in other situations. For example, on the Great Barrier Reef, abundance of prey species of coral trout (*Plectropomus leopardus*) doubled after removal of these key predators from reefs (Graham *et al.,* 2003); and in a seven-year manipulative study of the fish communities in two Canadian lakes, removal of piscivorous fish resulted in increased densities of all non-piscivorous fish, increased species richness of non-piscivorous fish and for some species a shift towards smaller body size, leading to the conclusion that top-down forces structured the fish communities (Demers *et al.,* 2001). Hypoxia has been found to affect trophodynamics of marine ecosystems, and could conceivably replace *k*-selected species with *r*-selected species and complex food chains with simple food chains (Wu, 2002). Once major structural changes have occurred in wetlands affected by hypoxia it is unknown how long it would take communities to recover from the impacts, if ever.

It is also concerning that one of two tanks of Utchee Creek rainbowfish ceased egg production during the course of an experiment where oxygen levels dropped to 10% DO saturation daily. In this study the second tank of rainbowfish did not cease to breed, but if these results were typical (which is uncertain because of low replication of treatments) a 50% reduction in spawning might be expected under similar oxygen conditions in the field. More research needs to be carried out to determine whether this is, in fact, the case. A 50% reduction in spawning biomass could have serious implications for a species like Utchee Creek rainbowfish with a very restricted

distribution and a small population size. The species is found only in one Pacific-draining catchment near Innisfail on the eastern coast of Queensland (McGuigan, 2001). The lowland streams occupied by the species are located in areas with a high level of agricultural activity, particularly in the production of banana and sugar cane crops. Both of these cropping activities have the potential to produce high levels of runoff with heavy nutrient loads (Khamsouk & Roose, 2003; Pearson *et al.*, 2003; McKergow *et al.*, 2004). Excess nutrients entering streams leads to excessive plant and algal growth (Hunt & Christiansen, 2000) and conceivably to a situation very similar to that presented in this study where oxygen levels undergo a diel cycle with very low DO saturations reached at dawn. A concomitant reduction in rate of Utchee Creek rainbowfish reproduction, "arguably the most important factor in determining species fitness and survival" (Wu, 2002) could be detrimental to small, range-restricted populations.

An increase in the incidence of hypoxia does not, however, necessarily spell the death of a fish species. Microevolutionary responses to hypoxic stress have been identified in some fish species, in areas where anthropogenic activities resulted in more frequent hypoxic episodes. *Haplochromis (Yssichromis) pyrrhocephalus*, a zooplanktonic haplochromine cichlid of Lake Victoria, showed incredible phenotypic plasticity in response to a changing environment. Apparently in response to increasing hypoxia, the average number of secondary gill lamellae in fish sampled from the lake increased by 25% between 1978 and 1999 (Witte *et al.*, 2000). Similarly, gill surface area has increased in *Rastrineobola argentea* (a zooplanktivorous cyprinid) between 1983 and 1988 (Wanink & Witte, 2000). Similar increases in gill surface area have also been found in laboratory studies on cichlids (Chapman *et al.*, 2000) and sea bass (Saroglia *et al.*, 2002).

This thesis presents what was essentially a baseline study of a broad range of sublethal effects of fluctuating hypoxia on several species of tropical freshwater fish from northern Queensland. More targeted research towards identifying critical levels of DO saturation below which fish condition and reproductive health are reduced for a larger variety of fish species is required to fully understand the impacts of fluctuating hypoxia on freshwater fish populations of the wetlands of tropical north Queensland. With increasing pressure from agricultural activities, as well as greater interest in

aquaculture enterprises and growing urban populations in the region now is the time to begin to understand the ecosystems our native fish fauna rely upon.

The duration of the experiments used for this study was chosen to identify short-term impacts of fluctuating hypoxia on fish. Greater impacts may have been identified if longer term experiments had been carried out. However short-term impacts of chronic hypoxia have been observed in other studies (e.g. Pearson *et al*., 2003), and thus both timescales are appropriate for further investigation.

Although some native fish species appear to be relatively resistant to hypoxia and have acquired the capacity to perform aquatic surface respiration and, in the case of rainbowfish embryos, to withstand fluctuating hypoxia regimes absolutely, not all species or even all life history stages will be as capable of coping with hypoxic stress. *Nematolosa erebi* (bony bream) is often the first fish species to die in hypoxic events in north Queensland (Pearson *et al*., 2003). Bony bream were not investigated in this study, due to the difficulty of maintaining sufficient numbers for experiments. If the problems associated with keeping the species in captivity could be overcome, it would be an ideal indicator species to use in identifying levels of hypoxia likely to affect fish communities in the region. Similarly, larval fish may be more affected by hypoxia than eggs or older fish of the same species. Experiments on critical levels and oxygen cycling regimes for indicator species or life stages such as bony bream and fish larvae, as well further analysis of the sublethal effects of hypoxia on these more vulnerable stream inhabitants would be valuable to help interpret the impact hypoxia might have on north Queensland's waterways.

Although it was not within the scope of this study, the synergistic effects of hypoxia on fishes' ability to withstand disease, parasites, pollutants and other reductions in water quality is important to understand if we wish to discover the true effect of hypoxia on fish communities. Hypoxia, when caused by agricultural runoff, occurs in tandem with increased dissolved carbon dioxide levels and slightly acidic waters as well as high sediment loads, high levels of nitrogenous wastes including in the extremely toxic form of ammonia, and pesticides (Pearson *et al*., 2003). It is possible that uptake rate of toxicants increases as a result of higher respiration rates during periods of low DO saturation. To begin to mitigate the effects of these products on

fish populations it is first necessary to understand the way they interact with each other and with individual fish; this topic warrants further investigation.

The species of fish investigated in this study proved to be quite tolerant to fluctuating hypoxia. Such resistance is frequently found in taxa that experience hypoxia naturally on a regular basis (Kramer, 1987). Despite a natural tolerance to hypoxic stress, fish kills attributed to hypoxic episodes still occur in the regions inhabited by these fish (e.g. Bishop, 1980; Townsend *et al.,* 1992; Pearson *et al*., 2003). Anthropogenic activities such as agriculture, aquaculture, industry and urban development in catchment areas exacerbate and increase the frequency and intensity of hypoxic episodes (e.g. Tucker & Burton, 1999; Collins *et al.*, 2000; Pearson *et al.,* 2003) such that even the most tolerant fish are affected.

Nevertheless, this study does suggest that even a small increase in oxygen saturation can have a large impact on improving the health of fish in tropical catchments. The positive attitudes of many rural industries and landowners towards increasing environmental sustainability and even rehabilitating water courses on their own land are to be commended, and stand to make a real difference to the survival of tropical wetlands. All the same, the situation for Australia's tropical wetlands is serious because of the limited range of some freshwater organisms and the relatively small area of suitable habitat that remains, most of which suffers impacts from agricultural and other contaminated runoff.

REFERENCES:

Aday, D.D., D.A. Rutherford & W.E. Kelso. 2000. Field and laboratory determinations of hypoxic effects on RNA-DNA ratios of bluegill. **American Midland Naturalist** 143: 433-442.

Alazemi, B.M., J.W. Lewis & E.B. Andrews. 1996. Gill damage in the freshwater fish *Gnathonemus ptersii* (Family: Mormyridae) exposed to selected pollutants: an ultrastructural study. **Environmental Technology** 17: 225-238.

Allen, G.R., S.H. Midgley & M. Allen. 2003. Field guide to the freshwater fishes of Australia, Western Australian Museum, Perth, 394 pp.

Almeida-Val, V.M.F, A.L. Val & I. Walker. 1993. Hypoxia tolerance in Amazon fishes status of an under-explored biological 'goldmine'. IN: P.W. Hochachka, P.L. Lutz, T. Sick, M. Rosenthal & G. Van den Thillart (Eds) Surviving Hypoxia Mechanisms of Control and Adaptation. CRC Press, Boca Raton. pp 435-445.

Arnim, S.S. 1935. A method for preparation of serial sections of teeth and surrounding structures of the rat. **Anatomical Records** 62: 321-330.

Araujo, F.G., R.G. Bailey & W.P. Williams. 1999. Spatial and temporal variations in fish populations in the upper Thames estuary. **Journal of Fish Biology** 55: 836-853.

Balon, E. K. 1975. Reproductive guilds of fishes: a proposal and definition. **Journal of the Fisheries Research Board of Canada** 32: 821-864.

Barrow, T.M. & E.J. Peters. 2001. Movements of rainbow trout in response to dissolved oxygen and food availability in Lake Ogallala, Nebraska. **Journal of Freshwater Ecology** 16(3): 321-329.

Bejda, A.J., B.A. Phelan & A.L. Studholme. 1992. The effect of dissolved oxygen on the growth of young-of-the-year winter flounder, *Pseudopleuronectes americanus*. **Environmental Biology of Fishes** 34: 321-327.

Bermudes, M. & A.J. Ritar. 1999. Effects of temperature on the embryonic development of the striped trumpeter (*Latris lineata*, Bloch and Schneider, 1801). **Aquaculture** 176: 245-255.

Bhujel, R.C., W.A. Turner, A. Yakupitiyage & D.C. Little. 2001. Impacts of environmental manipulation on the reproductive performance of the Nile tilapia (*Orechromis niloticus*). **Journal of Aquaculture in the Tropics** 16(3): 197-209.

Bishop, K.A. 1980. Fish kills in relation to physical and chemical changes in Magela Creek (East Alligator River System, Northern Territory) at the beginning of the tropical wet season. **Australian Zoology** 20: 485-500.

Blaxter, J.H.S. 1988. Pattern and variety in development. IN: W.S. Hoar & D.J. Randall (Eds) Fish Physiology Volume XIA: The Physiology of Developing Fish Part A: Eggs and Larvae. Academic Press Inc., California. pp 1-58.

Boeuf, G., D. Boujard & J. Person-Le Ruyet. 1999. Control of somatic growth in turbot. **Journal of Fish Biology** 55(Supplement A): 128-147.

Bolger, T. & P.L. Connolly. 1989. The selection of suitable indices for the measurement and analysis of fish condition. **Journal of Fish Biology** 34: 171-182.

Bonsdorff, E., E.M. Blomqvist, J. Matilla & A. Norkko. 1997. Coastal eutrophication: causes, consequences and perspectives in the archipelago areas of the northern Baltic Sea. **Estuarine, Coastal and Shelf Science** 44(Supplement A): 63-72.

Booth, D.T. 1995. Oxygen availability and embryonic development in sand snail (*Polinices sordidus*) egg masses. **Journal of Experimental Biology** 198(1): 241-247.

Breitburg, D.L. 1992. Episodic hypoxia in Chesapeake Bay: interacting effects of recruitment, behaviour, and physical disturbance. **Ecological Monographs** 62(4): 525-546.

Brett, J.R. 1979. Environmental factors and growth. IN: W.S. Hoar, D.J. Randall & J.R. Brett (Eds) Fish Physiology Volume VIII: Bioenergetics and Growth. Academic Press Inc, Florida. pp 599-675.

Bridges, C.R. 1988. Respiratory adapatations in intertidal fish. **American Zoologist** 28: 79-96.

Brooke, L.T. & P.J. Colby. 1980. Development and survival of embryos of lake herring at different constant oxygen concentrations and temperatures. **The Progressive Fish-Culturist** 42(1): 3-9.

Buentello, J.A., D.M. Gatlin III & W.H. Neill. 2000. Effects of water temperature and dissolved oxygen on daily feed consumption, feed utilization and growth of channel catfish (*Ictalurus punctatus*). **Aquaculture** 182: 339-352.

Caldwell, C.A. & J. Hinshaw. 1995. Tolerance of rainbow trout to dissolved oxygen supplementation and a *Yersinia ruckeri* challenge. **Journal of Aquatic Animal Health** 7: 168-171.

Carlson, A.R. & L.J. Herman. 1978. Effect of long-term reduction and diel fluctuation in dissolved oxygen on spawning of black crappie, *Pomoxis nigromaculatus*. **Transactions of the American Fisheries Society** 107: 742-746.

Carlson, A.R., J. Blocher & L.J. Herman. 1980. Growth and survival of channel catfish and yellow perch exposed to lowered constant and diurnally fluctuating dissolved oxygen concentrations. **The Progressive Fish-Culturist** 42: 73-78.

Cech, J.J. Jr., S.J. Mitchell, D.T. Castleberry & M. McEnroe. 1990. Distribution of California stream fishes: influence of environmental temperature and hypoxia. **Environmental Biology of Fishes** 29: 95-105.

Cech, J.J. Jr. & M.J. Massingill. 1995. Tradeoffs between respiration and feeding in Sacramento blackfish, *Orthodon microlepidotus*. **Environmental Biology of Fishes** 44: 157-163.

Cengiz, E.I. & E. Unlu. 2002. Histopathological changes in the gills of mosquitofish, *Gambusia affinis* exposed to endosulfan. **Bulletin of Environmental Contamination and Toxicology** 68: 290-296.

Cerqueira, C.C.C. & M.N. Fernandes. 2002. Gill tissue recovery after copper exposure and blood parameter responses in the tropical fish *Prochilodus scrofa*. **Ecotoxicology and Environmental Safety** 52: 83-91.

Chabot, D. & J.-D. Dutil. 1999. Reduced growth of Atlantic cod in non-lethal hypoxic conditions. **Journal of Fish Biology** 55: 472-491.

Chapman, L.J. & C.A. Chapman. 1998. Hypoxia tolerance of the Mormyrid *Petrocephalus catostoma*: implications for persistence in swamp refugia. **Copeia** 1998(3): 762-768.

Chapman, L.J., F. Galis & J. Shinn. 2000. Phenotypic plasticity and the possible role of genetic assimilation: Hypoxia-induced trade-offs in the morphological traits of an African cichlid. **Ecology Letters** 3: 387-393.

Chapman, L.J. & K.G. Hulen. 2001. Implications of hypoxia for the brain size and gill morphometry of mormyrid fishes. **Journal of the Zoological Society of London** 254: 461-472.

Chapman, L.J., L. Kaufman & C.A. Chapman. 1994. Why swim upside down?: a comparative study of two Mochokid catfishes. **Copeia** 1994(1): 130-135.

Chapman, L.J., L.S. Kaufman, C.A. Chapman & F.E. McKenzie. 1994. Hypoxia tolerance in twelve species of East African cichlids: potential for low oxygen refugia in Lake Victoria. **Conservation Biology** 9: 1274-1288.

Chapman, L. J., L. S. Kaufman, C.A. Chapman & F.E. McKenzie. 1995. Hypoxia tolerance in twelve species of East African cichlids: potential for low oxygen refugia in Lake Victoria. **Conservation Biology** 9(5): 1274-1288.

Chapman, L.J. & K.F. Liem. 1995. Papyrus swamps and the respiratory ecology of *Barbus neumayeri*. **Environmental Biology of Fishes** 44: 183-197.

Chippari-Gomes, A.R., N.P. Lopes, M. de Nazare Paula-Silva, A. Reis Oliveira & V.M.F. Almeida-Val. 2003. Hypoxia tolerance and adaptations in fishes: the case of Amazon cichlids. IN: A.L. Val & B.G. Kapoor (Eds) Fish Adaptations. Science Publishers Inc., Enfield (NH) USA. pp 37-54.

Close, P.G., C.G. Barlow & L.J. Rodgers. 2001. Early ontogeny of coal grunter from hormone-induced spawnings and laboratory-reared embryos and larvae. **Journal of Fish Biology** 58(4): 928-942.

Collins, M.R., S.G. Rogers, T.I.J. Smith & M.L. Moser. 2000. Primary factors affecting sturgeon populations in the southeastern United States: fishing mortality and degradation of essential habitats. **Bulletin of Marine Science** 66: 917-928.

Congleton, J.L. 1980. Observations on the responses of some southern California tidepool fishes to nocturnal hypoxia stress. **Comparative Biochemistry and Physiology A** 66A: 719-722.

Connolly, N.M., M.R. Crossland & R.G. Pearson. 2004. Effect of low dissolved oxygen on survival, emergence, and drift of tropical stream macroinvertebrates. **Journal of the North American Benthological Society** 23(2): 251-270.

Courtenay, S.C. & M.H.A. Keenleyside. 1983. Wriggler-hanging: a response to hypoxia by brood-rearing *Herotilapia multispinosa* (Teleostei, Cichlidae). **Behaviour** 85: 183-197.

Czerkies, P., P. Brzuzan, K. Kordalski & M. Luczynski. 2001. Critical partial pressures of oxygen causing precocious hatching in *Coregonus lavaretus* and *C. albula* embryos. **Aquaculture** 196(1-2): 151-158.

Dabrowski, K., J. Rinchard, J.S. Ottobre, F. Alcantara, P. Padilla, A. Ciereszko, M.J. De Jesus & C.C. Kohler. 2003. Effect of oxygen saturation in water on reproductive performances of Pacu *Piaractus brachypomus*. **Journal of the World Aquaculture Society** 34(4): 441-449.

Das, T., A.K.Pal, S.K. Chakraborty, S.M. Manush, R.S. Dalvi, K. Sarma & S.C. Mukherjee. 2006. Thermal dependence of embryonic development and hatching rate in *Labeo rohita* (Hamilton, 1822). **Aquaculture** 255: 536-541.

Davis, J.C. 1975. Minimal dissolved oxygen requirements of aquatic life with emphasis on Canadian species: a review. **Journal of the Fisheries Research Board of Canada** 32: 2295-2332.

Demers, E., D.J. McQueen, S.A. Popiel, A.M. Rocchi, C.W. Ramcharan & A. Perez-Fuentetaja. 2001. Changes in fish community structure. **Ergebnisse der Limnologie** 56: 23-48.

Dombeck, M.P., B.W. Menzel & P.N. Hinz. 1984. Muskellange spawning habitat and reproductive success. **Transactions of the American Fisheries Society** 113: 205-216.

Downing, K.M. 1954. The influence of dissolved oxygen concentration on the toxicity of potassium cyanide to rainbow trout. **The Journal of Experimental Biology** 31: 161-164.

Drewett, N. & P.D. Abel. 1983. Pathology of lindane poisoning and of hypoxia in the brown trout, *Salmo trutta* L. **Journal of Fish Biology** 23: 373-384.

Dunson, W.A. & D.B. Dunson. 1999. Factors influencing growth and survival of the killifish, *Rivulus marmoratus*, held inside enclosures in mangrove swamps. **Copeia** 1999: 661-668.

Dutta, H.M., A. Nath, S. Adhikari, P.K. Roy, N.K. Singh & J.S. Datta Munshi. 1994. Sublethal malathion induced changes in the ovary of an air-breathing fish, *Heteropneustes fossilis*: a histological study. **Hydrobiologia** 294: 215-218.

Fontenot, Q.C., D.A. Rutherford & W.E. Kelso. 2001. Effects of environmental hypoxia associated with the annual flood pulse on the distribution of larval sunfish and shad in the Atchafalaya River Basin, Louisiana. **Transactions of the American Fisheries Society** 130: 107-116.

Foster, A.R., D.F. Houlihan & S.J. Hall. 1993. Effects of nutritional regime on correlates of growth rate in juvenile Atlantic cod (*Gadus morhua*): comparison of morphological and biochemical measurements. **Canadian Journal of Fisheries and Aquatic Sciences** 50: 502-512.

Fry, F.E.J. 1951. A fractionating column to provide water of various dissolved oxygen contents. **Canadian Journal of Technology** 29: 144-146.

Fry, F.E.J. 1971. The effect of environmental factors on the physiology of fish. IN: W.S. Hoar & D.J. Randall (Eds) Fish Physiology Volume VI: Environmental Relations and Behaviour. Academic Press Inc., New York. pp 1-98.

Fukuda, Y., M. Maita, K. Satoh, H. Yamamoto, N. Okamoto & Y. Ikeda. 1997a. Effects of dissolved oxygen concentration on experimental horizontal transmission of induced by artificial infection with *Enterococcus seriolicida* in yellowtail. **Fish Pathology** 32(1): 43-49.

Fukuda, Y., M. Maita, K. Satoh, & N. Okamoto. 1997b. Influence of dissolved oxygen concentration on the mortality of yellowtail experimentally infected with *Enterococcus seriolicida*. **Fish Pathology** 32(2): 129-130.

Fulton, T.W. 1911. The Sovereignty of the Sea. London.

Furota, T. 1996. Life cycle studies on the introduced spider crab *Pyromaia tuberculata* (Lockington) (Brachyura:Majidae). II. Crab stage and reproduction. **Journal of Crustacean Biology** 16(1): 77-91.

Gee, J.H., R.F. Tallman & J.F. Smart. 1978. Reactions of some great plains fishes to progressive hypoxia. **Canadian Journal of Zoology** 56: 1962-1966.

Gee, J.H. & P.A. Gee. 1991. Reactions of gobioid fishes to hypoxia: buoyancy control and aquatic surface respiration. **Copeia** 1991(1): 17-28.

Gehrke, P.C. 1991. Avoidance of inundated floodplain habitat by larvae of golden perch (*Macquaria ambigua* Richardson): influence of water quality or flood distribution? **Australian Journal of Marine and Freshwater Research** 42: 707-719.

Gerald, J.W. & J.J. Cech. 1970. Respiratory responses of juvenile catfish (*I. punctatus*) to hypoxic conditions. **Physiological Zoology** 43: 47-54.

Giorgi, A.E. & J.L. Congleton. 1984. Effects of current velocity on development and survival of lingcod, *Ophiodon elongatus*, embryos. **Environmental Biology of Fishes** 10: 15-27.

Graham, N.A.J., R.D. Evans & G.R. Russ. 2003. The effects of marine reserve protection on the trophic relationships of reef fishes on the Great Barrier Reef. **Environmental Conservation** 30(2): 200-208.

Hamilton, S.K., S.J. Sippel, D.F. Calheiros & J.M. Melack. 1997. An anoxic event and other biogeochemical effects of the Pantanal wetland on the Paraguay River. **Limnology and Oceanography** 42: 257-272.

Hargrave, B.T., D.E. Duplisea, E. Pfeiffer & D.J. Wildish. 1993. Seasonal changes in benthic fluxes of dissolved oxygen and ammonium associated with marine cultured Atlantic salmon. **Marine Ecology Progress Series** 96: 249-257.

Heath, A.G. 1991. Effect of water-borne copper on physiological responses of bluegill (*Lepomis macrochirus*) to acute hypoxic stress and subsequent recovery. **Comparative Biochemistry and Physiology** 100C(3): 559-564.

Heath, A.G. 1995. Water Pollution and Fish Physiology 2nd ed. CRC Press, New York.

Henrique, M.M.F., M.F. Gouillou-Coustans & E. Gomes. 2002. Effect of dietary ascorbic acid supplementation and chronic hypoxia on sea bream growth and vitamin C status. **Journal of Fish Biology** 60: 442-452.

Hinton, D.E. & D.J. Lauren. 1990. Integrative histopathological approaches to detecting effects of environmental stressors on fishes. **American Fisheries Society Symposium** 8: 51-66.

Hogan, A.E. 1990. Notes on the production of sooty grunter (*Hephaestus fuliginosus*). Queensland Department of Primary Industries, Queensland. 12pp.

Hughes, G.M. 1973. Respiratory responses to hypoxia in fish. **American Zoologist** 13: 475-489.

Humphrey, C., D.W. Klumpp & R. Pearson. 2003. Early development and growth of the eastern rainbowfish, *Melanotaenia splendida splendida* (Peters) I. Morphogenesis and ontogeny. **Marine and Freshwater Research** 54: 17-25.

Hunn, J.B. 1969. Chemical composition of rainbow trout urine following acute hypoxic stress. **Transactions of the American Fisheries Society** 98: 20-22.

Hunt, R.J. & I.H. Christiansen. 2000. Understanding Dissolved Oxygen in Streams. CRC Sugar Technical Publication, Townsville, Queensland. 27 pp.

Huntingford, F. & C. Adams. 2005. Behavioural syndromes in farmed fish: implications for production and welfare. **Behaviour** 142: 1207-1221.

Ikejima, K. & M. Shimizu. 1999. Disappearance of a spring cohort in a population of the dragonet, *Repomucenus valenciennei*, with spring and autumn spawning peaks in Tokyo Bay, Japan. **Ichthyological Research** 46(3): 331-339.

Ingendahl, D. 2001. Dissolved oxygen concentration and emergence of sea trout fry from natural redds in tributaries of the River Rhine. **Journal of Fish Biology** 58: 325-341.

Jagoe, C. H. & T. A. Haines. 1983. Alterations in gill epithelial morphology of yearling sunapee trout exposed to acute acid stress. **Transactions of the American Fisheries Society** 112: 689-695.

Jensen, F.B., M. Nikinmaa & R.E. Weber. 1993. Environmental perturbations of oxygen transport in teleost fishes: causes, consequences and compensations. IN: J.C. Rankin & F.B. Jensen (Eds) Fish Ecophysiology. Chapman & Hall, London. pp 161-179.

Jobling, M. 1994. Fish Bioenergetics. Chapman and Hall, London. 294pp.

Jones, J.C. & J.D. Reynolds. 1999a. Oxygen and the trade-off between egg ventilation and brood protection in the common goby. **Behaviour** 136: 819-832.

Jones, J.C. & J.D. Reynolds. 1999b. Costs of egg ventilation for male common gobies breeding in conditions of low dissolved oxygen. **Animal Behaviour** 57: 181-188.

Jones, J.C. & J.D. Reynolds. 1999c. The influence of oxygen stress on female choice for male nest structure in the common goby. **Animal Behaviour** 57: 198-196.

Kalinin, A.L., F. Tadeu Rantin & M. Lesner Glass. 1993. Dependence on body size of respiratory function in *Hoplias malabaricus* (Teleostei, Erythrinidae) during graded hypoxia. **Fish Physiology and Biochemistry** 12(1): 47-51.

Keckeis, H., E. Bauer-Nemeschkal & E. Kamler. 1996. Effects of reduced oxygen level on the mortality and hatching rate of *Chondrostoma nasus* embros. **Journal of Fish Biology** 49: 430-440.

Keister, J.E., E.D. Houde & D.L. Breitburg. 2000. Effects of bottom-layer hypoxia on abundances and depth distributions of organisms in Patuxent River, Chesapeake Bay. **Marine Ecology Progress Series** 205: 43-59.

Khamsouk, B. & E. Roose. 2003. Runoff and water erosion processes of a tropical volcanic soil cultivated under the main cropping systems in Martinique. **Tropical Agriculture** 80(3): 168-174.

Kloepper-Sams, P.J., S.M. Swanson, T. Marchant, R. Schryer & J.W. Owens. 1994. Exposure of fish to biologically treated bleached-kraft effluent. 1. Biochemical, physiological and pathological assessment of Rocky Mountain whitefish (*Prosopium*

williamsoni) and longnose sucker (*Catostomus catostomus*). **Environmental Toxicology and Chemistry** 13(9): 1469-1482.

Knights, B.C., B.L. Johnson & M.B. Sandheinrich. 1995. Responses of bluegills and black crappies to dissolved oxygen, temperature, and current in backwater lakes of the upper Mississippi River during winter. **North American Journal of Fisheries Management** 15: 390-399.

Kramer, D.L. 1983. Aquatic surface respiration in the fishes of Panama: distribution in relation to risk of hypoxia. **Environmental Biology of Fishes** 8(1): 49-54.

Kramer, D.L. 1987. Dissolved oxygen and fish behaviour. **Environmental Biology of Fishes** 18: 81-92.

Kramer, D.L. & J.P. Mehegan. 1981. Aquatic surface respiration, an adaptive response to hypoxia in the guppy, *Poecilia reticulata* (Pisces, Poeciliidae). **Environmental Biology of Fishes** 6: 299-313.

Kramer, D.L. & M. McClure. 1982. Aquatic surface respiration, a widespread adaptation to hypoxia in tropical freshwater fishes. **Environmental Biology of Fishes** 7: 47-55.

Kramer, D.L., C.C. Lindsey, G.E.E. Moodie & E.D. Stevens 1978. The fishes and the aquatic environment of the central Amazon basin, with particular reference to respiratory patterns. **Canadian Journal of Zoology** 56: 717-729.

Kramer, D.L., D. Manley & R. Bourgeois. 1983. The effect of respiratory mode and oxygen concentration on the risk of aerial predation in fishes. **Canadian Journal of Zoology**. 61: 653 – 665.

Kreuger, R. 1994. Fish tissues - possible biomarkers for poor water conditions? International Symposium on Aquatic Animal Health, Davis, CA (USA).

Kumari, S.A. & N.S.R. Kumar. 1997. Histopathological alterations induced by aquatic pollutants in *Channa punctatus* from Hussainsagar lake (A.P.). **Journal of Environmental Biology** 18(1): 11-16.

Leamon, J.H., E.T. Schultz & J.F. Crivello. 2000. Variation among four health indices in natural populations of the estuarine fish, *Fundulus heteroclitus* (Pisces, Cyprinodontidea), from five geographically proximate estuaries. **Environmental Biology of Fishes** 57: 451-458.

Levels, P.J., R.E.M.B. Gubbels & J.M. Denuce. 1986. Oxygen consumption during embryonic development of the annual fish *Nothobranchius korthausae* with special reference to diapause. **Comparative Biochemistry and Physiology A** 84A: 767-770.

Mallat, J. 1985. Fish gill structural changes induced by toxicants and other irritants: a statistical review. **Canadian Journal of Fisheries and Aquatic Science** 42: 630-648.

Martin, B.A. & M.K. Saiki. 1999. Effects of ambient water quality on the endangered lost river sucker in Upper Klamath Lake, Oregon. **Transactions of the American Fisheries Society** 128: 953-961.

Mason, J.C. 1969. Hypoxial stress prior to emergence and competition among coho salmon fry. **Journal of the Fisheries Research Board of Canada Technical Report** 843: 27pp.

McClosky, J.R. & J.T. Oris. 1991. Effect of water temperature and dissolved oxygen concentration on the photo-induced toxicity of anthracene to juvenile bluegill sunfish (*Lepomis macrochirus*). **Aquatic Toxicology** 21(3-4): 145-156.

McCune, B. & M.J. Mefford. 1999. Multivariate analysis of ecological data. Version 4.37 MjM Software, Gleneden Beach, Oregon, USA.

McDonald, D.G. & B.R. McMahon. 1977. Respiratory development in Arctic char *Salvelinus alpinus* under conditions of normoxia and chronic hypoxia. **Canadian Journal of Zoology** 55: 1461-1467.

McGuigan, K.L. 2001. An addition to the rainbowfish (Melanotaeniidae) fauna of North Queensland. **Memoirs of the Queensland Museum** 46(2): 647-655.

McKergow, L.Z., I.P. Grayson, B. Rodger & D. Heiner. 2004. Performance of grass and rainforest riparian buffers in the wet tropics, Far North Queensland. 2. Water quality. **Australian Journal of Soil Research** 42(4): 485-498.

McNeil, D. 2004. Ecophysiology and behaviour of Ovens River Floodplain fish: hypoxia tolerance and the role of physicochemical environment in structuring billabong fish community. Doctoral Thesis, LaTrobe University, Bundoora, Victoria.

Miles-Richardson, S.R., S.L. Pierens, K.M. Nichols, V.J. Kramer, E.M. Snyder, S.A. Snyder, J.A. Render, S.D. Fitzgerald & J.P. Giesy. 1999. Effects of waterbourne exposure to 4-Nonylphenol and Nonylphenl Ethoxylate on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*)**. Environmental Research Section A** 80: S122-S137.

Mills, N.E., M.C. Barnhart & R.D. Semlitsch. 2001. Effects of hypoxia on egg capsule conductance in *Ambystoma* (Class Amphibia, Order Caudata). **Journal of Experimental Biology** 204(21): 3747-3753.

Monfils, R. 1998. The acute and sublethal effects of oil and oil dispersants on the estuarine glass perchlet, *Ambassis vachelli*. MS Thesis, James Cook University, Townsville, Queensland.

Moyle, P.B. & J.J. Cech Jr. 2004. Fishes: an introduction to ichthyology, 5th edition. Prentice Hall, New Jersey.

Mqolomba, T.N. & J.A. Plumb. 1992. Effect of temperature and dissolved oxygen concentration on *Edwardsiella ictaluri* in experimentally infected channel catfish. **Journal of Aquatic Animal Health** 4: 215-217.

Murugesan, A.G. & M.A. Hannita. 1992. Histopathological and histochemical changes in the oocytes of the air-breathing fish *Heteropneustes fossilis* (Bloch) exposed to textile-mill effluent. **Bulletin of Environmental Contamination and Toxicology** 48: 929-936.

Nath, K. & N. Kumar. 1989. Nickel-induced histopathological alterations in the gill architecture of a tropical freshwater perch, *Colisa fasciatus* (Bloch & Schn.) **The Science of the Total Environment** 80: 283-296.

Nestlerode, J.A. & R.J. Diaz. 1998. Effects of periodic environmental hypoxia on predation of a tethered polychete, *Glycera americana*: implications for trophic dynamics. **Marine Ecology Progress Series** 172: 185-195.

Oliveira, A.M. & B. Kjerfve. 1993. Environmental responses of a tropical coastal lagoon system to hydrological variability: Mundau-Manguaba, Brazil. **Estuarine, Coastal and Shelf Science** 37: 575-591.

Olowo, J.P. & L.J. Chapman. 1996. Papyrus swamps and variation in the respiratory behaviour of the African fish *Barbus neumayeri*. **African Journal of Ecology** 34: 211-222.

Oppen-Berntsen, D.O., A. Bogsnes & B. T. Walther. 1990. The effects of hypoxia, alkalinity and neurochemicals on hatching of Atlantic salmon (*Salmo salar*) eggs. **Aquaculture** 86: 417-430.

Örn, S., P.L. Andersson, L. Forlin, M. Tysklind & L. Norrgren. 1998. The impact on reproduction of an orally administered mixture of selected PCBs in zebrafish (*Danio rerio*). **Archives of Environmental Contamination and Toxicology** 35: 52-57.

Oseid, D.M. & L.L. Smith Jr. 1971a. Survival and hatching of walleye eggs at various dissolved oxygen levels. **The Progressive Fish-Culturist** 33(2): 81-85.

Oseid, D.M. & L.L. Smith Jr. 1971b. Survival and hatching of white sucker eggs at various dissolved oxygen levels. **The Progressive Fish-Culturist** 33(3): 158-159.

Oyen, F.G.F., L.E.C.M.M. Camps & S.E. Wendelaar Bonga. 1991. Effect of acid stress on the embryonic development of the common carp (*Cyprinus carpio*). **Aquatic Toxicology** 19(1): 1-12.

Papoutsglou, S.E. & G. Tziha. 1996. Blue tilapia (*Oreochromis aureus*) growth rate in relation to dissolved oxygen concentration under recirculated water conditions. **Aquacultural Engineering** 15(3): 181-192.

Pearson, R.G., M. Crossland, B. Butler & S. Manwaring. 2003. Effects of cane-field drainage on the ecology of tropical waterways. Report No. 03/04-1,2,3 to Sugar R&D Corporation, Townsville, Queensland, 3 volumes.

Perry, S.F. & B.L. Tufts (Eds). 1998. Fish Physiology Volume 17: Fish Respiration. Academic Press Inc., New York. 353pp.

Pichavant, K., Person-Le-Ruyet, N. Le Bayon, A. Severe, A. Le Roux, L. Quemener, V. Maxime, G. Nonnotte & G. Bouef. 2000. Effects of hypoxia on growth and metabolism of juvenile turbot. **Aquaculture** 188: 103-114.

Pichavant, K., J. Person-Le-Ruyet, N. Le Bayon, A. Severe, A. Le Roux & G. Boeuf. 2001. Comparative effects of long-term hypoxia on growth, feeding and oxygen consumption in juvenile turbot and European sea bass. **Journal of Fish Biology** 59: 875-883.

Pihl, L. 1994. Changes in the diet of demersal fish due to eutrophication-induced hypoxia in the Kattegat, Sweden. **Canadian Journal of Fisheries and Aquatic Science** 51: 321-336.

Pihl, L., S.P. Baden & R.J. Diaz. 1991. Effects of periodic hypoxia on distribution of demersal fish and crustaceans. **Marine Biology** 108: 349-360.

Pihl, L., S.P. Baden, R.J. Diaz & L.C. Schaffner. 1992. Hypoxia-induced structural changes in the diet of bottom-feeding fish and Crustacea. **Marine Biology** 112: 349-361.

Poleksic, V. & V. Karan. 1999. Effects of trifluran on carp: biochemical and histological evaluation. **Ecotoxicology and Environmental Safety** 43: 213-221.

Poulin, R., Wolf, N.G. & Kramer, D.L. 1987. The effect of hypoxia on the vulnerability of guppies (*Poecilia reticulata*, Poeciliidae) to an aquatic predator (*Astronotus ocellatus*, Cichlidae). **Environmental Biology of Fishes** 20: 285-292.

Pouliot, T. & J. de la Noue. 1989. Feed intake, digestibility and brain neurotransmitters of rainbow trout under hypoxia. **Aquaculture** 79: 317-327.

Pusey, B., M. Kennard & A. Arthington. 2004. Freshwater Fishes of North-Eastern Australia. CSIRO Publishing, Australia. 700 pp.

Rahel, F.J. & J.W. Nutzman. 1994. Foraging in a lethal environment: fish predation in hypoxic waters of a stratified lake. **Ecology** 75(5): 1246-1253.

Randall, D.J., W.W. Burggren, A.P. Farrell, & M.S. Haswell. 1981. The Evolution of Air-breathing in Vertebrates. Cambridge University Press, Cambridge.

Rantin, F.T. & K. Johansen. 1984. Responses of the teleost *Hoplias malabaricus* to hypoxia. **Environmental Biology of Fishes** 11(3): 221-228.

Rantin, F.T., A.L. Kalinin, M.L. Glass & M.N. Fernandes. 1992. Respiratory responses to hypoxia in relation to mode of life of two erythrinid species (*Hoplias malabaricus* and *Hoplias lacerdae*). **Journal of Fish Biology** 41: 805-812.

Reichenbach-Klinke, H.-H. 1973. Fish Pathology: a guide to the recognition and treatment of diseases and injuries of fishes, with emphasis on environmental and pollution problems. Hong Kong, T.F.H. Publications, Inc. Ltd.

Reynolds, J.D. & J.C. Jones. 1999. Female preference for preferred males is reversed under low oxygen conditions in the common goby (Pomatoschistus microps). Behavioural Ecology 10(2): 149-154.

Richardson, J., E.K. Williams & C.W. Hickey. 2001. Avoidance behaviour of freshwater fish and shrimp exposed to ammonia and low dissolved oxygen separately and in combination. **New Zealand Journal of Marine and Freshwater Research** 35: 625-633.

Robb, T. & M.V. Abrahams. 2003. Variation in tolerance to hypoxia in a predator and prey species: an ecological advantage of being small? **Journal of Fish Biology** 62: 1067-1081.

Rombough, P.J. 1988. Respiratory gas exchange, aerobic metabolism, and effects of hypoxia during early life. IN: W.S. Hoar & D.J. Randall (Eds) Fish Physiology Volume XIA: The Physiology of Developing Fish Part A: Eggs and Larvae. Academic Press Inc., California. pp 59-161.

Rosenthal, H. & D.F. Alderdice. 1976. Sublethal effects of environmental stressors, natural and pollutional, on marine fish eggs and larvae. **Journal of the Fisheries Research Board of Canada** 33: 2047-2065.

Ruggerone, G.T. 2000. Differential survival of juvenile sockeye and coho salmon exposed to low dissolved oxygen during winter. **Journal of Fish Biology** 56: 1013-1016.

Sabo, M.J., L.A. Brunet & D.S. Hickman. 1998. Physiological responses of centrarchid species that occupy hypoxic swamp habitats. IN: C. Kennedy & D. MacKinlay (Eds) Fish Responses to Toxic Environments. International Congress on the Biology of Fish. Towson University, Baltimore MD July 26-30, 1998.

Sagasti, A., L.C. Schaffner & J.E. Duffy. 2001. Effects of periodic hypoxia on mortality, feeding and predation in an estuarine epifaunal community. **Journal of Experimental Marine Biology and Ecology** 258: 257-283.

Saint-Paul, U. & G.M. Soares. 1988. Ecomorphological adaptation to oxygen deficiency in Amazon floodplains by serrasalmid fish of the genus *Mylossoma*. **Journal of Fish Biology** 32: 231-236.

Saroglia, M., G. Terova, A. De Stradis & A. Caputo. 2002. Morphometric adaptations of sea bass gills to different dissolved oxygen partial pressures. **Journal of Fish Biology** 60: 1423-1430.

Scott, A.L. & W.A. Rogers. 1980. Histological effects of prolonged hypoxia on channel catfish *Ictalurus punctatus* (Rafinesque). **Journal of Fish Diseases** 3: 305-316.

Secor, D.H. & T.E. Gunderson. 1998. Effects of hypoxia and temperature on survival, growth, and respiration of juvenile Atlantic sturgeon, *Acipenser oxyrinchus*. **Fishery Bulletin** 96: 603-613.

Sellers, T.J., B.R. Parker, D.W. Schindler & W.M. Tonn. 1998. Pelagic distribution of lake trout (*Salvelinus namaycush*) in small Canadian Shield lakes with respect to temperature, dissolved oxygen, and light. **Canadian Journal of Fisheries and Aquatic Science** 55: 170-179.

Sepulveda, A. 1994. Daily growth increments in the otoliths of European smelt *Osmerus eperlanus* larvae. **Marine Ecology Progress Series** 108: 33-42.

Siefert, R.E. & L.J. Herman. 1977. Spawning success of the black crappie *Pomoxis nigromaculatus* at reduced dissolved oxygen concentrations. **Transactions of the American Fisheries Society** 106: 376-378.

Siligato, S. & J. Bohmer. 2001. Using indicators of fish health at multiple levels of biological organization to assess effects of stream pollution in southwest Germany. **Journal of Aquatic Ecosystem Stress and Recovery** 8: 371-386.

Silver, S.J., C.E. Warren & P. Doudoroff. 1963. Dissolved oxygen requirements of developing steelhead trout and Chinook salmon embryos at different water velocities. **Transactions of the American Fisheries Society** 92(4): 327-345.

Sivarajah, K., C.S. Franklin & W.P. Williams. 1978. Some histopathological effects of Aroclor 1254 on the liver and gonads of rainbow trout, *Salmo gairdneri* and carp, *Cyprinus carpio*. **Journal of Fish Biology** 13: 411-414.

Skidmore, J.F. & P.W.A. Tovell. 1972. Toxic effects of zinc sulphate on the gills of rainbow trout. **Water Research** 6(3): 217-230.

Smale, M.A. & C.F. Rabeni. 1995. Influences of hypoxia and hyperthermia on fish species composition in headwater streams. **Transactions of the American Fisheries Society** 124: 711-725.

Sollid, J., P. De Angelis, K. Gundersen & G.E. Nilsson. 2003. Hypoxia induces adaptive and reversible gross morphological changes in crucian carp gills. **The Journal of Experimental Biology** 206: 3667-3673.

Spoor, W.A. 1977. Oxygen requirements of embryos and larvae of the largemouth bass, *Micropterus salmoides* (Lacepede). **Journal of Fish Biology** 11: 77-86.

Sprague, J.B. 1971. Measurement of pollutant toxicity to fish – III. Sublethal effects and "safe" concentrations. **Water Research** 5: 245-266.

Steffensen, J.F., J. P. Lomholt & K. Johansen. 1982. Gill ventilation and O_2 extraction during graded hypoxia in two ecologically distinct species of flatfish, the flounder (*Platichthys flesus*) and the plaice (*Pleuronectes platessa*). **Environmental Biology of Fishes** 7(2): 157-163.

Stephens, R.R. 1973. Twinning in *Porichthys notatus* Girard. **Transactions of the American Fisheries Society** 102(4): 846.

Stevens, E.D. & G.F. Holeton. 1978. The partitioning of oxygen uptake from air and from water by erythrinids. **Canadian Journal of Zoology** 56: 965-969.

Stewart, N.E., D.L. Shumway & P. Doudoroff. 1967. Influence of oxygen concentration on the growth of juvenile largemouth bass. **Journal of the Fisheries Research Board of Canada** 24: 475-494.

Steyermark, A.C., J.R. Spotila, D. Gillette & H. Isseroff. 1999. Biomarkers indicate health problems in brown bullheads from the industrialized Schuylkill River, Philadelphia. **Transactions of the American Fisheries Society** 128: 328-338.

Stierhoff, K.L., T.E. Targett & P.A. Grecay. 2003. Hypoxia tolerance of the mummichog: the role of access to the water surface. **Journal of Fish Biology** 63: 580-592.

Stockard, C.R. 1921. Developmental rate and structure expression: an experimental study of twins, double monsters and single deformities, and the interaction among embryonic organs during their origin and development. **American Journal of Anatomy** 28: 115-277.

Stouthart, X.J.H.X., M.A.J. Huijbregts, P.H.M. Balm, R.A.C. Lock & S.E. Wendelaar Bonga. 1998. Endocrine stress response and abnormal development in carp (*Cyprinus carpio*) larvae after exposure of the embryos to PCB 126. **Fish Physiology and Biochemistry** 18(4): 321-329.

Tallqvist, M., E. Sandberg-Kilpi & E. Bonsdorff. 1999. Juvenile flounder, *Platichthys flesus* (L.), under hypoxia: effects on tolerance, ventilation rate and predation efficiency. **Journal of Experimental Marine Biology and Ecology** 242: 75-93.

Tappin, A. 2003. *Melanotaenia splendida splendida*. **In Stream: Australia New Guinea Fishes Association Queensland Inc. Newsletter** 12(6): 11-17.

Taylor, M.H. 1999. A suite of adaptations for intertidal spawning. American Zoologist **39: 313-320.**

Taylor, J.C. & J.M. Miller. 2001. Physiological performance of juvenile southern flounder, *Paralichthys lethostigma* (Jordan and Gilbert, 1884), in chronic and episodic hypoxia. **Journal of Experimental Marine Biology and Ecology** 258: 195-214.

Teichert-Coddington, D. & B.W. Green. 1993. Tilapia yield improvement through maintenance of minimal oxygen concentrations in experimental grow-out ponds in Honduras. **Aquaculture** 118: 63-71.

Thetmeyer, H., U. Waller, K.D. Black, S. Inselmann & H. Rosenthal. 1999. Growth of European sea bass (*Dicentrarchus labrax* L.) under hypoxic and oscillating oxygen conditions. **Aquaculture** 174: 355-367.

Thompson, G.G. & P.C. Withers. 2002. Aerial and aquatic respiration of the Australian desert goby, *Chlamydogobius eremius*. **Comparative Biochemistry and Physiology A** 131(4): 871-879.

Timmerman, C.M. & L.J. Chapman. 2004. Behavioural and physiological compensation for chronic hypoxia in the sailfin molly (*Poecilia latipinna*). **Physiological and Biochemical Zoology** 77(4): 601-610.

Townsend, S.A., K.T. Boland & T.J. Wrigley. 1992 Factors contributing to a fish kill in the Australian wet/dry tropics. **Water Research** 26: 1039-1044.

Trippel, E.A. & H.H. Harvey. 1989. Missing opportunities to reproduce: an energy dependent or fecundity gaining strategy in white sucker (*Catostomus commersoni*)? **Canadian Journal of Zoology** 67: 2180-2188.

Tucker, K.A. & G.A. Burton Jr. 1999. Assessment of nonpoint-source runoff in a stream using in situ and laboratory approaches. **Environmental Toxicology and Chemistry** 18: 2797-2803.

Val, A.L., M.N.P. Silva & V.M.F. Almeida-Val. 1998. Hypoxia adaptation in fish of the Amazon: a never-ending task. **South African Journal of Zoology** 33(2): 107-114.

Verheyen, E., R. Blust & W. Decleir. 1994. Metabolic rate, hypoxia tolerance and aquatic surface respiration of some lacustrine and riverine cichlid fishes (Pisces: Cichlidae). **Comparative Biochemistry and Physiology A** 107A(2): 403-411.

Virgona, J.L. 1992. Environmental factors influencing the prevalence of a cutaneous ulcerative disease (red spot) in the sea mullet, *Mugil cephalus* L., in the Clarence River, New South Wales, Australia. **Journal of Fish Diseases** 15: 363-378.

von Westernhagen, H. 1988. Sublethal effects of pollutants on fish eggs and larvae. IN: W.S. Hoar & D.J. Randall (Eds) Fish Physiology Volume XIA: The Physiology of Developing Fish Part A: Eggs and Larvae. Academic Press Inc., California. pp 253-346.

Wajsbrot, N., A. Gasith, M.D. Krom & D.M. Popper. 1991. Acute toxicity of ammonia to juvenile gilthead seabream *Sparus aurata* under reduced oxygen levels. **Aquaculture** 92: 277-288.

Waller, U., E. Black, D. Burt, C. Groot & H. Rosenthal. 2000. The reaction of young coho *Oncorhynchus kisutch* to declining oxygen levels during long-term exposure. **Journal of Applied Ichthyology** 16: 14-19.

Wanink, J.H. & F. Witte. 2000. The use of perturbation as a natural experiment: effects of predator introduction on the community structure of zooplanktivorous fish in Lake Victoria. **Advances in Ecological Research** 31: 553-568.

Ware, D.M. 1975. Relationship between egg size, growth, and natural mortality of larval fish. **Journal of the Fisheries Research Board of Canada** 32: 2503-2512.

Watters, J.V. & J.J. Cech Jr. 2003. Behavioural responses of mosshead and woolly sculpins to increasing environmental hypoxia. **Copeia** 2003(2): 397-401.

Webb, A.C. 2005. The ecology of invasions of non-indigenous freshwater fishes in northern Queensland. Unpublished PhD thesis, James Cook University, Townsville, Queensland.

Weber, J.-M. & D.L. Kramer. 1983. Effects of hypoxia and surface access on growth, mortality, and behaviour of juvenile guppies, *Poecilia reticulata*. **Canadian Journal of Fisheries and Aquatic Science** 40: 1583-1588.

Wedemeyer, G.A., F.P. Meyer & L. Smith. 1976. Environmental stress and fish diseases, Book 5. IN: S.F. Snieszko & H.R.Axelrod (Eds) Diseases of Fishes. T.H.F. Publ. Inc., New Jersey. pp 1-192.

Wells, R.M.G., J. Baldwin, R.S. Seymour & R.E. Weber. 1997. Blood oxygen transport and hemoglobin function in three tropical fish species from northern Australian billabongs. **Fish Physiology and Biochemistry** 16: 247-258.

Whitfield, A.K. & A.W. Paterson. 1995. Flood-associated mass mortality of fishes in the Sundays Estuary. **Water SA** 21: 385-389.

Whitworth, W.R. 1968. Effects of diurnal fluctuations of dissolved oxygen on the growth of brook trout. **Journal of the Fisheries Research Board of Canada** 25(3): 579-584.

Wieland, K., U. Waller & D. Schnack. 1994. Development of Baltic cod eggs at different levels of temperature and oxygen content. **Dana** 10: 163-177.

Winemiller, K.O. 1989. Development of dermal lip protuberances for aquatic surface respiration in South American Characid fishes. **Copeia** 1989(2): 382-390.

Winn, R.N. & D.M. Knott. 1992. An evaluation of the survival of experimental populations exposed to hypoxia in the Savannah River estuary. **Marine Ecology Progress Series** 88: 161-179.

Witte, F., B.S. Msuku, J.H. Wanink, O. Seehausen, E.F.B. Katunzi, P.C. Goudswaard & T. Goldschmidt. 2000. Recovery of cichlid species in Lake Victoria: an examination of factors leading to differential extinction. **Reviews in Fish Biology and Fisheries** 10: 233-241.

Wolf, N.G. & D.L. Kramer. 1987. Use of cover and the need to breathe: the effects of hypoxia on vulnerability of dwarf gouramis to predatory snakeheads. **Oecologia** 73: 127-132.

Wu, R.S.S. 2002. Hypoxia: from molecular responses to ecosystem responses. **Marine Pollution Bulletin** 45: 35-45.

Wu, R.S.S.; B.S. Zhou; D.J. Randall; N.Y.S Woo & P.K.S. Lam. 2003. Aquatic hypoxia is an endocrine disruptor and impairs fish reproduction. **Environmental Science & Technology** 37(6): 1137-1141.

Yamamoto, T. & U.G. Reinhardt. 2003. Dominance and predator avoidance in domesticated and wild masu salmon *Oncorhynchus masou*. **Fisheries Science** 69: 88-94.

Yoshiyama, R.M., C.J. Valpey, L.L. Schalk, N.M. Oswald, K.K. Vaness, D. Lauritzen & M. Limm. 1995. Differential propensities for aerial emergence in intertidal sculpins (Teleostei: Cottidae). **Journal of Experimental Marine Biology and Ecology** 191: 195-207.

APPENDIX 1

Use of aquatic plants to create fluctuating hypoxia in an experimental environment

Introduction

Hypoxia (low dissolved oxygen saturation of water) is a major cause of fish deaths and reduced fish diversity worldwide (e.g., Bishop 1980, Townsend et al. 1992, Whitfield & Paterson 1995, Hamilton et al. 1997). Although not limited to freshwater, hypoxia is commonly encountered in lakes and streams, and it is in these confined waters where the greatest damage to ecosystems and populations is likely to occur. Hypoxia occurs naturally, but can be exacerbated by anthropogenic nutrient sources such as agricultural runoff (e.g., Oliveira & Kjerfve 1993, Bonsdorff et al. 1997, Martin & Saiki 1999, Collins et al. 2000), urban runoff (Tucker & Burton 1999), industrial effluents (Winn & Knott 1992), or wastes from aquaculture facilities (Hargrave et al. 1993, Bonsdorff et al. 1997). An increase in nutrients encourages growth of plants, algae and microbes which all consume oxygen through respiration.

The effects of hypoxia on marine and freshwater fish have been widely studied in cold and temperate regions, and both lethal and sublethal conditions have been identified (e.g. Kramer 1987, Miller et al. 2002). However, little information exists on the sublethal effects of hypoxia in tropical freshwater systems, with some notable exceptions from South America (e.g., Fernandes et al. 1995, Rantin et al. 1992),

Africa (e.g. Chapman et al. 1994, Chapman & Chapman 1998, Schofield & Chapman 2000) and northern Australia (Pearson et al. 2003[9]).

In some freshwater systems, dissolved oxygen saturation follows a diel cycle, falling at night due to the respiration of aquatic organisms, and rising during the day, through production of oxygen by plant photosynthesis. The upper and lower saturations reached during the cycle vary with conditions that include nutrient status, plant abundance, abundance of particulate and dissolved organic material, temperature and flow. In agricultural regions in tropical north Queensland, diurnally fluctuating hypoxia occurs commonly (Pearson et al. 2003[1]). Studies on responses of fish to fluctuating hypoxia require techniques that produce a cost-efficient, low maintenance system for oxygen depletion and replacement.

Previous experimental studies on the effects of hypoxia on fish have used a variety of methods to achieve depleted oxygen levels, including addition of nitrogen gas, vacuum degassing, addition of sodium sulphite, insertion of cages or mesocosms into low-oxygen environments in the field, and sealing the experimental containers so the fish's own respiration removes oxygen from the water (Table 1).

The most common method of oxygen depletion is by bubbling of nitrogen gas into the experimental water body (61% of 59 papers sampled) to remove oxygen by displacement. This technique is very effective and has the advantage that nitrogen gas is biologically inert. However some aspects of this method are unnatural, including the presence of a 'nitrogen atmosphere' above the water's surface, preventing fish from effectively employing adaptations such as aquatic surface respiration (ASR – Kramer & Mehegan 1981) or facultative air breathing. Furthermore, the change in pH that occurs when nitrogen gas is pumped into water for substantial periods of time is

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[9] Pearson, R.G., M. Crossland, B. Butler & S. Manwaring. 2003 "Effects of cane-field drainage on the ecology of tropical waterways". Report No. 03/04-1,2,3 to Sugar R&D Coroporation, Townsville, QLD, 3 volumes.

opposite to the change caused by natural oxygen uptake by plants and animals. In natural waterways, fluctuating hypoxia is often caused by abundant macrophyte growth, encouraged by high light or nutrient levels (Kaenel et al. 2000). During the night, respiration by organisms in the water body removes oxygen from the water and replaces it with carbon dioxide, causing pH levels to drop (except in extremely hard water) (Burnett 1997). Adding nitrogen gas to water, however, causes the reverse effect, and pH levels rise as oxygen concentration decreases. This situation is clearly not ideal given that low pH due to high levels of dissolved carbon dioxide (hypercapnia) can disrupt the acid-base balance and gas transfer across fish gills (Cruz-Neto & Steffensen 1997), thereby lowering the efficiency of oxygen uptake (Dahlberg et al. 1968). Hence, in studies which have used methods such as nitrogen bubbling or vacuum degassing to reduce dissolved oxygen saturation, harmful effects of hypoxia on fish survival or fitness may be underestimated due to the lack of the naturally occurring synergistic effect of low pH. This confounding factor may be avoided by the additional bubbling of carbon dioxide gas into the water, or by altering the pH using chemicals, but the disadvantage is that such methods make experiments more expensive, time-consuming and difficult to control.

Here we demonstrate the use of aquatic plants to create conditions of hypoxia, as occur in eutrophic waterways, thereby exposing test organisms to diurnal cycling of oxygen and pH which replicates natural environments and avoids some of the problems associated with other methods. We are unaware of any previous published study that has attempted to use plants to reduce the dissolved oxygen saturation of water. We present an extremely cost effective laboratory method, which can be deployed over long periods of time with minimal effort in comparison to traditional oxygen reduction methods.

Table 1. Experimental oxygen depletion methods used in marine and freshwater studies from a sample of 59 publications. All references were either technical methods papers, or examined the effects of hypoxia on fish.

Technique	Studies using this technique	Location of study (*or location of fish source)	Marine/ freshwater (M/F)	Type of hypoxia: chronic/ fluctuating/ gradually altered to a maximum or minimum (C/F/G)	Lethal/ sublethal (L/S)
Addition of Nitrogen gas (36 of 59)	Fry 1951	Toronto	-	-	-
	Downing 1954	UK	F	G	L
	Downing & Merkins 1955	UK	F	G	L
	Whitmore et al. 1960	Oregon	F	C	S
	Davis et al. 1963	Oregon	F	C	S
	Dahlberg et al. 1968	Oregon	F	C	S
	Siefert & Spoor 1974	Minnesota	F	C	L & S
	Swift & Lloyd 1974	UK	F	C	S
	Johnston 1975	UK	F	C	S
	McDonald & McMahon 1977	Canada	F	C	S
	Kramer & Mehegan 1981	*Trinidad	F	C	L & S
	Drewett & Abel 1983	UK	F	C	L
	Petersen & Petersen 1990	Denmark	M	C	L & S
	Pihl et al. 1991	Virginia	M	C	L & S
	Kaufman & Wieser 1992	Austria	F	C	S
	Schurmann & Steffensen 1994	Denmark	M	G	S
	Cech & Massingill 1995	California	F	G	S
	Fernandes et al. 1995	Brazil	F	C	S
	Gee & Gee 1995	Australia	M	C	S
	Thomason et al. 1996	UK	M	C	S
	Crocker & Cech 1997	California	F	C	S
	Schurmann & Steffensen 1997	Denmark	M	G	S
	Dalla Via et al. 1998	*Italy	M	G	S
	Plante et al. 1998	Quebec	M	C	L
	Chabot & Dutil 1999	Quebec	M	C	S
	Jones & Reynolds 1999a, 1999b, 1999c	UK	M	C	S
	Renshaw & Dyson 1999	Australia	M	C	S
	Tallqvist et al. 1999	Finland	M	C	L & S
	Geiger et al. 2000	Florida	M	C	S
	Pichavant et al. 2000	France	M	C	S
	Richardson et al. 2001	New Zealand	F	C	S
	Taylor & Miller 2001	North Carolina	M	C & F	S
	Pichavant et al. 2001	France	M	C	S
	Pearson et al. 2003[1]	Australia	F	C	L & S
Vacuum degassing (7 of 59)	Mount 1961	Ohio	F	G	L
	Carlson et al. 1980	Minnesota	F	C & F	L & S
	Scott & Rogers 1980	Alabama	F	C	S
	Bejda et al. 1987	New Jersey	M	G	S
	Pouliot et al. 1988	Quebec	F	C	S
	Pouliot & De La Noue 1989	Quebec	F	C	S
	Miller et al. 2002	East USA	M	C	L
Sodium sulphite	Chapman et al. 1994	*Lake	F	G	S

		Victoria			
(including when used in conjunction with nitrogen gas) (4 of 59)	Chapman & Chapman 1998	*Lake Nabugabo	F	G	S
	Schofield & Chapman 2000	*Uganda	F	G	S
	Melnychuk & Chapman 2002	*Africa	F	G	S
Cages in the field (3 of 59)	Moore 1942	Minnesota	F	C	L
	Dunson & Dunson 1999	Florida	M	Not stated	S
	Ruggerone 2000	Alaska	F	C	L
Sealing tank & fish respiration alone (9 of 59)	Hunn 1969	Wisconsin	F	G	S
	Carlson & Herman 1978	*Wisconsin	F	C & F	S
	Courtenay & Keenleyside 1983	*Central America	F	G	S
	Vig & Nemcsok 1989	Hungary	F	G	S
	Kakuta et al. 1992	Japan	F	G	S
	Kakuta & Murachi 1992	Japan	F	G	S
	van Raaij et al. 1994	Netherlands	F	G	S
	Thetmeyer et al. 1999	Germany	M	C & F	S
	Waller et al. 2000	*British Columbia	M	C	S

Material and Methods

We used *Ceratophyllum demersum,* a common aquatic macrophyte in Australia and other parts of the world, as our test species. *C. demersum* is a non-rooted plant that absorbs nutrients from the surrounding water, has high photosynthetic performance (Blum et al., 1997) and produces increasing oxygen saturation with increasing light intensity (Pearson et al. 2003[1]). It is commonly used as a spawning substrate for native fish, is not cyanogenic and is not known to accumulate metals such as aluminium (Watson & Dallwitz 1992[10]). *C. demersum* grows quickly in high nutrient waters, can tolerate a wide range of water hardness, and can become a problem weed in areas where it is exotic (Anon. 2003[11]). Plants were collected from weir pools in

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[10] Watson, L. & M.J. Dallwitz. 1992 onwards. The families of flowering plants: descriptions, illustrations, identification, and information retrieval. Version: 14th December 2000. Internet address: http://biodiversity.uno.edu/delta

[11] Anonymous. 2003. Native freshwater plants: coontail – a freely floating native plant. Washington State Department of Ecology: Water Quality Program. Updated: 24th February 2003. Internet address: http://www.edy.wa.gov/programs/wq/plants/native/coontail.html

Ross River (19°18'S, 146°45'E) and held in carbon-filtered (0.5-μm) tap water in 500-l white plastic mesocosms at James Cook University campus in open sunlight. Plants were maintained with a nutrient mix of aquarium plant food ('Aquamaster' plant food), sodium nitrate and potassium dihydrogen orthophosphate, and kept outside for five days before use in experiments.

The trials were conducted in a heat- and temperature-controlled room at a constant water temperature of 29°C using 30-l glass aquaria filled with 25 l of carbon-filtered water. Each aquarium was sealed with a PVC plastic lid and high vacuum silicon grease. Plant volume was measured as the amount of water displaced in a graduated measuring cylinder. Different treatments consisted of different volumes of plant being added to the aquaria (Table 2). Only healthy parts of the plants were used, especially the dense green tips. Stringy stems with little foliage were not used. A submersible pump (Resun SP-600: 5 watts; 220 volts; 60/50 hertz; 250 l h-1 delivery) in each aquarium maintained water circulation. Circulation was found be to be effective, with variation within aquaria less than 0.5% saturation.

Trials with fish used juvenile barramundi (*Lates calcarifer*). Barramundi inhabit coastal drainages in northern Australia (Allen et al. 2002), and are a popular target for recreational and commercial fishers. Juvenile fish live in freshwater, while adults are generally found in or near estuaries (Allen et al. 2002). Previous studies on hypoxic tolerance suggest juveniles of the species are reasonably hypoxia tolerant, with a lethal concentration of approximately 12-13% at 29°C over 24 h for fish of 85-105 mm total length (Pearson et al. 2003[1]).

Table 2. Examples of changes in dissolved oxygen saturation (% and mg l-1 at 29°C) and pH in aquaria with various amounts of *Ceratophyllum demersum*, or with four barramundi (*Lates calcarifer*) fingerlings sealed and kept in darkness for 18 h.

Volume of plant material or number of fish	Reduction in level of dissolved oxygen saturation in two aquaria (% and mg l-1)		Change in pH in two aquaria	
	Aquarium 1	Aquarium 2	Aquarium 1	Aquarium 2
300 ml plant	96 → 7 % 7.3 → 0.5 mg l-1	95→ 14 % 7.2 → 1.0 mg l-1	7.00 → 6.4	7.02 → 6.68
200 ml plant	99 → 32 % 7.5 → 2.4 mg l-1	100 → 46 % 7.6 → 3.5 mg l-1	7.05 → 6.58	7.00 → 6.70
150 ml plant	98 → 40 % 7.4 → 3.0 mg l-1	99 → 52 % 7.5 → 4.0 mg l-1	7.23 → 6.69	7.30 → 6.70
100 ml plant	103 → 62 % 7.8 → 4.7 mg l-1	99 → 66 % 7.5 → 5.0 mg l-1	7.23 → 6.71	7.31 → 6.85
50 ml plant	94 → 80 % 7.1 → 6.1 mg l-1	100 → 81 % 7.6 → 6.2 mg l-1	7.00 → 6.82	7.02 → 6.69
4 x 35 mm fish	98 → 89 % 7.4 → 6.7 mg l-1	98 → 92 % 7.4 → 7.0 mg l-1	7.98 → 7.45	7.90 → 7.41

In all trials, dissolved oxygen, pH and temperature were regularly monitored using a WTW (Wissenschaftlich-Technische Werkstatten) pH/Oxi 340i meter, in combination with a WTW CellOx 325-3 dissolved oxygen probe and WTW SenTix pH probe. Both probes were calibrated daily. The readings were taken by inserting the probes through a resealable opening in the lid of each aquarium.

Three series of tests are presented here: one using various amounts of plant material to decrease oxygen saturation overnight; one using fish alone to decrease oxygen saturation overnight; and a final test of the effectiveness of the plants to reduce oxygen levels overnight in the presence of fish for prolonged time periods.

4.2   Tests with plants

C. demersum was used to deplete oxygen levels in the aquaria over 18 h, during which time aquaria were kept in complete darkness. During the 6-h 'day', when overhead fluorescent lights were switched on in the experimental room, additional

'sunlights' (Hagen Aquaglo – 55 Lux, 18000 K) were switched on behind the aquaria, closest to the plants, to encourage photosynthesis, and compressed air was provided to boost oxygen renewal.

4.3   Tests with fish

Reduction in dissolved oxygen saturation due to fish respiration was tested using two sealed aquaria each containing four barramundi (*Lates calcarifer*) of 40-50 mm total length. To duplicate tests using plants and provide a comparable test result, this trial was also carried out in darkness for 18 h, after which measurements of dissolved oxygen were recorded. Compressed air was provided during the 6-h light period to boost oxygen renewal.

4.4   Tests with fish and plants

To test the capacity of plants to remove oxygen in the presence of fish, four barramundi (40-50 mm total length) were placed into each of two experimental aquaria, each of which also contained 275 ml of *C. demersum*. Aquaria were sealed and kept in the dark for 16 h, during which time one was bubbled with compressed air, as a control, and one was not. In initial pilot trials it was found that 18 h darkness resulted in excessive oxygen depletion using fish and plants together, so 16-h dark periods were employed, in contrast to the previous tests using plants and fish separately.   During times when the overhead fluorescent lights were on in the aquarium room, additional Aquaglo 'sunlights' were used to supplement light reaching the plants, and oxygen renewal was boosted by bubbling compressed air. For

20 days, oxygen, pH and temperature were recorded every morning (when lights switched on at 9am) and evening (before lights switched off at 5pm) in both tanks. The four fish in each aquarium were kept separated by means of plastic mesh divisions. Maintenance of fish during this time included daily feeding as lights came on at 9am, and a daily 50% water change using carbon filtered tap water (dissolved oxygen saturation > 90%), which was carried out one hour before lights were switched off. By this time oxygen levels had returned to ~100% (normoxia). *C. demersum* in experimental aquaria was replaced every three days with fresh material that had been kept in the outdoor mesocosm for at least five days as previously described.

Results

C. demersum alone reduced the concentration of dissolved oxygen in aquaria substantially, and percent decrease in dissolved oxygen saturation increased logarithmically with increased volume of plant material (Figure 1). Corresponding changes in pH showed a decrease from evening to dawn of about 0.5 units in each aquarium (Table 2).

Figure 1. Reduction in dissolved oxygen saturation (%) in experimental aquaria at 29°C, containing various volumes of *Ceratophyllum demersum*, and no fish. Equation of the logarithmic curve is: $y = 36.766\text{Ln}(x) - 130.17$, which fits the data with $R^2 = 0.937$.
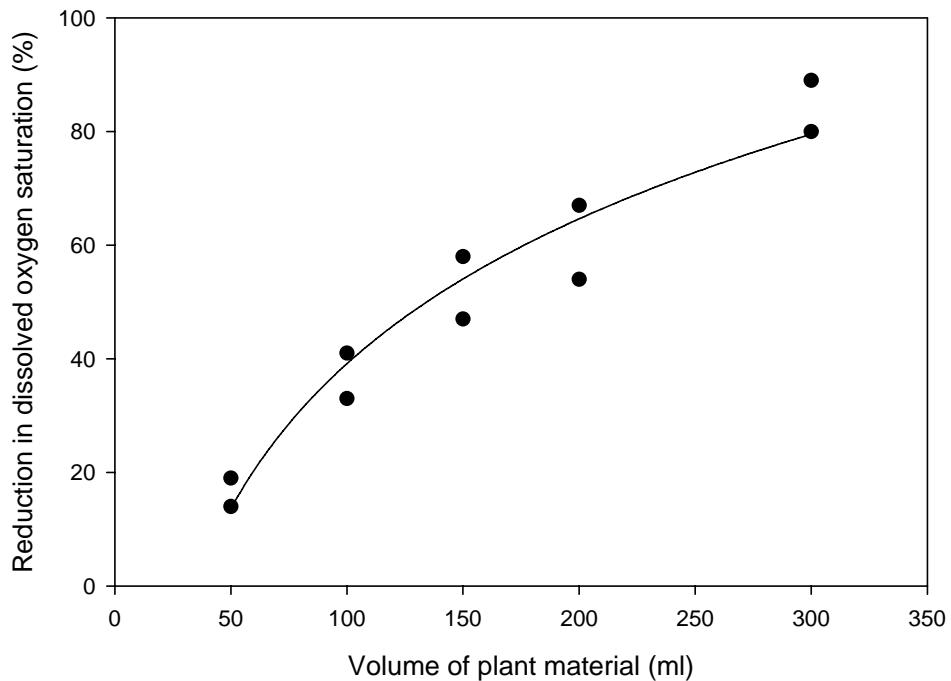
The respiration of four barramundi (total length 40-50 mm) depleted oxygen saturation in the sealed experimental aquaria by a maximum of 9%, with a similar change in pH to that found in the aquaria containing only plants (Table 2).

When 275 ml *C. demersum* was sealed in an aquarium containing four barramundi, resulting dissolved oxygen reduction was reasonably similar each day for 20 days. The lowest minimum ('dawn') dissolved oxygen level reached over the 20 days of oxygen cycling was 1.3% saturation (0.1 mg l-1 at 29°C), and the highest dawn reading was 12.0% (0.9 mg l-1) (Figure 2), with an average of 4.8% (0.4mg l-1) minimum dissolved oxygen saturation over the duration. The average change in pH was a decrease from evening to dawn of 0.88 (Figure 3).

Figure 2. Dissolved oxygen readings from morning and evening in two aquaria each containing four 40-50 mm barramundi (*Lates calcarifer*) fingerlings, and 275 ml *Ceratophyllum demersum*. The treatment aquarium was sealed and not aerated for 16 h overnight and then aerated for 8 h during the day. The control aquarium was lightly sealed and aerated continuously. Note that readings were taken in the morning and evening, and continuous lines are for illustrative purposes only.
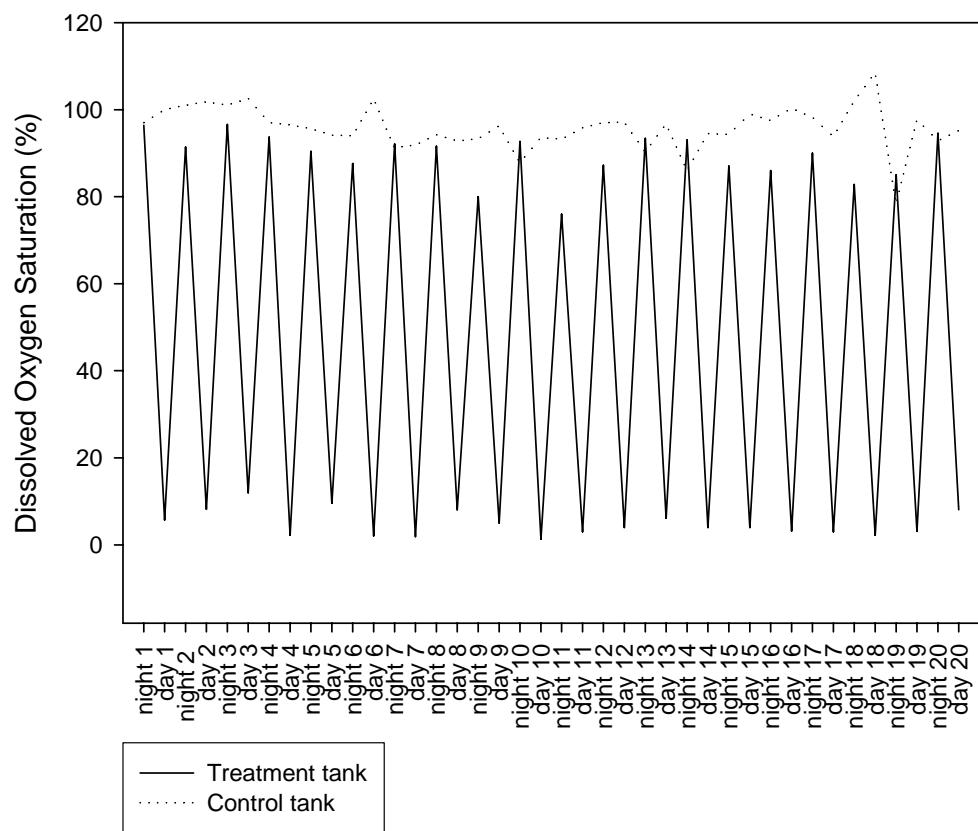
Figure 3. pH readings from morning and evening in two aquaria each containing four 40-50 mm barramundi (*Lates calcarifer*) fingerlings and 275 ml *Ceratophyllum demersum*. The treatment aquarium was sealed and not aerated for 16 h overnight, and then aerated for 8 h during the day. The control aquarium was lightly sealed and aerated continuously. The absence of data at day 6 was due to equipment failure. Note that readings were taken in the morning and evening, and continuous lines are for illustrative purposes only.
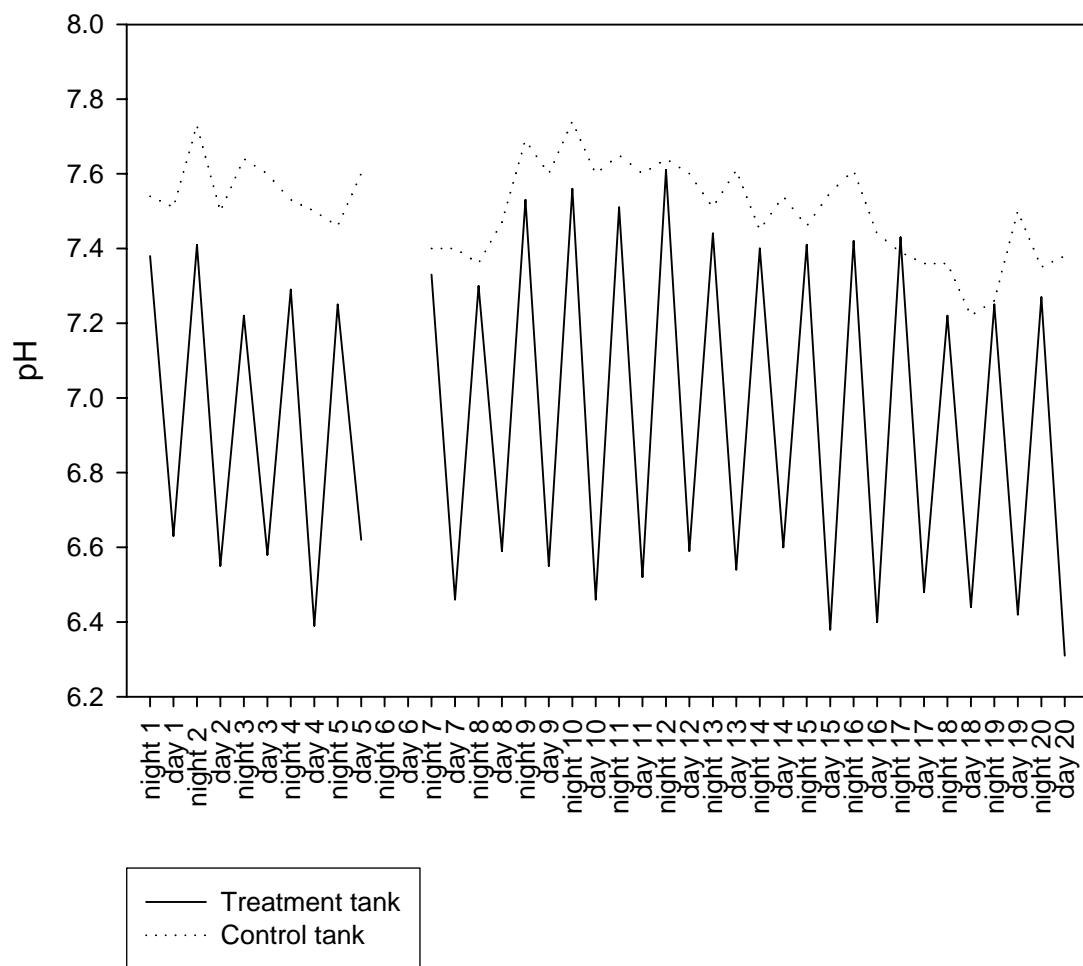
Discussion

In experimental situations, it is desirable to apply methods that produce results as similar to the natural environment as possible. Many of the experiments carried out on the effects of hypoxia on fish to date have been poor simulations of conditions found in eutrophic environments such as those often found in the floodplains of tropical

Australia. Plants can be used to induce hypoxia in freshwater aquaria containing fish in a way that replicates natural rhythms of oxygen and pH fluctuation in aquatic environments.

In this study, aquaria containing barramundi alone achieved minimal oxygen reduction overnight, with a depletion of only 10% dissolved oxygen saturation. Greater density of fish per tank, as is typical of other studies, would be required to cause substantial oxygen depletion, but this might cause additional stress to fish through density effects on behaviour and physiology.

When plants were added to aquaria containing barramundi, dissolved oxygen depletion was rapid and followed a naturalistic pattern. The oxygen cycling seen in the treatment aquarium containing barramundi and *Ceratophyllum demersum* was similar to field conditions in areas where there is a high level of plant and algal material in still water. For example, in a lentic habitat in north Queensland, Pearson et al. (2003)[1] found that dissolved oxygen levels frequently cycled between 2% and 85% saturation over 24 hours.

It should be mentioned that the very low dissolved oxygen levels survived by juvenile barramundi in the results presented here are not typical. Previous studies suggest a lethal concentration of about 12-13% dissolved oxygen saturation (Pearson et al. 2003[1]). During the present study animals had access to the water's surface, and were probably able to access more highly oxygenated water at the surface-water interface compared to the minimum levels measured within the water column. In pilot trials carried out prior to this study, most barramundi died at 4% dissolved oxygen saturation. In field situations barramundi are unlikely to inhabit such hypoxic waters successfully. In the Burdekin region of north Queensland, barramundi seldom persist in waters where dissolved oxygen saturation is very low (less than ~10% saturated,

0.8 mg l-1 at 27°C). Although barramundi have been captured in lagoons where saturation reached 5% (0.45mg l-1 at 20°C) at dawn, the fish were not seen conducting ASR, which indicates they may have found gradients of higher dissolved oxygen saturation within the lagoon. Additionally, in the areas surveyed, barramundi died off before the next sampling round several months later (C. Perna, pers. comm.[12]).

Aquaria containing both plants and fish displayed greater variability in minimum oxygen value reached in a set time, especially at the intermediate levels tested (unpublished data), than did plants alone. The use of aquaria larger than the 30-l aquaria we used would probably rectify this problem, and also make measurements more precise. Additionally, conducting experiments under natural light may increase plant productivity and reduce the amount of plant required to deplete oxygen levels to a required concentration, and reduce the need to regularly replace used plants.

The technique described here is easily implemented and could replace other methods as an oxygen depletion mechanism. It is a particularly appropriate method to use to replicate diurnal oxygen cycling. The method simulates patterns observed in natural aquatic environments more efficiently than simply using the fish themselves to deplete oxygen, especially when fish to be examined are small. Use of plants also allows natural fluctuations of carbon dioxide and hence pH, and enables some access to surface waters to allow fish to utilize such adaptations as ASR and facultative air breathing. This contrasts strongly with current methods commonly used to create hypoxic conditions, such as vacuum degassers and nitrogen replacement.

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References cited

Allen, G.R., S.H. Midgley & M. Allen. 2002. Freshwater fishes of Australia. Western Australian Museum, Perth. 394 pp.

Bejda, A.J., A.L. Studholme & B.L. Olla. 1987. Behavioural responses of red hake, *Urophycis chuss*, to decreasing concentrations of dissolved oxygen. Environmental Biology of Fishes 19: 261-268.

Bishop, K.A. 1980. Fish kills in relation to physical and chemical changes in Magela Creek (East Alligator River System, Northern Territory) at the beginning of the tropical wet season. Australian Zoology 20: 485-500.

Blum, V., H. Hollander-Czytko & D. Voeste. 1997. The closed equilibrated biological aquatic system: general concept and aspects of botanical research. Planta 203: S201-S208.

Bonsdorff, E., E.M. Blomqvist, J. Matilla & A. Norkko. 1997. Coastal eutrophication: causes, consequences and perspectives in the archipelago areas of the northern Baltic Sea. Estuarine, Coastal and Shelf Science 44(Supplement A): 63-72.

Burnett, L.E. 1997. The challenges of living in hypoxic and hypercapnic aquatic environments. American Zoologist 37: 633 - 640.

Carlson, A.R. & L.J. Herman. 1978. Effect of long-term reduction and diel fluctuation in dissolved oxygen on spawning of black crappie, *Pomoxis nigromaculatus*. Transactions of the American Fisheries Society 107: 742-746.

Carlson, A.R., J. Blocher & L.J. Herman. 1980. Growth and survival of channel catfish and yellow perch exposed to lowered constant and diurnally fluctuating dissolved oxygen concentrations. The Progressive Fish-Culturist 42: 73-78.

Cech, J.J. Jr. & M.J. Massingill. 1995. Tradeoffs between respiration and feeding in Sacramento blackfish, *Orthodon microlepidotus*. Environmental Biology of Fishes 44: 157-163.

Chabot, D. & J.-D. Dutil. 1999. Reduced growth of Atlantic cod in non-lethal hypoxic conditions. Journal of Fish Biology 55: 472-491.

Chapman, L.J., L.S. Kaufman, C.A. Chapman & F.E. McKenzie. 1994. Hypoxia tolerance in twelve species of East African cichlids: potential for low oxygen refugia in Lake Victoria. Conservation Biology 9: 1274-1288.

Chapman, L.J. & C.A. Chapman. 1998. Hypoxia tolerance of the Mormyrid *Petrocephalus catostoma*: implications for persistence in swamp refugia. Copeia 1998: 762-768.

Collins, M.R., S.G. Rogers, T.I.J. Smith & M.L. Moser. 2000. Primary factors affecting sturgeon populations in the southeastern United States: fishing mortality and degradation of essential habitats. Bulletin of Marine Science 66: 917-928.

Courtenay, S.C. & M.H.A. Keenleyside. 1983. Wriggler-hanging: a response to hypoxia by brood-rearing *Herotilapia multispinosa* (Teleostei, Cichlidae). Behaviour 85: 183-197.

Crocker, C.E. & J.J. Cech Jr. 1997. Effects of environmental hypoxia on oxygen consumption rate and swimming activity in juvenile white sturgeon, *Acipenser transmontanus*, in relation to temperature and life intervals. Environmental Biology of Fishes 50: 383-389.

Cruz-Neto, A.P. & J.F. Steffensen. 1997. The effects of acute hypoxia and hypercapnia on oxygen consumption of the freshwater European eel. Journal of Fish Biology 50: 759 - 769.

Dahlberg, M.L., D.L. Shumway & P. Doudoroff. 1968. Influence of dissolved oxygen and carbon dioxide on swimming performance of largemouth bass and coho salmon. Journal of the Fisheries Research Board of Canada 25: 49-70.

Dalla Via, J., G. Van den Thillart, O. Cattani & P. Cortesi. 1998. Behavioural responses and biochemical correlates in *Solea solea* to gradual hypoxic exposure. Canadian Journal of Zoology 76: 2108-2113.

Davis, G.E., J. Foster, C.E. Warren & P. Doudoroff. 1963. The influence of oxygen concentration on the swimming performance of juvenile Pacific salmon at various temperatures. Transactions of the American Fisheries Society 92: 111-124.

Downing, K.M. 1954. The influence of dissolved oxygen concentration on the toxicity of potassium cyanide to rainbow trout. The Journal of Experimental Biology 31: 161-164.

Downing, K.M. & J.C. Merkens. 1955. The influence of dissolved-oxygen concentration on the toxicity of un-ionized ammonia to rainbow trout (*Salmo gairdnerii* Richardson). Annals of Applied Biology 43: 243-246.

Drewett, N. & P.D. Abel. 1983. Pathology of lindane poisoning and of hypoxia in the brown trout, *Salmo trutta* L. Journal of Fish Biology 23: 373-384.

Dunson, W.A. & D.B. Dunson. 1999. Factors influencing growth and survival of the killifish, *Rivulus marmoratus*, held inside enclosures in mangrove swamps. Copeia 1999: 661-668.

Fernandes, M.N., W.R. Barrionuevo & F.T. Rantin. 1995. Effects of thermal stress on respiratory responses to hypoxia of a South American Prochilodontid fish, *Prochilodus scrofa*. Journal of Fish Biology 46: 123-133.

Fry, F.E.J. 1951. A fractionating column to provide water of various dissolved oxygen contents. Canadian Journal of Technology 29: 144-146.

Gee, J.H. & P.A. Gee. 1995. Aquatic surface respiration, buoyancy control and the evolution of air-breathing in gobies (Gobiidae: Pisces). The Journal of Experimental Biology 198: 79-89.

Geiger, S.P., J.J. Torres & R.E. Crabtree. 2000. Air breathing and gill ventilation frequencies in juvenile tarpon, *Megalops atlanticus*: responses to changes in dissolved oxygen, temperature, hydrogen sulfide, and pH. Environmental Biology of Fishes 59: 181-190.

Hamilton, S.K., S.J. Sippel, D.F. Calheiros & J.M. Melack. 1997. An anoxic event and other biogeochemical effects of the Pantanal wetland on the Paraguay River. Limnology and Oceanography 42: 257-272.

Hargrave, B.T., D.E. Duplisea, E. Pfeiffer & D.J. Wildish. 1993. Seasonal changes in benthic fluxes of dissolved oxygen and ammonium associated with marine cultured Atlantic salmon. Marine Ecology Progress Series 96: 249-257.

Hunn, J.B. 1969. Chemical composition of rainbow trout urine following acute hypoxic stress. Transactions of the American Fisheries Society 98: 20-22.

Johnston, I.A. 1975. Studies on the swimming musculature of the rainbow trout. II. Muscle metabolism during severe hypoxia. Journal of Fish Biology 7: 459-467.

Jones, J.C. & J.D. Reynolds. 1999a. Oxygen and the trade-off between egg ventilation and brood protection in the common goby. Behaviour 136: 819-832.

Jones, J.C. & J.D. Reynolds. 1999b. Costs of egg ventilation for male common gobies breeding in conditions of low dissolved oxygen. Animal Behaviour 57: 181-188.

Jones, J.C. & J.D. Reynolds. 1999c. The influence of oxygen stress on female choice for male nest structure in the common goby. Animal Behaviour 57: 198-196.

Kaenel, B.R., H. Buehrer & U. Uehlinger. 2000. Effects of aquatic plant management on stream metabolism and oxygen balance in streams. Freshwater Biology 45: 85-95.

Kakuta, I. & S. Murachi. 1992. Renal response to hypoxia in carp, *Cyprinus carpio*: changes in glomerular filtration rate, urine and blood properties and plasma cataecholamines of carp exposed to hypoxic conditions. Comparative Biochemistry and Physiology 103A: 259-267.

Kakuta, I., K. Namba, K. Uematsu & S. Murachi. 1992. Effects of hypoxia on renal function in carp, *Cyprinus carpio*. Comparative Biochemistry and Physiology 101A: 769-774.

Kaufmann, R. & W. Wieser. 1992. Influence of temperature and ambient oxygen on the swimming energetics of cyprinid larvae and juveniles. Environmental Biology of Fishes 33: 87-95.

Kramer, D.L. 1987. Dissolved oxygen and fish behaviour. Environmental Biology of Fishes 18: 81-92.

Kramer, D.L. & J.P. Mehegan. 1981. Aquatic surface respiration, an adaptive response to hypoxia in the guppy, *Poecilia reticulata* (Pisces, Poeciliidae). Environmental Biology of Fishes 6: 299-313.

McDonald, D.G. & B.R. McMahon. 1977. Respiratory development in Arctic char *Salvelinus alpinus* under conditions of normoxia and chronic hypoxia. Canadian Journal of Zoology 55: 1461-1467.

Martin, B.A. & M.K. Saiki. 1999. Effects of ambient water quality on the endangered lost river sucker in Upper Klamath Lake, Oregon. Transactions of the American Fisheries Society 128: 953-961.

Melnychuk, M.C. & L.J. Chapman. 2002. Hypoxia tolerance of two haplochromine cichlids: swamp leakage and potential for interlacustrine dispersal. Environmental Biology of Fishes 65: 99-110.

Miller, D.C., S.L. Poucher & L. Coiro. 2002. Determination of lethal dissolved oxygen levels for selected marine and estuarine fishes, crustaceans, and a bivalve. Marine Biology 140: 287-296.

Moore, W.G. 1942. Field studies on the oxygen requirements of certain fresh-water fishes. Ecology 23: 319-329.

Mount, D.I. 1961. Development of a system for controlling dissolved oxygen content of water. Transactions of the American Fisheries Society 90: 323-327.

Oliveira, A.M. & B. Kjerfve. 1993. Environmental responses of a tropical coastal lagoon system to hydrological variability: Mundau-Manguaba, Brazil. Estuarine, Coastal and Shelf Science 37: 575-591.

Petersen, J.K. & G.I. Petersen. 1990. Tolerance, behaviour and oxygen consumption in the sand goby, *Pomatoschistus minutus* (Pallas), exposed to hypoxia. Journal of Fish Biology 37: 921-933.

Pichavant, K., Person-Le-Ruyet, N. Le Bayon, A. Severe, A. Le Roux, L. Quemener, V. Maxime, G. Nonnotte & G. Bouef. 2000. Effects of hypoxia on growth and metabolism of juvenile turbot. Aquaculture 188: 103-114.

Pichavant, K., J. Person-Le-Ruyet, N. Le Bayon, A. Severe, A. Le Roux & G. Boeuf. 2001. Comparative effects of long-term hypoxia on growth, feeding and oxygen consumption in juvenile turbot and European sea bass. Journal of Fish Biology 59: 875-883.

Pihl, L., S.P. Baden & R.J. Diaz. 1991. Effects of periodic hypoxia on distribution of demersal fish and crustaceans. Marine Biology 108: 349-360.

Plante, S., D. Chabot & J.-D. Dutil. 1998. Hypoxia tolerance in Atlantic cod. Journal of Fish Biology 53: 1342-1356.

Pouliot, T., J. de la Noue & A.G. Roberge. 1988. Influence of diet and hypoxia on brain serotonin and catecholamines in rainbow trout (*Salmo gairdneri*). Comparative Biochemistry and Physiology 89C: 57-64.

Pouliot, T. & J. de la Noue. 1989. Feed intake, digestibility and brain neurotransmitters of rainbow trout under hypoxia. Aquaculture 79: 317-327.

Rantin, F.T., A.L. Kalinin, M.L. Glass & M.N. Fernandes. 1992. Respiratory responses to hypoxia in relation to mode of life of two erythrinid species (*Hoplias malabaricus* and *Hoplias lacerdae*). Journal of Fish Biology 41: 805-812.

Renshaw, G.M.C. & S.E. Dyson. 1999. Increased nitric oxide synthase in the vasculature of the epaulette shark brain following hypoxia. NeuroReport 10: 1707-1712.

Richardson, J., E.K. Williams & C.W. Hickey. 2001. Avoidance behaviour of freshwater fish and shrimp exposed to ammonia and low dissolved oxygen separately and in combination. New Zealand Journal of Marine and Freshwater Research 35: 625-633.

Ruggerone, G.T. 2000. Differential survival of juvenile sockeye and coho salmon exposed to low dissolved oxygen during winter. Journal of Fish Biology 56: 1013-1016.

Schofield, P.J. & L.J. Chapman. 2000. Hypoxia tolerance of introduced Nile perch: implications for survival of indigenous fishes in the Lake Victoria basin. African Zoology 35: 35-42.

Schurmann, H. & J.F. Steffensen. 1994. Spontaneous swimming activity of Atlantic cod *Gadus morhua* exposed to graded hypoxia at three temperatures. The Journal of Experimental Biology 197: 129-142.

Schurmann, H. & J.F. Steffensen. 1997. Effects of temperature, hypoxia and activity on the metabolism of juvenile Atlantic cod. Journal of Fish Biology 50: 1166-1180.

Scott, A.L. & W.A. Rogers. 1980. Histological effects of prolonged hypoxia on channel catfish *Ictalurus punctatus* (Rafinesque). Journal of Fish Diseases 3: 305-316.

Siefert, R.E. & W.A. Spoor. 1974. Effects of reduced oxygen on embryos and larvae of the white sucker, coho salmon, brook trout, and walleye. pp. 487-495. In: J.H.S. Blaxter (ed.) The Early Life History of Fish, Springer-Verlag, New York.

Swift, D.J. & R. Lloyd. 1974. Changes in urine flow rate and haematocrit value of rainbow trout *Salmo gairdneri* (Richardson) exposed to hypoxia. Journal of Fish Biology 6: 379-387.

Tallqvist, M., E. Sandberg-Kilpi & E. Bonsdorff. 1999. Juvenile flounder, *Platichthys flesus* (L.), under hypoxia: effects on tolerance, ventilation rate and predation efficiency. Journal of Experimental Marine Biology and Ecology 242: 75-93.

Taylor, J.C. & J.M. Miller. 2001. Physiological performance of juvenile southern flounder, *Paralichthys lethostigma* (Jordan and Gilbert, 1884), in chronic and episodic hypoxia. Journal of Experimental Marine Biology and Ecology 258: 195-214.

Thetmeyer, H., U. Waller, K.D. Black, S. Inselmann & H. Rosenthal. 1999. Growth of European sea bass (*Dicentrarchus labrax* L.) under hypoxic and oscillating oxygen conditions. Aquaculture 174: 355-367.

Thomason, J.C., J. Davenport & E. Le Comte. 1996. Ventilatory mechanisms and the effect of hypoxia and temperature on the embryonic lesser spotted dogfish. Journal of Fish Biology 49: 965-972.

Townsend, S.A., K.T. Boland & T.J. Wrigley. 1992 Factors contributing to a fish kill in the Australian wet/dry tropics. Water Research 26: 1039-1044.

Tucker, K.A. & G.A. Burton Jr. 1999. Assessment of nonpoint-source runoff in a stream using in situ and laboratory approaches. Environmental Toxicology and Chemistry 18: 2797-2803.

van Raaij, M.T.M., E. Bakker, M.C. Nieveen, H. Zirkzee & G.E.E.J.M. van den Thillart. 1994. Energy status and free fatty acid patterns in tissues of common carp (*Cyprinus carpio*, L.) and rainbow trout (*Oncorhynchus mykiss*, L.) during severe oxygen restriction. Comparative Biochemistry and Physiology 109A: 755-767.

Vig, E. & J. Nemcsok. 1989. The effects of hypoxia and paraquat on the superoxide dismutase activity in different organs of carp, *Cyprinus carpio* L. Journal of Fish Biology 35: 23-25.

Waller, U., E. Black, D. Burt, C. Groot & H. Rosenthal. 2000. The reaction of young coho *Oncorhynchus kisutch* to declining oxygen levels during long-term exposure. Journal of Applied Ichthyology 16: 14-19.

Whitfield, A.K. & A.W. Paterson. 1995. Flood-associated mass mortality of fishes in the Sundays Estuary. Water SA 21: 385-389.

Whitmore, C.M., C.E. Warren & P. Doudoroff. 1960. Avoidance reactions of salmonid and centrarchid fishes to low oxygen concentrations. Transactions of the American Fisheries Society 89: 17-26.

Winn, R.N. & D.M. Knott. 1992. An evaluation of the survival of experimental populations exposed to hypoxia in the Savannah River estuary. Marine Ecology Progress Series 88: 161-179.

APPENDIX 2

POLICY IMPLICATIONS SUMMARY FOR:
"Growing the Smart State 2003: A PhD Research Funding Program"

"Fish health and water quality in agricultural landscapes"

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Introduction:
Low dissolved oxygen (hypoxia) is one of the greatest killers of freshwater fish in Australia and around the world. In tropical Australian rivers, dissolved oxygen levels are affected naturally by seasonal floods, and run-off from nutrient-rich soil. Adding nutrients to the water (e.g., via fertilisers) initiates increases in plant and algal abundance. These plants and algae release oxygen during the day, by the process of photosynthesis, but also use oxygen in respiration during the night. For this reason, creeks affected by eutrophication (excess nutrient load) show strong daily patterns of dissolved oxygen cycling, where oxygen levels are high in the evening but drop during the night to a minimum at dawn. This process is known as "fluctuating hypoxia".

Although fish kills occur in Australian coastal waters, very little information is available on how Australian freshwater fish are affected by sublethal levels of hypoxia. None is available for species in the tropics. My project aimed to address this issue by experimentally identifying levels of fluctuating hypoxia that adversely affect fish condition. Condition of fish was assessed in this study by measures of reproductive health, behaviour and growth, with additional information on effects of hypoxia on organ health and fish eggs. Four species of fish were used – barramundi (*Lates calcarifer*), eastern rainbowfish (*Melanotaenia splendida splendida*), Utchee Creek rainbowfish (*Melanotaenia utcheensis*) and sooty grunter (*Hephaestus fuliginosus*). These species were chosen for their diverse ecological characteristics, as well as for their importance to Queensland industries.

Research Project Outcomes:
Results were gathered through experiments exposing fish of various life history stages to diurnally fluctuating hypoxia. The treatments were differentiated by the lowest oxygen level reached each day.
    Juvenile barramundi
Juvenile barramundi were adversely affected in terms of growth and food consumption in treatments where dissolved oxygen (DO) reached less than 20% saturation, at 28°C (Figure 1). They also showed signs of stress under this level and were forced to breathe at the water's surface when DO saturation reached about 7%. Barramundi died at about 3% DO saturation.
    Juvenile eastern rainbowfish
Eastern rainbowfish died when DO saturation reached 7%, 4% higher than barramundi. However in the field, both species can perform aquatic surface respiration (ASR) and access highly oxygenated water at the air:water interface to survive even lower oxygen concentrations for short periods of time.

FIGURE 1: Change in weight (% increase) of barramundi in each DO treatment.
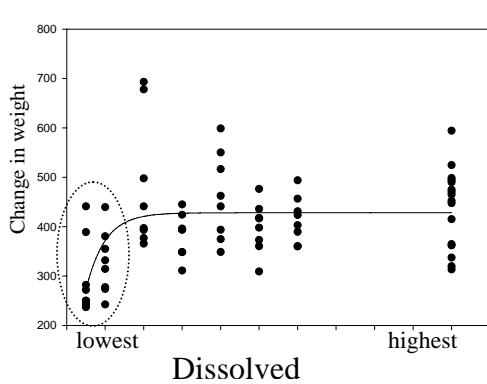
FIGURE 2: Change in weight (% increase) of sooty grunter in each DO treatment.
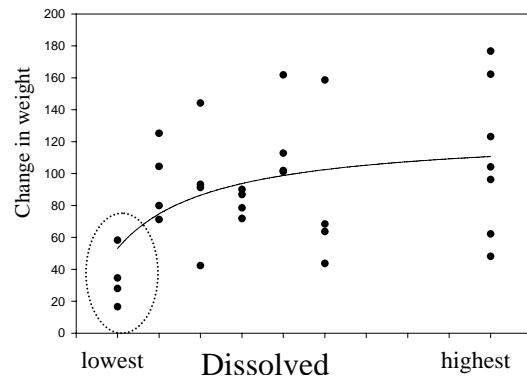
Juvenile sooty grunter

Sooty grunter died at 7% DO saturation, and did not attempt ASR, suggesting this is their absolute lethal DO limit and if waters dropped to this level in the field fatalities would result. In the lowest surviving DO treatment, sooty grunter grew at a slower rate than those in other treatments (Figure 2).

Adult Utchee Creek rainbowfish

Reproductive health of Utchee Creek rainbowfish was tested under hypoxia treatments. Rainbowfish died at 7% DO (as was the case for eastern rainbowfish), and some fish stopped laying eggs after 18 days of diurnal oxygen cycling to 10% each day. Health of fish gonads did not appear to be affected by the hypoxic treatments, but there was a trend for lower egg production over the duration of the experiment in tanks with lower oxygen levels (Figure 3).

Eggs of Utchee Creek rainbowfish and eastern rainbowfish

The eggs of Utchee Creek rainbowfish and eastern rainbowfish (Figure 4) were found to be remarkably tolerant to hypoxia. Eggs were able to survive and produce viable larvae even after cycling to DO saturations lower than those would kill their parents (lowest tested was 5% at 28°C). This is not likely to be the case for other species.

Policy Implications:

The results of this study have implications for water quality management, particularly in northern Queensland. The fish of the Australian tropics appear to be remarkably tolerant to hypoxia when compared to their counterparts in, for example, Europe or North America, where much more research has been carried out on the topic. However, hypoxia does impact on fish health, growth and reproduction, and can cause widespread fish kills if levels are low enough. It would appear that a level of 20% DO saturation is an important threshold, although more extensive data will be needed to confirm this. If oxygen levels were chronically low, rather than fluctuating to a minimum before returning to normal saturations (as was the case for my experiments), or if oxygen levels declined more rapidly than in these experiments, the thresholds for fish death and for sublethal effects would be much higher.

FIGURE 3: Total number of eggs laid by Utchee Creek rainbowfish in each treatment.
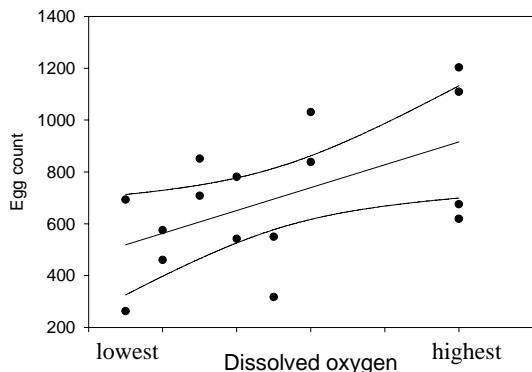
FIGURE 4: Proportion of larvae form each DO treatment that hatched alive and healthy.

My results can be used to assist in policy making including in the "State Coastal Management Plan", the "Sustainable River Management" research area, the "Coastal Planning" research area and the "Sustainability Indicators" research area for the sugar industry.

Included amongst the "State Coastal Management Plan's" 48 policies are issues such as "the values of coastal landscapes and the need to conserve coastal wetlands... and coastal biodiversity... including coastal estuaries... and catchment areas of waterways flowing to the coast". My results can contribute to this Plan, by addressing the question of how the ecology of coastal waterways may be affected by poor water quality, to allow us to improve guidelines for management and conservation of these disappearing and priceless areas.

The state government's "Sustainable River Management" research area aims to "investigate the processes for setting and using end of river water quality targets". The information required to set these targets includes how inhabitants of rivers are affected by degradation of water quality. My results assist in determining the levels of dissolved oxygen that are too low to sustain a healthy population of fish in tropical rivers, and in improving management of agricultural areas.

In addition, the results are of interest to the Premier's "Coastal Planning" research interest, which includes amongst its aims "develop a framework for monitoring ecological changes to RAMSAR sites". The knowledge of how fish health and viability are affected by low oxygen levels, amongst other water quality parameters, is vital for development of this framework.

Because of the financial link between this project and the sugar industry, through funding by the Sugar Research and Development Corporation (SRDC), this project is also of relevance to the Premier's "Sustainability Indicators" research area, which aims to "develop a suite of sustainability indicators for the sugar industry". The sponsorship of this project by SRDC indicates a desire for knowledge from the sugar industry, on how their own practices can affect natural surroundings. Cooperative projects between industry and academia are vital for the sharing of knowledge between both parties, and act as a catalyst to initiate more sustainable and environmentally friendly farming practices.