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Fish Physiology, Toxicology, and Water Quality

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ABSTRACT

Scientists from Europe, North America and South America convened in Capri, Italy, April 24-28, 2006 for the Ninth International Symposium on Fish Physiology, Toxicology, and Water Quality. The subject of the meeting was "Eutrophication: The toxic effects of ammonia, nitrite and the detrimental effects of hypoxia on fish." These proceedings include 22 papers presented over a 3-day period and discuss eutrophication, ammonia and nitrite toxicity and the effects of hypoxia on fish with the aim of understanding the effects of eutrophication on fish. The ever increasing human population and the animals raised for human consumption discharge their sewage into rivers and coastal waters worldwide. This is resulting in eutrophication of rivers and coastal waters everywhere. Eutrophication is associated with elevated ammonia and nitrite levels, both of which are toxic, and the water often becomes hypoxic. Aquatic hypoxia has been shown to reduce species diversity and reduce total biomass.

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ACKNOWLEDGEMENTS

The 9th International Symposium was organized by Drs. Rosemarie Russo and Brenda Rashleigh, US Environmental Protection Agency (EPA) Ecosystems Research Division, Athens, Georgia, Drs. Colin Brauner and David Randall from the Department of Zoology, University of British Columbia, Dr. Bruno Tota, University of Calabria, Italy, and Dr. Göran Nilsson, University of Oslo, Norway. The meeting was held in the Hotel St. Michele on the Isle of Capri, Italy and managed by Ms. Laurajean Carbonaro who did an excellent job. The meeting was funded by the U.S. EPA (EPA-ORD-25489) and the University of British Columbia, Canada, the "A. Dohrn Zoological Station of Naples" and the Doctorate School of Animal Biology of the University of Calabria, Italy. We were advised throughout by Drs. Brenda Rashleigh and Rosemarie Russo. Ms. Kim Suvajdzic assisted greatly in the preparation of this document and Dan Baker, Clarice Fu, Matthew Regan, and Jodie Rummer helped with many of the reviews. Finally, I attribute the success of this Symposium to the many researchers who came to Capri to present their findings.

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Colin Brauner

FOREWORD

Symposia on Fish Physiology, Toxicology, and Water Quality; a Brief History

Vance Thurston and Rosemarie Russo were good friends long before I knew them, and I have known Vance since the 1970's. We would go to Russia together and we had a number of joint research projects. In the early 1980's Rose and I were driving from Athens to Atlanta when she told me she had been asked to organize a science exchange program with several research institutes in China. She asked if I knew anyone in China because she knew I had been a guest of the central government in China and had worked at Zhongshan University in Guangzhou, PRC for several months. The end result was that UBC held the cooperative agreement to encourage research collaboration between environmental scientists from the Peoples' Republic of China and scientists from North America and Europe, under the USA-PRC environmental protection agreement.

The First Symposium was held at Zhongshan University, Guangzhou, PRC, in September 1988, with the help of Professor Lin Hao-ran of Zhongshan University, and attracted scientists from Europe, Canada, and the U.S., as well as many scientists from the PRC and Hong Kong. This was the beginning of the series of international symposia organized by Vance, Rose, and myself, sponsored by the US Environmental Protection Agency through the Athens Laboratory. The Guangzhou Symposium was memorable for its audio equipment: the sound was such that the lectures could be heard by people on boats passing down the Pearl River. The Second Symposium was held two years later in September 1990 in Sacramento, California, with the help of Professor Joe Cech. We had an excellent dinner in the Train Museum; the positive response from all participants illustrated the world wide acceptance of Californian cuisine. The location for the Third Symposium was Nanjing University in Nanjing, PRC, in November 1992 and this time we had the skilled help of Professor Jin Hong-jun. Vance, the great entertainer, sang songs during dinner and as usual brought us together as a group. Vance made a special effort for the Fourth Symposium, held in Bozeman, Montana, in September 1995, to encourage participation of scientists from Europe, as well as from the PRC, North America, and Mexico. Vance drove a van from Bozeman to San Francisco with Chinese delegates on board to show them various sites of ecological interest in North America. We returned to China for the Fifth Symposium, which was held at the City University of Hong Kong, in November 1998 with the able help of Professor Rudolf Wu. The Sixth Symposium was held in La Paz, Mexico, in January 2001. In addition to attracting a large audience from the Mexican scientific community, the 30 papers accepted for presentation represented 15 countries, more than any previous Symposium. The Seventh Symposium, in Tallinn, Estonia, was affectionately dedicated to the memory of Robert Vance Thurston, who died unexpectedly on February 16th, 2002, at the age of 75. Dr. Arvo Tuvikene was a great help in putting that Symposium together, along with Gretchen Rupp from Montana State University. Vance was very active in international environmental research projects in the Baltic republics, the former Soviet Union, and Mexico. He was practically an honorary citizen of Lithuania for the many projects he had there and the computers and other equipment he provided to their scientists. Rose and I organized the Eighth Symposium in Chongqing, China in October 2004 in association with Dr. George

Bailey of Athens EPA and the able help of Professor Gao Yuqi of the 3rd Military Medical University in Chongqing.

The functions of these Symposia are twofold, the first to exchange scientific information and the second to remove political barriers between scientists from different countries and promote collaboration. This has been achieved. Now we come to the Ninth Symposium in this long-standing series, to be held in Capri, Italy with the able assistance of Professor Bruno Tota and Laurajean Carbonaro.

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Attendees of the Ninth International Symposium on Fish Physiology, Toxicology, and Water Quality, Capri, Italy, April 24-28, 2006.

Environmental eutrophication and its effects on fish of the Amazon

by

A.L. Val[1], M.N. Paula da Silva and V.M.F. Almeida-Val

Introduction

Eutrophication is a natural process whereby lakes, estuaries and slow-moving streams receive excess nutrients as a consequence of weathering of rocks and soils from the surrounding watershed. Increased nutrient inputs, particularly phosphorus and nitrogen, result in increased growth of aquatic plants and organic production of the water body. Young water bodies (lakes and man made reservoirs) usually are oligotrophic as they have low levels of nutrients and correspondingly low levels of biological activity. In contrast, old water bodies possess high biological activity as a consequence of high nutrient levels. These are referred to as eutrophic water bodies. The natural time scale from being oligotrophic to eutrophic is in the order of thousands of years, depending on the levels of encrusted minerals and on the rate of watershed weathering, among other environmental characteristics (Wetzel, 1975). These terms were first applied to lakes by Naumann in early 1900 (Naumann, 1919, 1927) noting that oligotrophic lakes contained modest levels of algae and were often found in igneous rock areas while eutrophic lakes contained high amounts of algae and were found in more fertile lowland regions. The author concluded that within a normal thermal range, levels of phosphorus, nitrogen and calcium are the primary determining factors of lake trophic status.

There is no single or simple definition of eutrophy or oligotrophy. These states are the extremes along an axis defining the trophic state of a given water mass. Based on phosphorus concentrations, Mueller and Helsel (1999) classified lakes with concentrations below10µgP/L as oligotrophic, those with concentrations between10-20µgP/L as mesotrophic, and those lakes with concentrations exceeding 20µg/L as eutrophic. The relationship between mineralization and production is also an important tool defining the trophic state of water bodies as, in general, in oligotrophic lakes production and mineralization are closely coupled, while in eutrophic lakes production far exceeds mineralization resulting in accumulation of organic matter and a depletion of oxygen (Niell *et al*., 2005). Regardless of the definition employed, the evolution of a water body from oligotrophic to eutrophic state is characterized by an overload of nutrients, mainly nitrogen and phosphate, that displaces the system from the equilibrium.

In 1934, Alfred Redfield proposed that the N:P ratio in the interior of all major oceans were remarkably similar, based on empirical observation. This ratio is 16:1 (N:P) and is similar to that of plankton from all over the world. Today, the residence time of

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these elements relative to the ocean's circulation time is though to be the basis of the Redfield ratio. Large lakes, though much more variable due to the effects of surrounding ecosystems, display similar elemental composition ratios (Falkowski and Davis, 2004). Indeed, some variability is expected due to several internal processes such as recycling, sedimentation, resuspension or release from the bottom, nitrogen fixation, temperature, among other factors, in both oceans and lakes (Tundisi and Matsumura-Tundisi, 1984; Howarth, 1988).

More recently, eutrophication rates have increased dramatically as a consequence of alterations in nutrient cycles related to land-use changes, i.e., related to human inputs of urban and agricultural waste, including sewage and fertilizers. Analyses of rivers in the temperate zone indicate an increase of 3-20 fold of river nitrogen export in developed areas since industrialization. Since 1960, our population has doubled (we were six billion people in 2000) which has necessitated a doubling of food production. However, the use of N and P has increased at a much higher rate: the use of N as fertilizer increased 8.8 fold (from 10 to 88 million metric tons) and the use of P increased 4.4 fold (from 9 to 40 million metric tons). There is a projection of a further 50% increase in the use of these fertilizers in decade of 2030-2040 (Vance, 2001). These changes lead to the concept of cultural eutrophication where sudden environmental changes (10 years or less, a time frame that contrasts to that of natural eutrophication of 1,000-10,000 years) displace the ecosystem into a state of un-compensated disequilibrium (Stirn, 1987).

In contrast to temperate water bodies, nutrient fluxes in tropical water bodies are less well documented, limiting projections of aquatic disturbances caused by anthropogenic factors such as increased silt, deforestation, nutrient loads and land-use perturbations. In the Amazon, these perturbations are exacerbated by two important environmental factors: a) increased incidence of ultraviolet radiation, and b) increased temperature. The purpose of this review is to analyze the current status of eutrophication in the Amazon, both natural and cultural in origin, and the effects of eutrophication on fish of the Amazon.

Eutrophication in the Amazon

In general, there is a clear trend between pristine N-export and water runoff which is much higher for tropical rivers compared to temperate ones. There are two exceptions: the Rio Negro, a tributary of the Amazon, that is exceptionally low in sediment load, rich in dissolved organic carbon, poor in ions and behaves as most pristine temperate rivers; and Mackenzie and Lena Rivers that are large high latitude rivers carrying large sediment loads and behaving as most tropical pristine rivers (Sioli, 1984; Furch and Junk, 1997; Downing *et al*., 1999).

The major ecological driving force in the Amazon is the annual river water level oscillation that affects nearly all organic-aquatic environment interactions (Fink and Fink, 1979; Val and Almeida-Val, 1995). The annual regular flood pulse (Junk *et al*., 1989) extends over significant parts of nearby rivers flooding a myriad of *ria* and *varzea* lakes. As the flooding occurs for few months every year, the lakes respond with bursts of

production which are swept into the rivers (Rai and Hill, 1984). However, as the flood pulses occur at different periods of the year within the region, the overall production in the main river channel is low.

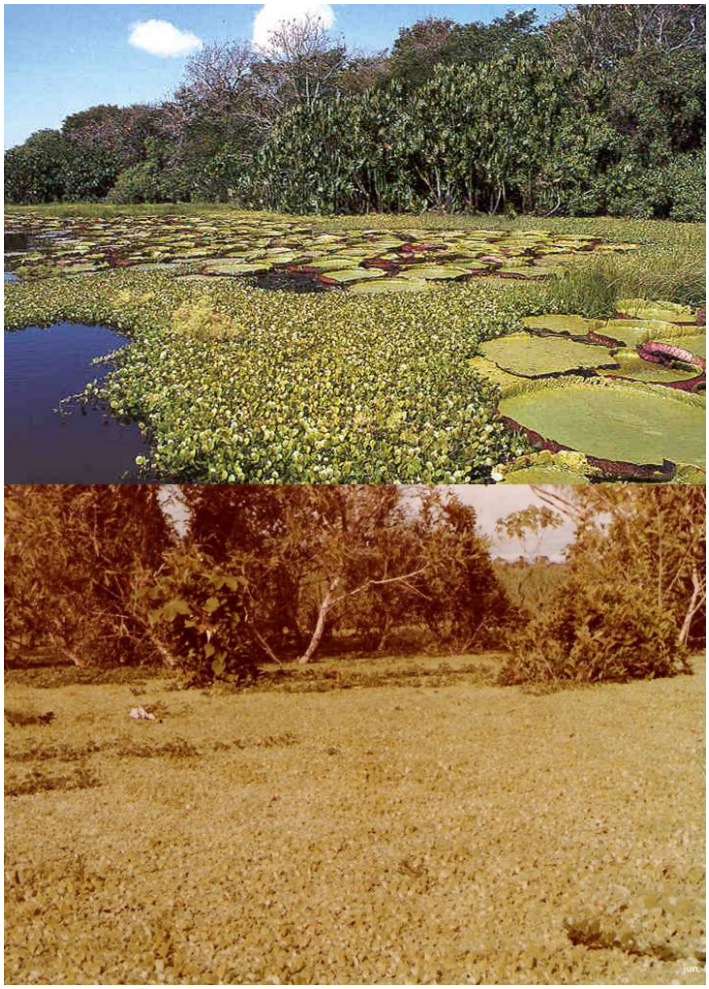

Figure 1. The nutrients unloaded into the *várzea* as the river water level increases result in an extensive propagation of aquatic plants that cover extensive areas water surface limiting light penetration and so causing reduced photosynthesis. As the water recedes the aquatic plants decomposes what results in a further deterioration of water quality.

As the river water level increases it floods extensive areas unloading its nutrient rich sediment which causes excessive growth of phytoplankton, algae and rooted aquatic plants (macrophytes). These plants cover the entire water surface in many places (Fig. 1) reducing light levels and rates of photosynthesis in the water column. When the water stops flowing into these areas, there is a decrease in available nutrients causing extensive plant decay. Subsequently, the water recedes leaving behind an enormous amount of dead aquatic plants that lead to anoxia, high levels of hydrogen sulfide, methane and ammonia. The naturally fertilized soil left behind is then used by locals for production of vegetables

before the next flood. So, the cycles of natural eutrophication in the pristine areas of the Amazon have many social, economical and ecological implications (Junk, 1984; Val and Almeida-Val, 1995; Junk, 1996).

In general, tropical freshwaters are more nitrogen limited than temperate freshwaters while phosphorus is frequently more limiting in tropical marine systems (Downing *et al*., 1999). These authors suggest that disturbances to pristine tropical land will lead to profound freshwater disturbances. They have analyzed the effects of cultural eutrophication, from deforestation, a typical disturbance of all phases of disturbances in tropical regions, up to urban and industrial development and hypothesized for tropical aquatic systems, including the Amazon, a significant increase of N-export and a decrease of N:P ratio (see Downing *et al*., 1999).

Eutrophication, regardless of origin, causes significant changes within aquatic communities. Fish for example experience a change in food availability as the food web is dramatically affected by changes in water quality. Deterioration of water quality imposes additional physiological challenges to fish that are naturally exposed to periodic episodes of hypoxia, hydrogen sulfide and ammonia. In some cases the animals are exposed to extreme conditions, hypoxia and hyperoxia, for example, within short periods of time. In other cases, eutrophication may over expose fish to uncommon environmental conditions, as ultraviolet, as under hypoxia many fish species breathe at the water-air interface.

Effects of eutrophication on fish of the Amazon

Natural eutrophication is a cyclic process that occurs every year in the Amazon. Amazonian fish have evolved a myriad of adjustments to survive such environmental conditions.

Oxygen

The fish of the Amazon face low dissolved oxygen, a regular environmental constraint since the formation of the Amazon basin. In fact, oxygen levels below the present atmospheric levels existed when the major fish groups appeared during the Devonian (Acanthopterygii), the Triassic (Teleostei), and the Jurassic (Euteleostei) (Dudley, 1998). Adaptations to low O_2 levels occur at the behavioral, morphological, physiological, and biochemical level and adjustments can be made as soon as the animal senses hypoxia. Oxygen sensing has been analyzed under a variety of conditions and in a diversity of animals and plants (Hochachka, 1996; Sundin *et al*., 2000; Bailey-Serres and Chang, 2005). However, we are far from a clear picture of this issue for fish of the Amazon.

The first line of defense against hypoxia is behavioral. At least two behavioral changes have been observed among the fish of the Amazon: a position change within the water column and lateral migration. When exposed to hypoxia, many fish species move to the upper region of the water column, close to the air-water interface, where more

dissolve oxygen is naturally available. While some fish species expand their lower lip to facilitate surface skimming, as many species of serrasalmids, others gulp air and water into the digestive system, as some loricariids (Gradwell, 1971), to aid in oxygen uptake (Val, 1995). If access to the upper region of the water column is denied, there is a significant decrease of blood oxygenation as observed for *Pterygoplichthys multiradiatus* (Val, 1995). Lateral migration refers to a movement between the main river channel, where dissolved oxygen is more stable, and the flooded forest, where food is available (Junk *et al*., 1983). The sunset is accompanied by a significant decrease in dissolved oxygen due to a reduction of photosynthesis and an increase of respiration and serves as cue for many fish species to migrate back to the main river. Early in the morning these fish migrate again back to the flooded forest to feed. Lateral migration has been described for fish species in the Amazon (Lowe McConnell, 1987), Pantanal (Antunes de Moura, 2000), and in Africa (Bénech and Quensière, 1982). These behavioral changes have been reported for several fish species and have been described as adaptive convergence (Brauner and Val, 2006).

Air-breathing for fish means independence from fluctuations of dissolved oxygen. In addition to the obligatory air-breathers, such as *Arapaima* and *Lepidosiren*, there are several groups of facultative air-breathers (Table I). Facultative air-breathers use many structures to take up oxygen directly from air and are able to switch from water- to air-breathing according to dissolve oxygen availability. In general, under normoxia these animals are aquatic breathers while under hypoxia they rely on some degree of aerial respiration, using a variety of air-breathing organs (ABO) (Val, 1999). Indeed, facultative air-breathers switching to air-breathing experiences some extra physiological adjustments related to blood chemistry, as blood oxygenation occur at the ABO and carbon dioxide excretion takes place at the gills. Aerial exposure may also increase the risk of predation.

Table I. Major air-breathing fish families of the Amazon. O=obligatory air-breather; F-facultative air-breather; L=lung; SB=swim bladder; Sk=skin; S/I=stomach and intestine; PBM=pharyngeal, branchial and mouth diverticula.

Fish Family	Type	ABO
Lepidoseriniformes	O	L
Arapaimidae	O	SB
Erythrinidae	F	Sk, SB, S/I
Doradidade	F	S/I
Callichthyidae	F	S/I
Loricariidae	F	S/I
Rhamphichthyidae	F	PBM
Electrophoridae	O	PBM
Synbranchidae	F	PBM

If hypoxia cannot be avoided, fish make adjustments either directed towards increasing oxygen transfer to tissues or reducing oxygen consumption through metabolic depression. Adjustments to blood characteristics are very common when fish are exposed to stress which includes adrenergically mediated red blood cell swelling and release of red blood cells from the spleen (Val, 1993; Randall and Perry, 1994) as reported in tambaqui (Moura, 1994). In general, hypoxia causes a reduction of spontaneous activity and metabolic consumption of oxygen (Brauner *et al*., 1995; Almeida-Val *et al*., 2000), an increase in gill ventilation rate (Rantin *et al*., 1992) and bradycardia (Rantin *et al*., 1995).

In addition to behavioral and morphological adjustments, fish are able to increase blood-oxygen affinity by adjusting the levels of organic phosphates within the erythrocytes (Val, 2000). ATP and GTP are the major organic phosphates detected in fish erythrocytes and both are negative modulators of Hb-O_2 affinity. The concentration of these phosphates within erythrocytes is regulated according to dissolved oxygen, i.e., the lower the oxygen availability, the lower the levels of these phosphates. The reduction of erythrocytic levels of ATP and GTP results in an increase of Hb-O_2 affinity, safeguarding oxygen loading at the gills. In general the regulation of GTP is faster than the regulation of ATP. In addition to ATP and GTP, other phosphates have been detected in the erythrocytes of fish of the Amazon, namely 2,3DPG in *Hoplosternum littorale*, IPP in *Arapaima gigas*, and IP_2 (inositol diphosphate) and UTP (uridine-5'-triphosphate), in *Lepidosiren* (Val, 2000).

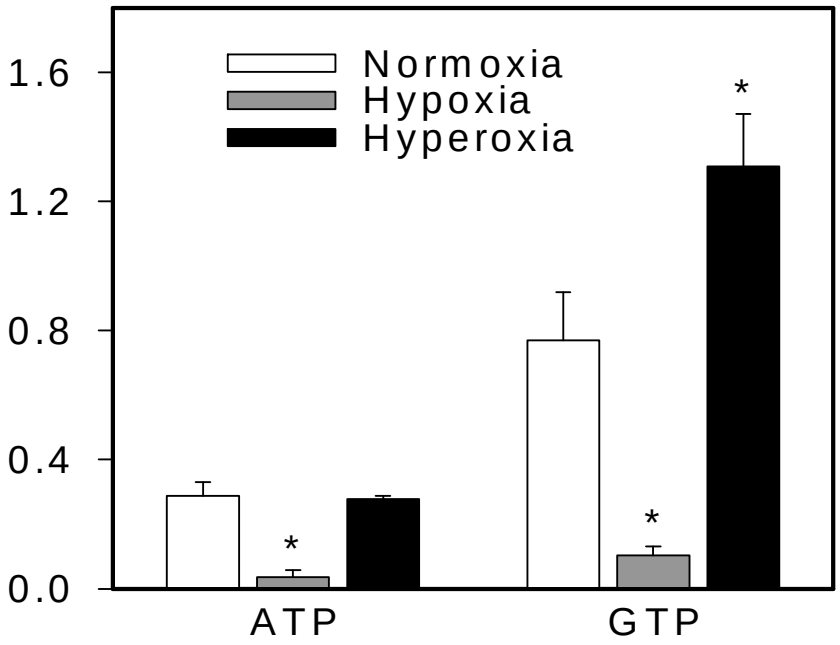

Figure 2. Erythrocytic ATP and GTP levels in specimens of *Pygocentrus nattereri*, a species of piranha, exposed to normoxia, hypoxia and hyperoxia.

Eutrophication may lead some water bodies to oxygen super-saturation during the day; contrasting to hypoxia caused by increased respiration at night. To prevent tissue damage caused by excess oxygen, *Pygocentrus nattereri*, a species of piranha, is able to increase the concentration of erythrocytic ATP and GTP (Fig. 2), decreasing Hb-O_2 affinity. This daily oxygen oscillation (over-saturation during the day and anoxia at night) imposes rapid adjustments directed towards oxygen transfer to tissues. Analysis of ATP and GTP levels over time in the erythrocytes of the same species of piranha above mentioned revealed a 40% reduction of these organic phosphates during the first ten minutes of exposure to deep hypoxia.

So, fish of the Amazon have developed a suite of adjustments to face low oxygen that can be used during cyclic periods of natural eutrophication. Cultural eutrophication processes, however, induce additional challenges that need to be further analyzed.

Ammonia/Nitrite

In addition to changes in available oxygen, eutrophication causes significant accumulation of ammonia, inorganic phosphorus, nitrate and nitrite, particularly with poor tidal flushing and high stocking density (Wu *et al*., 1994; Gonzalez *et al*., 2004). The toxic effect of ammonia on temperate fish has been extensively discussed elsewhere (Tomasso *et al*., 1980; Randall and Wright, 1987; Shingles *et al*., 2001) but is unknown in fish of the Amazon. The ammonia released to water is readily converted to nitrite by *Nitrosomonas*. Nitrite together with other nitrogen compounds increases in *várzea* lakes as result of plant decomposition and diffuses across the fish gills and then into the erythrocytes, there converting hemoglobin to methemoglobin. Methemoglobin is unable to bind reversibly to oxygen which further aggravates the effects of eutrophication on fish.

Hydrogen sulfide

Exceptionally high levels of hydrogen sulfide (HS) occur at the *várzea* lakes due to the circulation of the water column, particularly after eutrophication and plant decay. Together with HS, oxygen poor water is displaced from the bottom. Hydrogen sulfide inhibits a series of enzymes, including many related to oxidative phosphorylation, causing metabolic impairment and generation of oxygen radicals (Nichols and Kim, 1982; Hill *et al*., 1984). Thus, fishes exposed to HS are unable to maintain regular biological activities. As hydrogen sulfide occurs together with low oxygen, many fish alter conditions to increase oxygen transfer to tissues, resulting in an even greater HS transfer to tissues (Val, 1999).

In general, the great majority of fish that survive in *varzea* lakes during periods of high HS/low oxygen are air-breathers. Analysis of the effect of HS on the facultative air-breather *Hoplosternum littorale* indicate that air-breathing represents an important adaptation to reduce HS transfer to tissues in this animal (Brauner *et al*., 1995). The authors reported that animals exposed to acidic water (pH 3.8, Po_2=155 mmHg) increased air-breathing frequency to levels displayed by animals exposed to mild hypoxia (28 air

breaths/h (ab/h)). Interestingly, air-breathing frequency increased up to 40ab/h in animals exposed to buffered HS, suggesting that water-air breathing transition may be an adaptation to acidic and high HS waters. In specimens denied access to air, low levels of HS are lethal.

Anthropogenic activities and eutrophication

Cultural eutrophication is a world wide matter of concern. In the Amazon, though it does not represent a major issue due to the shear volume of the system, many anthropogenic activities are taking place, all potentially able to displace the system from equilibrium. Deforestation, fire, siltation, mineral and petroleum mining, river damming and urbanization are, among others, causes of concern. These activities result in an increase of nutrients and energy being transported to the water systems increasing aquatic plant growth and changing water quality. In conjunction with other environmental changes, cultural eutrophication is potentially dangerous to fish. For example, deforestation and fire causes a removal of natural protection against solar radiation (shadowing) in addition to increased siltation. As many fish species of the Amazon respond to low dissolved oxygen by switching from water- to air-breathing, which may increase UV exposure. Exposure to UV causes massive kills and DNA breakdown. Using the comet assay we have observed a significant increase of DNA damage over time of exposure of tambaqui to UVR (Groff *et al*., unpublished data). Crude oil spills close to eutrophic water bodies also create large challenges for fish. The water-air breathing transition results in an increase of crude oil taken in inducing a series of physiological disturbances. Thus, while the fish of the Amazon areprepared to face the regular constraints caused by natural eutrophication, they are not prepared to face cultural eutrophication, in particular when it is associated with other extreme environmental changes such as increased UV and crude oil.

Conclusions

Organisms are expected to respond to novel events as if they are familiar events. Fish of the Amazon have developed a suite of adjustments to face natural constraints of their environment, including those related to cyclic eutrophication process. Hypoxia, for example, is a familiar event in the Amazon and so fish respond to it with adaptations shaped over their existence, but in the case of anthropogenic pressures these adaptations may have negative consequences. Cultural eutrophication is becoming a matter of concern, particularly around the major cities of the Amazon and its evolution should be followed over the next decades to support regional procedures.

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Biochemical responses to hypoxia: The case of amazon fishes

by

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The Amazon basin is the result of geological and climatic phenomena that took place during the different eras. Hypoxic and anoxic conditions were prevalent in the aquatic environment during the Cambrian period, owing to the low atmospheric oxygen levels at that time. Since the Cambrian geological period, oxygen depletion has been a limiting factor for aquatic life in general (Randall *et al*., 1981; Almeida-Val and Farias, 1996; Almeida-Val *et al*., 1999). After the break up in the southern hemisphere that caused the appearance of South America and Africa during the Cretaceous period, the main geological phenomenon causing the Amazonian hydrographic basin formation was the Andean Mountains uplift, occurring in the Tertiary period. This fact caused an enormous change in the region, cutting off the Pacific Ocean drainage of the upper tributaries of Amazon River and changing the whole orientation of the Amazon basin towards the Atlantic Ocean (Reviewed by Val and Almeida-Val, 1995). Natural episodes of hypoxia occur globally and have different causes and effects. The poorly oxygenated waters of the Amazon basin result from a number of phenomena. Annual flood pulses produce an average crest of 10 meters between November and June in central Amazonia and the flooding of the jungle results in a complete new set of habitats becoming available each year, causing large changes in water physical-chemical parameters, including pH, water density, conductivity, temperature, and dissolved oxygen. Currently, these annual flood pulses cause oscillations in oxygen availability with interspersed episodes of severe hypoxia and anoxia (Fig. 1).

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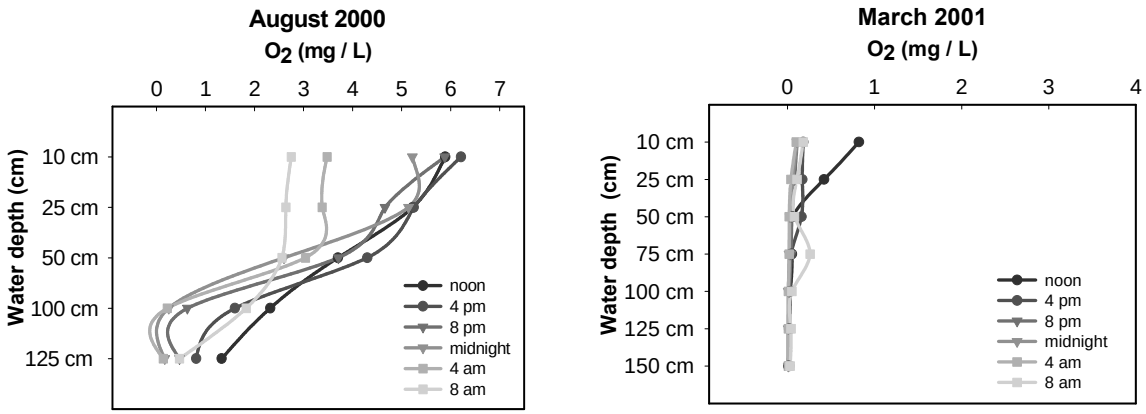

Figure 1. Natural changes in dissolved oxygen in Catalão Lake, located in front of Manaus, where the Negro and Solimoes rivers mix. Panels A and B show diurnal and spatial changes from high and low water level seasons, respectively.

Besides the natural phenomena, there are other causes for the changes in dissolved oxygen, which are common in many water bodies and are caused mainly by human activities. Acute pollution episodes may cause mortality or permanent damage to aquatic organisms. Constant pollution activities also may induce a chronic decrease in oxygen availability, which may result in alterations in species distribution or their occurrence, or cause severe decreases in population size. Interestingly, animals that survive such conditions can adapt to these new hypoxic environments once their evolutionary histories have provided them with many adaptive traits to survive hypoxic conditions. For instance, fish of the Amazon have developed a series of coordinated metabolic adjustments, which, combined with morphological and anatomical changes, have resulted in a number of solutions to avoid or minimize the stress caused by hypoxia (Val and Almeida-Val, 1995). In addition, long and short-term changes in oxygen are determinants of fish distribution in Amazonian water bodies (Almeida-Val *et al*., 1999; Fig. 2).

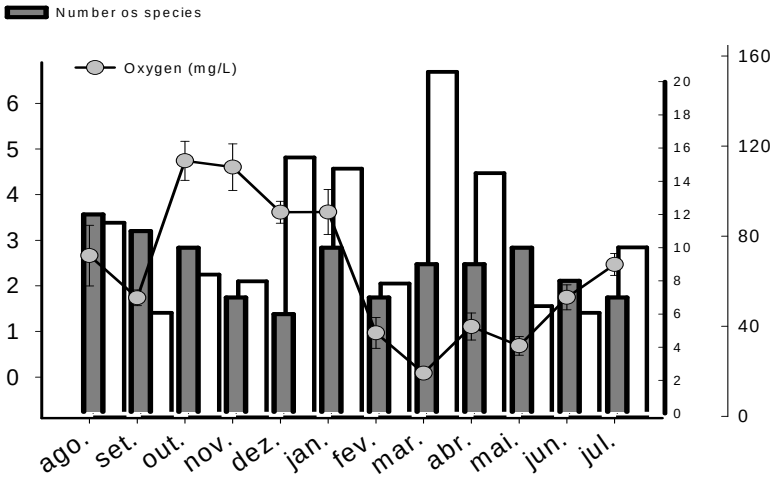

Figure 2. Relationship between oxygen distribution (circle symbols) during the year, August, 2000 to July, 2001 (values obtained at noon) and the abundance (number of fishes – empty bars) and numbers of cichlid species (gray bars) captured near Catalão lake.

Hypoxic episodes may be devastating for most ecosystems because it may cause mass mortality, defaunation of benthic populations, declines in fisheries production, changes in community composition, and, as an ultimate consequence, a decrease in animal diversity. In the Amazon, chronic hypoxic situations are common, and have resulted in fish adaptation at different levels of biological organization, thereby inducing increased species diversity. Seasonal changes in species composition may occur as the result of different oxygen distribution in the environment. Junk *et al*. (1983) showed that low oxygen levels are coincident with a selective occurrence of air-breathing fish species in a varzea lake. The hypoxia tolerant cichlids are among the water-breathing fish remaining in the lake during low oxygen periods.

Some field studies have suggested that fish distribution in aquatic ecosystems of the Amazon is the consequence of aquatic oxygen contents (Junk *et al*., 1983; Crampton, 1998; Chippari-Gomes, 2002). Low oxygen environments are known to be the ideal places for hypoxia tolerant species to avoid predation, competition for food, and other constraints, since during low oxygen season, these places hold few species. Nevertheless, várzea lakes, which are commonly hypoxic, are considered the main nursery site for several Amazon fish species due to the presence of high levels of organic matter. The high abundance of fish fingerlings and juveniles in varzea lakes are, thus, a paradox. To address this issue, we have investigated one of the most anoxia tolerant fish ever described in a tropical region (Muusze *et al*., 1998): the Oscar, *Astronotus ocellatus* (Cichlid: Perciformes).

Studies conducted in our laboratory have demonstrated that both anaerobic power (the ability of the animal to activate anaerobic metabolism, as reflected by LDH absolute levels in skeletal muscle) and hypoxia survivorship are a function of body mass (Almeida-Val *et al*., 1999; 2000). As fish size increases, the ability to survive under severe hypoxia increases as well. The total amount of time required to reach disequilibrium (loss of orientation that precedes death) increases as the animal increases in size, suggesting that the Oscar shows an increase in hypoxia tolerance with age (Fig. 3).

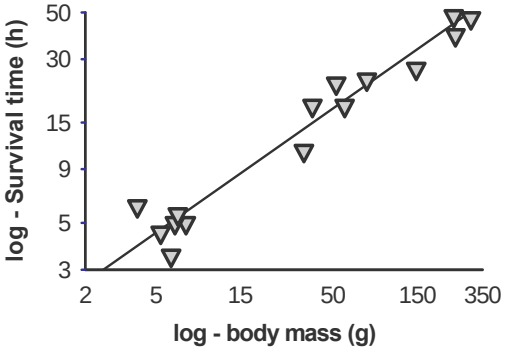

Figure 3. Relationship between body mass (g) of *Astronotus ocellatus* and its ability to survive hypoxia (hours). The log-log regression shows a close relationship (r=0.98) (Almeida-Val *et al*., 1999).

Recent studies comparing the behavior of small and adult Oscars when exposed to hypoxia revealed different strategies between the two groups (Sloman *et al*., 2006). Specific metabolic rates are higher in small animals (Almeida-Val *et al*., 1999, 2000; Sloman *et al*., 2006). Adult Oscars are able to regulate their oxygen consumption by adjustments in respiration and circulation to a lower oxygen threshold than juveniles (50 Torr compared to 70 Torr in the latter). Adult Oscars also have a much greater ability to survive exposure to extreme hypoxia as already mentioned. Fish around 16 g in weight (equivalent to 'small') survived extreme hypoxia for about 9 h (Almeida-Val *et al*., 2000) and the respective larger individuals weighing 230 g, survived for approximately 35 h. Thus while adult Oscars appear to tolerate falling PO_2 slightly better than their juvenile counterparts this difference is magnified considerably once extreme hypoxia is reached and anaerobic metabolism becomes necessary. The greater anaerobic potential of adults, as indicated by higher concentrations of lactate dehydrogenase and malate dehydrogenase, (Almeida-Val *et al*., 2000) also fits with this scenario. Thus, unlike other temperate species (e.g. yellow perch, *Perca flavescens*, Robb and Abrahams, 2003; largemouth bass, *Micropterus salmoides*; Burleson *et al*., 2001) the Oscar shows a positive relationship between physiological tolerance of hypoxia and size.

Besides the increase in anaerobic potential as animals become bigger (Almeida-Val *et al*., 2000), Oscars also have a low metabolic rate, undoubtedly lower than most fish species, as demonstrated by Almeida-Val *et al*. (1999). Among Amazon fishes, metabolic rates of exclusively water-breathing fishes vary related to a spectrum of sluggish to "athletic" type behavior patterns. As would be expected, comparisons between Amazon and temperate fish species suggest that the more sluggish the fish the less the amount of oxygen consumed per unit weight (reviewed by Val and Almeida-Val, 1995).

Metabolic rate plotted *versus* total fish mass shows the relationship between body mass (g) and organism oxygen uptake (mg oxygen per fish per hour). The allometric relationship is described as: $\mathbf{VO_2} = \boldsymbol{a}\mathbf{M}^{\boldsymbol{b}}$**,** where $\boldsymbol{a}$ = log mass coefficient, $\boldsymbol{M}$ = log body mass and $\boldsymbol{b}$ = mass exponent. The value of the exponent b obtained for Oscar is 0.52. Reviewing this exponent for several species of tropical fishes, Hammer and Purps (1996) reported a mean value of 0.73. Compared to other tropical fishes, Oscar has one of the lowest exponents reported. Former studies with temperate fish species described an exponent of 0.86 (Glass, 1969). It is evident that tropical fishes have lower metabolic rates, in general, than temperate fishes, and that, among troical fishes, Oscars have the lowest metabolic rate among all species studied (Fig. 4).

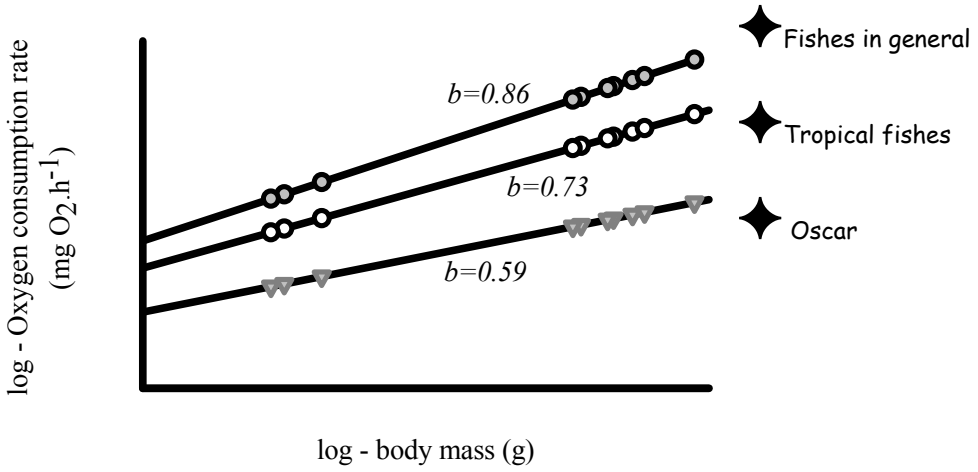

Figure 4. Relationship between body mass (g) and whole organism oxygen uptake of *Astronotus ocellatus* (Oscar) compared with the same relationship for tropical fishes and fishes in general.

We can summarize the Oscar's responses to hypoxia as follows: 1) escaping the hypoxic water or skimming the water surface; 2) reducing metabolic rate; 3) activating anaerobic glycolysis; 4) depressing metabolic rate below standard rates; 5) gene regulation and signal transduction.

Most studies on whole animals conducted in our laboratory with Amazon fish species subjected to some level of oxygen depletion (acute hypoxia, graded hypoxia or anoxia). These studies revealed that the animals showed alterations in plasma glucose and lactate levels resulting from glucose reserve mobilization and anaerobic-based lactate production (Fig. 5 and 6). From this data, it becomes clear that anaerobic glycolysis takes place in most species. This response can be combined with metabolic depression in some species, mainly in those that are already known to be hypoxia-tolerant, such as the cichlids, the group that includes the Oscar.

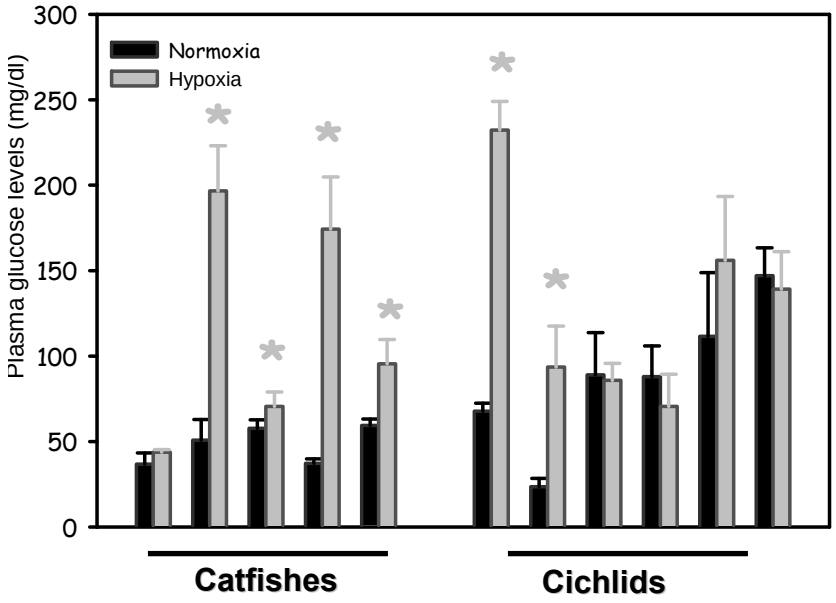

Figure 5. Plasma glucose levels from different species under normoxia and acute hypoxia. The two groups are composed of species of catfishes (Siluriformes) and cichlids (Perciformes) (Data obtained from Almeida-Val *et al*., 2005).

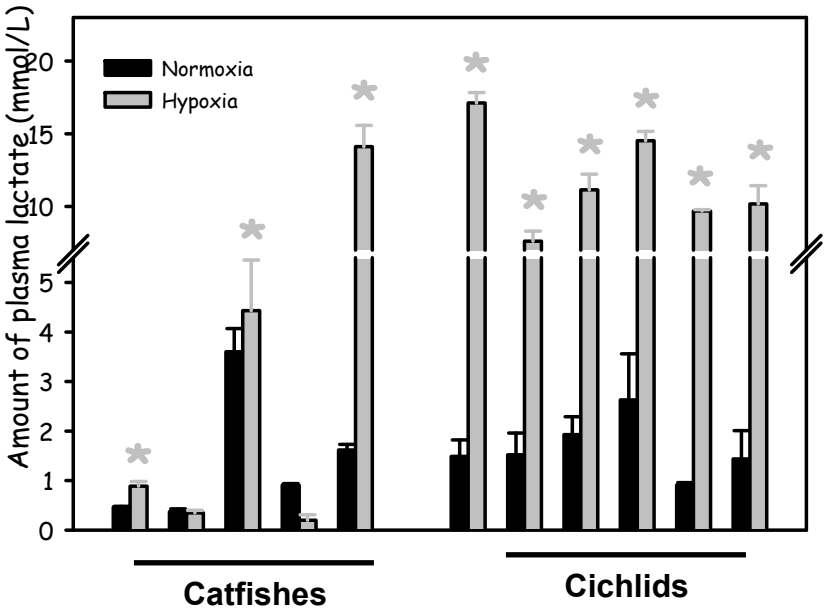

Figure 6. Plasma lactate levels from different species under normoxia and acute hypoxia. The two groups are composed of species of catfishes (Siluriformes) and cichlids (Perciformes). (Data obtained from Almeida-Val *et al*., 2005)

Glucose mobilization may occur even in facultative air-breathers such as the armored catfish *Glyptoperychthys gibbceps*, which is not necessarily related to activation of anaerobic metabolism, since lactate levels are significantly decreased (Lopes, 2003, Fig. 6). When denied aerial respiration under hypoxia in laboratory aquaria, the armored catfish *Glyptoperychthys gibbceps*, increased gill ventilation rates, but no alteration was detected in heart rate, suggesting that bradycardia is not one of their strategies against hypoxia. Thus, it is not possible to affirm that all species respond to hypoxia with similar metabolic adjustment, i.e., the generalization that anaerobic metabolism is activated and aerobic metabolism is suppressed during hypoxia is not true, at least not to all fish species from the Amazon. Nevertheless, cichlids are uniform in their responses.

The ability of the organisms to deal with environmental change depends on the magnitude of the change, the time frame in which the change occurs, and the individual genetic constitution, which may be altered over generations by the selection of genetic variants that are better suited to cope with the new environmental situation. As a consequence, environmental stress has been considered to be among the most important triggers of change in biological organization and functioning during evolution (Almeida-Val *et al*., 1999). Wilson (1976) called attention to the importance of regulatory genes in the evolution of plants and animals. This author stated that "although definitive conclusions are not possible at present, it seems likely that evolution at the organism level depends predominantly on regulatory gene mutations. Structural gene mutations may have a secondary role in organism evolution". So, changes in form, color, morphology, physiology, and metabolism of many organisms may occur according to environmental changes and the investigations about the kind of genetic (or metabolic) control over phenotypes under different environmental conditions have revealed that some genes are turned on or off accordingly (Walker, 1979; Smith, 1990; De Jong, 1995; Land and Hochachka, 1995; Hochachka, 1996; Walker, 1997; Hochachka *et al*., 1998). As we have mentioned on different occasions (Almeida-Val *et al*., 1993; 1999), long-term adaptive responses to low-oxygen environments involves oxidative metabolic suppression in fish of the Amazon, as first suggested by Hochachka and Randall (1978) and corroborated by Driedzic and Almeida-Val (1996) and West *et al*. (1999). However the immediate hypoxia responses from fish of the Amazon have been barely studied from the evolutionary point of view (Almeida-Val *et al*., 1999).

Oxygen sensing and its physiological and biochemical consequences in cells are not fully understood yet, despite the fact that some mechanisms have been extensively studied in isolated cell models. There are many reviews on this subject (Gracey *et al*., 2001; Yu *et al*., 2001). Genes are coordinated and individually regulated during hypoxia by a variety of hypoxia-responsive transcription factors including HIF-1α (Webster, 2003). This system regulates many glycolytic genes, inducing anaerobic glycolysis and down regulating many genes of aerobic metabolism. Investigations of this system indicates that this pathway developed in the Silurian period, 500 MYA, when highly mobile sea and land species were evolving. In fact, this period is coincident with the high DNA duplication rates (polyploidy) which induced the radiation of vertebrates and the appearance of new duplicated genes, giving rise to most gene families and pathway systems currently known to exist in vertebrates. The gene family of LDH is one of the

best studied gene groups, at both the functional and molecular levels. LDH-A* is one of the genes that is up regulated under hypoxia. Studies with cDNA microarrays in fish (*Gillichthys mirabilis*) by Gracey and co-workers (2001) revealed which genes were up-regulated and which were down-regulated. Based on that, they suggested that hypoxia survival may involve three molecular strategies: (i) down-regulating genes for protein synthesis and locomotion to reduce energy consumption; (ii) up-regulating genes for anaerobic ATP production and gluconeogenesis, and (iii) suppressing cell growth and channeling energy to essential metabolic processes. Observed changes in gene expression was tissue-specific and reflected metabolic roles.

Our first experiments conducted with LDH-A* regulation in Oscar showed that hypoxia may induce or suppress its expression according to the size of the animal. Changes in absolute LDH activities may be the result of gene expression and post translational processes that take place after the protein is synthesized.
The first results of LDH-A* expression in response to different oxygen tensions and anoxia have showed some variation in LDH-A* mRNA levels (Fig. 7). Changes in LDH-A* expression may also be related to animal size, tissue sample, and pre-acclimated condition.

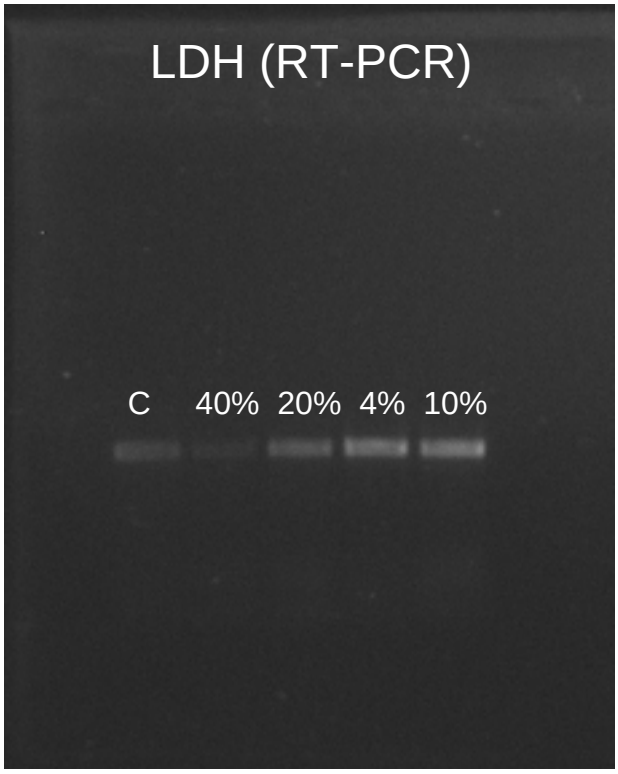

Figure 7. mRNA expressed for LDH-A* levels in skeletal muscle of Oscar juveniles submitted to different levels of hypoxia for 2 hours. Animals were pre-acclimated to normoxia for 24 hours.

Concluding remarks

In summary, we conclude that the ability of many fishes of the Amazon to survive daily extreme changes in oxygen, especially if they spend part of their life cycle in floodplain areas, known as varzea, is the result of behavioural, physiological, biochemical, and molecular adaptations to hypoxia. These responses are integrated in order to respond and survive to environmental oscillations. Nowadays we can recognize an enormous diversity of adaptations to hypoxia in fish of the Amazon. Air-breathing, aquatic surface respiration (ASR), and metabolic depression, are all related to the machinery that connects environmental changes with metabolism through a series of signals that promote responses at biochemical, physiological, and molecular levels. The survivorship of fishes exposed to hypoxia and anoxia such as the water-breathing Oscar, which do not have the morphological changes to breathe air, depends upon the coordination of aquatic surface respiration and metabolic depression, affecting a whole suite of strategies to optimize oxygen use. This species can be used as an anaerobic model in further studies of oxygen sensing and molecular gene regulation. The comparison of young and adults have shown several adaptive responses related to their size; young animals are less tolerant than adult animals, which may stand anoxia for 6 hours at 28^{o}C. The perfect combination of metabolic depression and activation of anaerobic metabolism allows the animals to increase their tolerance to hypoxia as they grow. Anaerobic machinery is activated through a series of gene regulation, which is similar in all vertebrates. Analysis of LDH-A * gene expression in Oscars exposed to hypoxia and anoxia revealed that changes in muscle LDH levels are due to gene regulation, but possible posttranscriptional changes due to other endogenous conditions such as the substrate amount in muscle tissue still remains unknown.

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Swimming performance as a practical and effective biomarker of pollution exposure in fish

by

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Introduction

It is commonly accepted that physiological adaptation by fishes to their environment will influence the success with which they can colonise particular habitats (Fry, 1947; 1971; Prosser, 1950). Fish species that pursue an active lifestyle perform sustained aerobic exercise to forage, to migrate and to maintain position against currents. This requires the coordinated activity of systems at various levels of organismal organisation (Brett, 1958; Randall, 1982; Moyes and West, 1995) and a single unifying trait known as maximum sustainable aerobic swimming speed (U_{crit}, as conceived by Brett, 1964) has proven to be sensitive to many environmental stressors (Randall and Brauner 1991) including pollutants such as low pH (Ye and Randall, 1991; Butler *et al*., 1992), dissolved metals (e.g. Waiwood and Beamish, 1978; Wilson *et al*., 1994; Beaumont *et al*., 1995a,b; 2003), ammonia (Shingles *et al*., 2001; Wicks *et al*., 2002; McKenzie *et al*., 2003), and various other toxic chemicals such as organo-phosphate pesticides (Peterson, 1974).

Another complex trait that has been proposed as a potentially valuable indicator of sub-lethal pollution is routine aerobic metabolic rate, measured as rates of oxygen consumption (Sprague, 1971; Fry, 1971; Rice, 1990). Metabolic rate can be considered the unifying currency of adaptation to the environment (Wikelski and Rickleff, 2001) and can be linked to the increased energetic costs associated with occupying polluted habitats (Rice, 1990). There is much evidence to indicate that increased metabolic rate is a general indicator of stress in fish (Wendelaar Bonga, 1997) and studies have shown it to be raised by exposure to various pollutants such as organophosphate pesticides (Holmberg and Saunders, 1979; Farrell *et al*., 1998), methylmercury (Rodgers and Beamish, 1981) and various specific herbicides (Johansen and Geen, 1990; Janz *et al*., 1991).

The literature investigating the effects of pollutants upon fish exercise performance and metabolic rates is, however, almost exclusively laboratory-based, and mainly comprises the exposure of salmonid species to single toxicants. Relatively little is known about the potential physiological effects of exposure to polluted natural environments (Farrell *et al*., 2004). Many aquatic habitats are continuously loaded with mixtures of chemicals released by human communities and industries, and many of these pollutants have been shown to exert adverse impacts upon the resident biota. The last few decades have, therefore, seen an increasing interest in the use of "biomarkers" to

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establish early-warning signals of exposure and toxic effects of specific pollutants or pollutant classes (reviewed by van der Oost *et al*., 2003). This term is most commonly used to refer to measurements in body fluids, cells or tissues, which are indicative of bioaccumulation of toxic chemicals, biochemical and cellular modifications provoked by specific toxicants, or secondary responses of host tissues to these toxicants (van der Oost *et al*., 2003). The assumption is that such modifications at these lower orders of biological organisation are indicative of, or directly linked to, modifications in systemic and organismal function which, in turn, lead to changes in the populations and communities that comprise the ecosystem.

These assumptions, and in particular the link between expression of such biomarkers and the functional integrity of the whole organism remain, however, to be proven (van der Oost *et al*., 2003). Both swimming performance and metabolic rate may be valuable in this sense because they directly reflect the functional integrity of fish and are also of immediate relevance to their ecology (Sprague, 1971; Rice, 1990; MacKinnon and Farrell, 1992).

The current study used custom-built portable swim-tunnel respirometers to compare exercise performance (Ucrit) and associated aerobic metabolism of fish exposed in cages for three to four weeks at either clean or polluted sites on three urban European river systems in different seasons (spring, summer, and winter). The rivers studied were the Lambro (Milan, Italy), the Blythe/Cole/Tame confluence (Birmingham, United Kingdom), and the Amstel (Amsterdam, The Netherlands). Two species of cyprinid were chosen as models, the chub (*Leuciscus cephalus*) was studied in Italy and the UK whereas carp (*Cyprinus carpio*) were studied in the Netherlands. Jain *et al*. (1998) demonstrated that the ability of the fish to perform two sequential exercise tests with a brief intervening recovery interval could provide significantly more sensitive information about fish health and water quality than a single exercise test alone. This "repeat-exercise" protocol was, therefore, adopted in the current study. Measurements were made of routine rates of oxygen uptake under standardised conditions of sub-maximal exercise, to investigate sensitivity of this trait to the prevailing water chemical quality. Other metabolic traits, such as the maximum metabolic rate, measured as oxygen uptake, during exercise and aerobic metabolic scope, were also derived during the swim tests, to gain insight into proximate physiological mechanisms that underlie impaired exercise performance (Beamish, 1978; Wilson *et al*., 1994; McKenzie *et al*., 2003; Pane *et al*., 2004).

Methods

River sites

Italy

The river Lambro rises in the foothills of the Alps and flows in a southerly direction through the provinces and cities of Monza and Milan, which are major centres of population and industry. Two sites were studied: a relatively clean upstream site at

Merone and a polluted downsteam site just north of the city of Milan at Brugherio. Water sampling campaigns used diffusive gradient in thin film (DGT) and semi-permeable membrane devices (SPMD) passive samplers to monitor total bioavailable heavy metal and organic pollutants respectively (Garofalo *et al*., 2004; Garofalo and Ceradini, unpublished observations). The Brugherio site was polluted by bioavailable copper, nickel and zinc plus bioavailable organics such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs). There were no differences in water temperature, pH, dissolved oxygen and conductivity between sites. During the reported campaign in September 2001 water temperature varied closely around 20^{o}C.

United Kingdom

The confluent Blythe, Cole and Tame rivers lie within the Birmingham conurbation in the West Midlands, a major centre of industry and population with a consequent legacy of pollution. They have a history of relatively good, intermediate and poor chemical water qualities respectively (Winter *et al*., 2004; 2005). The Tame is polluted with a complex mixture of metals, largely copper, nickel and zinc; the Cole was also polluted with these metals but to a lesser extent, whereas the Blythe exhibited only a low level of bioavailable zinc (Garofalo *et al*., 2004). Sampling of bioavailable organics with SPMDs revealed that the Tame had four to five fold higher levels of PAHs, PCBs and OCPs than the Blythe, which had low levels of all these contaminants. The Cole exhibited similar levels of PAHs to the Tame but levels of PCBs and OCPs intermediate between the Tame and the Blythe (Winter *et al*., 2005). During the sampling campaign in June/July, 2002 water temperatures were 13-15^{o}C.

The Netherlands

The Amstel River is 40 km long, running south to north from a rural area into and through the centre of Amsterdam where it becomes heavily polluted by industrial and domestic effluents . A number of study sites on the river have been described in detail by Verweij *et al*. (2004). Three were identified for the current investigation: a clean site in the southern rural area, at Vrouwenakker; a polluted site in downtown Amsterdam and a heavily polluted landfill site, Volgermeerpolder. These sites presented very little bioavailable heavy metal pollution (Garofalo *et al*., 2004) but extremely high levels of organic pollution (Verweij *et al*., 2004). Water temperature was 18-19^{o}C.

Experimental animals

Two species of cyprinid were studied. The chub, *Leuciscus cephalus*, is common in running water throughout Europe and is native to the rivers that were studied in Italy and the UK. It is an omnivorous species, eating invertebrates and some plant material when young and becoming an active piscivore as it grows to maturity. The carp, *Cyprinus carpio*, is native to Eastern Europe and Asia but has been introduced in many other countries, including the Netherlands. It occupies ponds, lakes and slow-flowing rivers

such as the river Amstel. It is also an omnivorous species, feeding on bottom-dwelling invertebrates and plant material.

Italy

Wild chub were captured by electrofishing in the river Lambro, at the clean Merone site. The fish were transported live to the La Casella Fluvial Hydrobiology Station (via Argine del Ballottino, 29010 Sarmato [PC], Italy), where they were stocked in $4m^2$ fibreglass tanks provided with a flow of water within a recirculating biofiltered system (vol. 90 m^3), at prevailing seasonal ambient temperatures and photoperiods. The animals were maintained under these conditions for two weeks and fed daily with pelleted feed prior to use in any experiments.

United Kingdom

Farm-reared chub were obtained from the Environment Agency fish farm (Calverton, Nottinghamshire) and transported to the animal holding facilities at the School of Biosciences, University of Birmingham. The fish were held in laboratory aquaria in biofiltered dechlorinated Birmingham tapwater for at least two weeks prior to use in experiments, at prevailing seasonal temperatures and photoperiod and were fed daily with a pelleted feed.

The Netherlands

Caging experiments (see below) were performed with genetically identical male carp from a cultured F1 hybrid fish line produced and maintained at the Agricultural University of Wageningen (van der Oost, 1998; Verweij *et al*., 2004). This experimental group offered the clear advantage of reduced variability between individuals in the toxicity tests.

Caging exposures

The caging of fish on the bed of the river Lambro in Italy was described by McKenzie *et al.* (2006) and for the rivers in the UK by Winter *et al*. (2005). Briefly, two cages were transported to each river site and anchored on the riverbed, in areas of gentle flow. Ten chub were placed in each cage for at least 3 weeks during which time they had access to both the water column and the riverbed. The fish were observed visually to feed upon naturally available food. Data are reported for caging experiments performed in Italy in September 2001 and in the UK in June/July 2002. In the Netherlands, caging experiments were performed as described by van der Oost *et al.* (1998). Briefly, twenty carp were placed in submerged cages anchored in the water column at each site, for at least 3 weeks. No attempt was made to feed the fish during the exposure protocol. Data are reported for a caging experiment performed in September 2002 (summer).

Exercise respirometry

Exercise performance and metabolism were measured with custom-built portable swimming respirometers, designed to exercise individual fish in a non-turbulent water flow with a uniform velocity profile (Steffensen *et al*., 1984). One respirometer constructed of PVC, with a cross-sectional area of 225 cm^2 to the swimming chamber and a respirometric volume of 49.0 L, has been described in detail previously (McKenzie *et al*., 2001). The second was of similar design but had a cross-sectional area of 100 cm^2 to the swim chamber, a respirometric volume of 13.4 L and was constructed of Plexiglass. In each, water flow was generated by a stainless steel propeller attached to a variable speed DC permanent magnet motor. Motor speed was controlled by a PC and Labview software (National Instruments Inc.), calibrated to deliver water velocities in cm s^{-1} and, hence, swimming speeds corrected for the solid blocking effect of the fish (Bell and Terhune, 1970). The respirometer chambers were thermostatted by immersion in larger outer tanks that received a constant flow of the appropriate source of water.

Use of these respirometers in the field was described in detail by McKenzie *et al*. (2006). Fish were transferred to the respirometer without air-exposure, then permitted 4h recovery from handling stress while swimming gently at a current speed of 20 cm s^{-1}, prior to testing their repeat swimming performance (Jain *et al*., 1998). All fish were exercised by progressive increments in swimming speed of 10 cm s^{-1} every 30 min until fatigue. Fatigue was unambiguous in both the chub and the carp, they swam vigorously until they collapsed against the back screen and would not resume in response to gentle prodding or further increases in current velocity. The fish were then allowed 40 min recovery from the first swim test (T1) after which they were exposed to exactly the same protocol a second time (T2). Maximum sustainable aerobic swimming speed (Ucrit) for both T1 and T2 was calculated in BL s^{-1} as described by Brett (1964). The repeat performance ratio was calculated as T2/T1 (Jain *et al*., 1998).

Instantaneous O_2 uptake (M_{O2}) was measured at each swimming speed by intermittent flow-through respirometry (Steffensen, 1989) controlled by a PC and Labview software as described in detail by McKenzie *et al*. (2001). For both T1 and T2, active (maximum) metabolic rate (AMR) was estimated as the M_{O2} measured at highest swimming speed immediately prior to fatigue (Fry, 1971; Beamish, 1978). For both T1 and T2, an estimate of functional aerobic scope for activity was also calculated by subtracting rates of O_2 uptake measured at the lowest swimming speed (20 cms^{-1}, defined as routine metabolic rate, RMR) from the measured AMR.

Statistics

To analyse the effect of the repeated exercise protocol upon measured variables, these were compared between groups by two-way analysis of variance (ANOVA) for repeated samples, where the interacting factors were the group (i.e. caging sites for the field studies) and the repetition of the exercise protocol. To compare the repeat performance ratio between groups a T-test was used to compare the two Italian sites, whereas a one-way ANOVA was used to compare three sites in the UK and the

Netherlands. Holm-Sidak post-hoc tests were used to identify differences amongst means. In all cases, $p < 0.05$ was taken as the fiducial level for statistical significance.

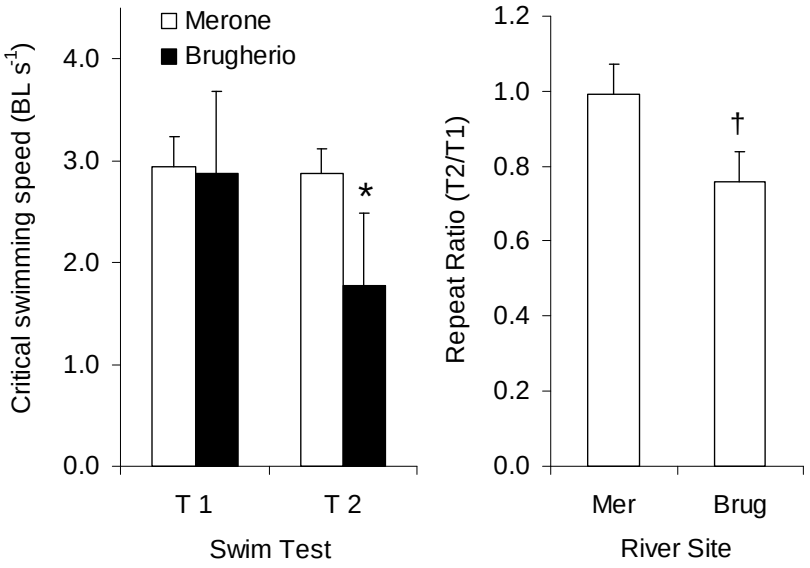

Figure 1. Swimming performance of chub (*Leuciscus cephalus*) following exposure in submerged cages to two sites on the river Lambro, Italy, in summer 2001. The graphs show mean (± SEM) critical swimming speed (Ucrit) as measured twice, with the second swim test (T2) measured 40 min following fatigue in the first swim test (T1), and the corresponding repeat performance ratio (Ucrit T2 / Ucrit T1). n = 6 in all cases, * denotes a significant difference between T1 and T2 for that river site, † denotes a significant difference between the two sites for the relevant variable, Mer, Merone; Brug, Brugherio.

Results

Italy

Figure 1 shows the swimming performance of chub in Merone and Brugherio, in late summer at a water temperature of 20°C. At both sites, the fish (chub, mean mass 200 ± 25g) swam equally well in T1. The fish from the clean site at Merone were able to repeat this performance in T2 but those from the polluted site at Brugherio were not, and exhibited a significant decline in their Ucrit. As a result, the fish from Merone had a repeat ratio that was not significantly different from 1, whereas the fish from Brugherio had a significantly lower ratio of around 0.7 (Fig. 1).

Figure 2 shows the respirometric measures taken during these swim tests. Prior to T1, RMR was not significantly different between Merone and Brugherio. In both groups, T1 caused a significant increase in metabolic rate, with AMR and functional aerobic scope for activity being similar in both groups (Fig. 2). During T2 the fish from Merone

showed a similar AMR to that in T1 and, therefore, a functional aerobic scope in T2 that was similar to that measured in T1. The chub from Brugherio exhibited a reduced AMR during T2, and a highly significant decline in functional aerobic scope for activity in T2 when compared with T1 (Fig. 2).

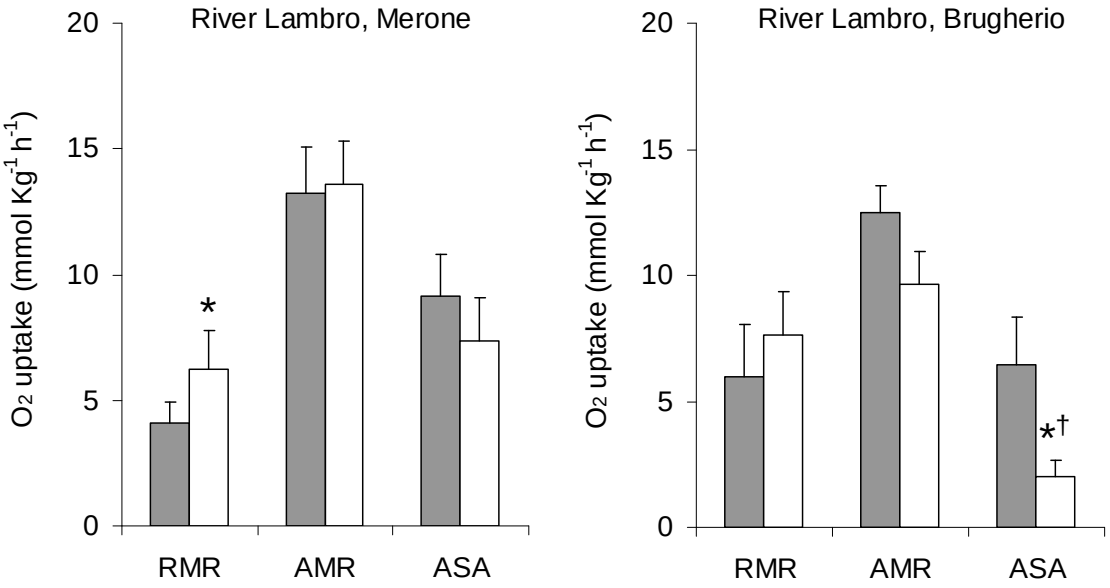

Figure 2. Swimming respirometry of chub (*Leuciscus cephalus*) following exposure in submerged cages to two sites on the river Lambro, Italy, in summer 2001. The graphs show mean (± SEM) routine metabolic rate (RMR) of fish swimming steadily at 20 cm s^{-1} prior to the exercise challenge; the active metabolic rate achieved during exercise (AMR) and the functional aerobic scope for activity (ASA) calculated as AMR-RMR. Each variable was measured twice, with the second swim test (T2, filled columns) measured 40 min following fatigue in the first swim test (T1, open columns). n = 6 in all cases, * denotes a significant difference between T1 and T2 for that river site, † denotes a significant difference between the two sites for the relevant variable.

United Kingdom

Figure 3 shows the swimming performance of chub (mean mass 62 ± 7g) caged in the Blythe, Cole and Tame, as measured in summer at water temperatures between 13 and 15 ^{o}C. At all sites, the fish swam equally well in T1. The fish from the clean Blythe site were able to repeat this performance in T2. Those from the polluted Cole and Tame sites were not, and exhibited a significant decline in their Ucrit relative to T1**.** As a result, the fish from the Blythe had a repeat ratio that was not significantly different from 1, whereas the fish from the Cole and Tame had a significantly lower ratio of around 0.7.

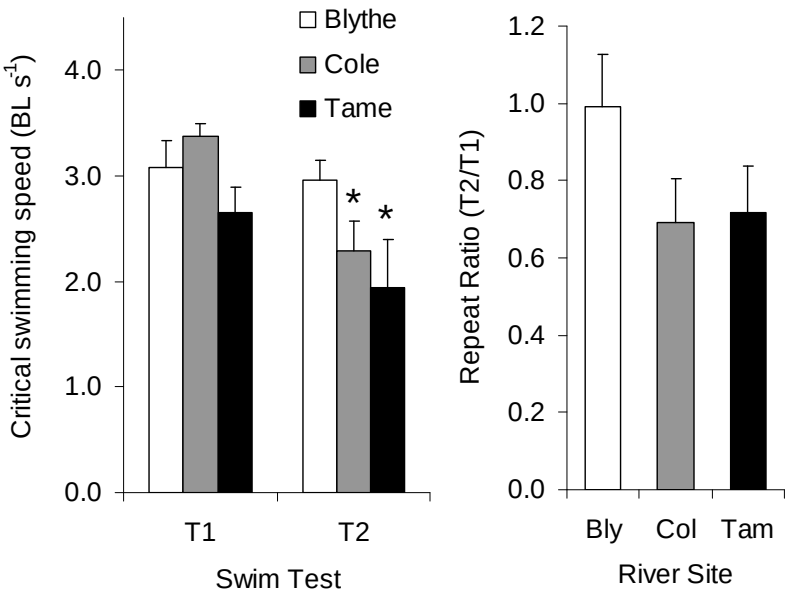

Figure 3. Swimming performance of chub (*Leuciscus cephalus*) following exposure in submerged cages to three river sites on the confluent Blythe, Cole and Tame rivers in the UK, in summer 2002. The graphs show mean (± SEM) critical swimming speed (Ucrit) as measured twice, with the second swim test (T2) measured 40 min following fatigue in the first swim test (T1), and the corresponding repeat performance ratio (Ucrit T2 / Ucrit T1). n = 6 in all cases, * denotes a significant difference between T1 and T2 for that river site; Bly, Blythe; Col, Cole; Tam, Tame.

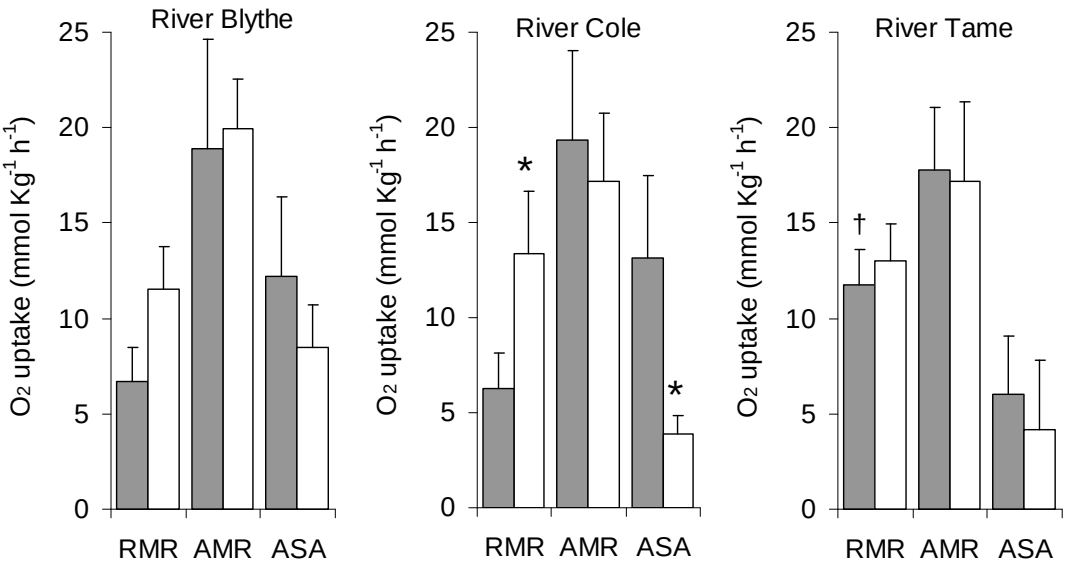

Figure 4. Swimming respirometry of chub following exposure in submerged cages to three river sites on the confluent Blythe, Cole and Tame rivers in the UK, in summer 2002. Graphs show mean routine metabolic rate (RMR) of fish swimming steadily at 20 cm s^{-1} prior to the exercise challenge; active metabolic rate achieved during exercise (AMR) and functional aerobic scope for activity (ASA). Each variable was measured twice, with the second swim test (T2, filled columns) measured 40 min following fatigue in the first swim test (T1, open columns). n = 6 in all cases, * denotes a significant difference between T1 and T2 for a river site, † denotes a significant difference between the Tame and the other sites for that variable.

Figure 4 shows the respirometric measures taken during these performance tests. Prior to T1, the fish from the Tame exhibited elevated RMR relative to those from the Blythe. All fish, however, showed a statistically similar AMR and functional aerobic scope in T1, although the mean value appears visibly lower in the Tame, where there was much variability in exercise metabolism amongst the fish. In T2, chub from the Blythe achieved similar AMRs and functional aerobic scopes to those measured in T1. The fish from the Cole and Tame, however, showed significant declines in their functional scope in T2 relative to T1 (Fig. 4).

The Netherlands

There were no differences in performance between the carp caged in the Vrouwenakker, Amsterdam and Volgermeerpolder sites with different pollution status (data not shown). All animals exhibited a repeat performance ratio that was statistically identical to 1. Despite the absence of any differences in swimming performance between the groups, there were differences in their respiratory metabolism (Fig. 5). Prior to T1 the groups differed in their RMR, which was lower at the Vrouwenakker and Amsterdam sites when compared to the severely polluted Volgermeerpolder site. Despite this

apparent metabolic loading in the animals in the heavily polluted Volgermeerpolder site, there were no differences in AMR or functional aerobic scope for activity at any site, in either T1 or T2.

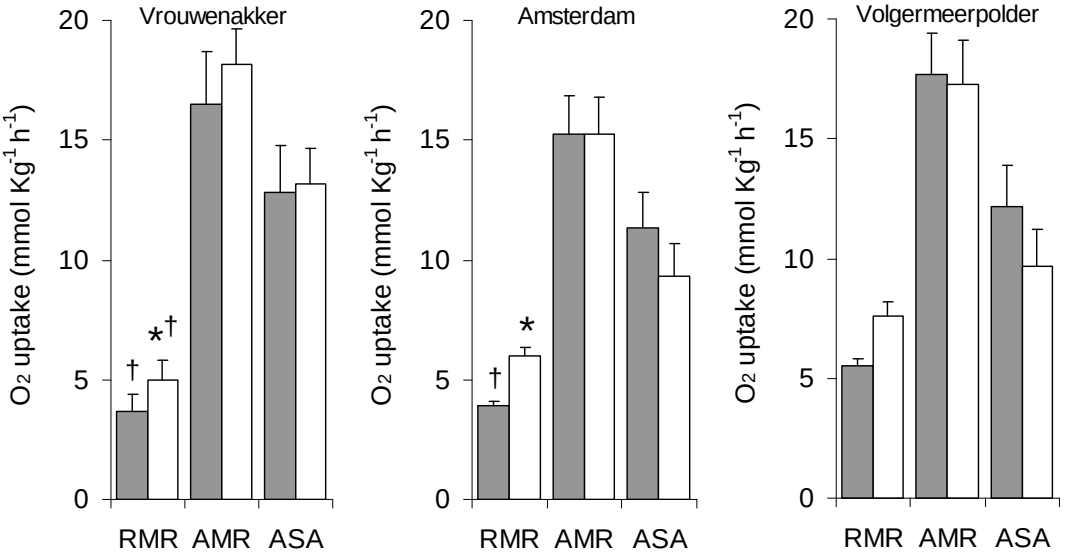

Figure 5. Swimming respirometry of chub following exposure in submerged cages to three river sites on the confluent Blythe, Cole and Tame rivers in the UK, in summer 2002. Graphs show mean routine metabolic rate (RMR) of fish swimming steadily at 20 cm s^{-1} prior to the exercise challenge; active metabolic rate achieved during exercise (AMR) and functional aerobic scope for activity (ASA). Each variable was measured twice, with the second swim test (T2, filled columns) measured 40 min following fatigue in the first swim test (T1, open columns). n = 6 in all cases, * denotes a significant difference between T1 and T2 for that river site, † denotes a significant difference from the Volgermeerpolder site for the relevant variable.

Discussion

The caging studies demonstrated that measured values of the exercise performance and metabolic rate of fish vary with the recorded conditions in their sites of exposure so that these indices of physiological performance can be used to demonstrate sub-lethal effects of the complex mixtures of chemicals which prevail in polluted urban rivers. In both Italy and the UK, chub exposed to sites polluted with bioavailable heavy metals (primarily Cu, Ni, and Zn) and organics (PAHs, PCBs, and OCPs) exhibited impairments to their exercise physiology, revealed as a reduced ability to repeat their swimming performance in a standard Ucrit test. Such impairments were not observed in the carp exposed in the Netherlands to sites that were heavily polluted with bioavailable organics. In both chub and carp, however, there was evidence of metabolic disruption following exposure to polluted sites, with fish exhibiting elevated RMR. However, there

was no measurable effect of water chemical quality upon exercise performance or exercise metabolism of chub during the winter campaigns in both Italy and the UK (McKenzie *et al.*, 2006), possibly because the sub-lethal toxic effects of the pollutants at each site were less pronounced when physico-chemical activities and fish metabolism were depressed by low water temperatures (Beaumont *et al*., 1995b; Taylor *et al*., 1996). These results indicate that traits of performance such as the ability to repeat a swim test, and traits of metabolism such as routine metabolic rate during sustained low-intensity aerobic exercise, can be employed as physiological biomarkers of sub-lethal aquatic pollution. This utility may be confined to spring and summer, when pollution events are likely to be most critical, though more work is required to examine responses to winter spates when maximum swimming speeds may be exceeded.

The swimming performance studies upon the chub in Italy and the UK provide strong support for the proposal of Jain *et al*. (1998) that a protocol of repeated exercise performance can provide more sensitive information about fish health and water quality than a standard single Ucrit test. Evidence that this is the case and the proximate mechanisms underlying the impaired repeat performance in chub exposed to polluted river sites was discussed by McKenzie *et al*. (2006).

In brown trout, *Salmo trutta*, copper impairs performance by interfering with ammonia excretion, causing plasma ammonia accumulation and a consequent depolarisation of white muscle (Beaumont *et al*., 2000a,b), which compromises white muscle recruitment and therefore swimming performance at the highest speeds (Beaumont *et al*., 2003; McKenzie *et al*., 2003). Zinc may have a similar mode of action (Alsop *et al*., 1999). The absence of any impairments to the exercise performance of carp exposed to the polluted sites on the river Amstel may have been due to the absence of significant bioavailable metals. An impact of pollution was, however, visible in both chub and carp as an increase in metabolic rate during low-level sustained aerobic exercise at a water speed of 20 cm s^{-1} (defined as RMR for the purposes of the current study). The elevated RMR that was measured prior to T1 in the chub and carp exposed to polluted sites may have derived from a metabolic load imposed upon fish exposed to polluted sites. All of the polluted sites at which fish exhibited elevated RMR also had significant bioavailable OCP levels, particularly the Vogermeerpolder site in the Netherlands (Verweij *et al*., 2003; Winter *et al*., 2005; Garofalo and Ceradini, unpublished observations). Exposure to pentachlorophenol leads to profound increases in routine oxygen consumption in the American eel, *Anguilla rostrata* (Holmberg and Saunders, 1979) and in sockeye salmon (Farrell *et al*., 1998). There is also quite a large body of evidence to suggest that elevated metabolic rates in fish can be an indicator of chronic aspecific stress (Schreck,1990; Wendelaar Bonga, 1996).

Whatever the mechanisms by which exposure to polluted river sites caused a reduced ability to repeat strenuous exercise and/or elevated routine metabolic rates, these traits do seem to offer some potential as physiological biomarkers of sublethal toxic stress. McKenzie *et al*. (2006) considered the extent to which these measures of swimming performance satisfied the set of six criteria used to evaluate the strengths or weaknesses of a biomarker, proposed by Stegeman *et al*. (1992) and van der Oost *et al*.

(2003). They concluded that most were met so that this approach can be recommended to agencies responsible for maintaining the health of rivers and their fish populations.

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Management of eutrophication

by

M.J. Gromiec[1]

Introduction

Introduction of excess nutrients, as well as other pollutants, into water bodies are causing many changes in aquatic environments, since they accelerate the process of eutrophication. The term "eutrophication", usually refers to the natural or artificial addition of nutrients to water bodies and to the effects of these added nutrients. It is possible that either nitrogen or phosphorus will be the limiting nutrient controlling the process of eutrophication in a water body. It is recognized that phosphorus is typically the limiting nutrient in freshwaters, whereas nitrogen is typically limiting in estuarine or marine waters. However, the relationships are more complex than this. Environmental conditions in most water bodies are dominated by many factors, including seasonal changes and interactions with bottom sediment. The dynamics of limiting nutrients are not well known in most water bodies. Currently, the best nutrient management policy is based on simultaneous reduction of both nitrogen and phosphorus inputs to water bodies.

Causes and effects of eutrophication

The causes of eutrophication are related to meteorological and climatic status, anthropogenic causes and features and characteristics of water bodies.
The anthropogenically induced nutrient loads represent the main problem and include the indirect loads which originate from atmospheric deposition or rivers and wastewaters including that from agriculture which represent the main point sources. A fourth cause is an internal nutrient load, which is related to release from sediments during hypoxic and anoxic conditions. Increased concentrations of nitrogen and phosphorus are the main primary causes of eutrophication. However, substances other than inorganic phosphorus and nitrogen compounds can also contribute to eutrophication. It should be stressed that the whole population is contributing to the problem of eutrophication by our life style.

The complex relation between natural and anthropogenic processes in relation to nutrient dynamics make it difficult to understand cause and effect relationships. Therefore, only some examples of eutrophication are presented below. The primary effects of eutrophication are related to biological, chemical and physical disturbances. The increasing production of algae biomass, the decreasing amount of silica, and the increasing turbidity of the water, are well known examples. Cyanobacterial blooms may cause damage to organisms and result in odour problems. Sedimentation associated with primary production and occurrence of hypoxia and anoxia are well known examples of secondary and tertiary effects caused by eutrophication (Lundberg, 2005).

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Water basin policy and nutrient management

It is well recognised that river basin management is the key for sustainability in water and land use management. In the European Union (EU), a Water Framework Directive (WFD) is the most significant legislative instrument of policy in the water management field (Chave, 2001). This legal instrument provides a framework for each member state to develop a common basis for the sustainable use of water and for the protection of water. The main requirement of the WFD is that EU member states ensure that all waters are in good status by the end of 2015.

The most important features of WFD are that it aims to manage the water environment as a whole on a river basin basis. In addition, it calls to use a combined approach to pollution control, setting limit values to control emissions from individual point sources, and establishing water quality standards to limit the cumulative impact of emissions and diffuse sources of pollution.

Therefore, in nutrient management it is necessary to assess the impact of human activities on water bodies in each river basin, taking into consideration nutrient inputs from point sources and from diffuse sources and other human activities that may impact water status. Furthermore, it is necessary to establish and implement a legally binding program of measures. This program to achieve the defined quality objectives will have to follow the above mentioned combined approach, using the setting of emission limit values and water quality standards.

Water quality criteria and effluent standards for nutrient management

Population growth in cities has resulted in an effort to reduce nutrient loads in municipal wastewaters on receiving water bodies. A number of options exist for removal of nitrogen and phosphorus from wastewaters. The strategies to achieve low concentrations of nitrogen and phosphorus are usually based on water quality criteria and effluent standards (Barnard and Steichen, 2006).

In 2001, the United States Environmental Protection Agency published water quality criteria with stringent nitrogen and phosphorus requirements. Depending on the eco-region, the water quality criteria are as follows (USEPA, 2001):

- total nitrogen (TN) — from 0.12 mg TN/l
  to 2.18 mg TN/l
- total phosphorus (TP) — from 10 µg TP/l
  to 76 µg TP/l

These water quality criteria for 17 eco-regions of the USA are currently being evaluated. There are also stringent requirements for low levels of effluent nutrient concentrations in many regions of the world. In Europe, according to the EU urban wastewater treatment directive (91/271/EC) the effluents also have to meet guidelines on the content of

nitrogen and phosphorus. However, when a territory is designated as a sensitive area, more advanced treatment of wastewater with nutrient removal should be provided according to article 5.4 of the directive. This guideline restricts the following nutrients concentrations in the effluent, for the sensitive areas, (where p.e. (population equivalent) is equal to 60 g BOD_5/d):

- total nitrogen — 15 mg TN/l for 10 000-100 000 p.e.
  10 mg TN/l for >100 000 p.e.
- total phosphorus — 2 mg TP/l for 10 000-100 000 p.e.
  1 mg TP/l for >100 000 p.e.

The minimum percentage reduction of the overall load of phosphorus is at least 80% and a minimum of 70 to 80% for nitrogen. However, there is presently a great emphasis on reaching very low levels of effluent phosphorus. For example, in Germany (Berlin area), limiting phosphorus removal to 0.05 mg TP/l is required for the larger plants to further reduce eutrophication of the local surface bodies.

The above criteria and requirements require technologies to achieve low nitrogen and phosphorus concentrations. Obviously, there are some limits to obtaining very low total nitrogen concentrations in the treated effluents. Presently, a challenge is to remove the dissolved organic nitrogen from wastewaters. Since the residual effluent dissolved organic nitrogen (DON) is composed of non-biodegraded nitrogen forms, a method is needed to assess the biodegradability of effluent DON (Pagilla *et al.*, 2006). It should be stressed, however, that the biological and chemical technologies for nutrient removal are rather well established. A problem still exists with efficiently controlling nutrient loads from diffused sources.

Non-point nutrient management in a catchment- An example

Non-point source nutrients resulting from agricultural practices can be analyzed with the assistance of mathematical models. The application of these models should be based on reliable, complete and correct data. The project "Controlling non-point pollution in Polish catchments" deals with control measures in relation to agricultural practices and management that minimizes nutrient leakage from non-point sources (DHI/IMWM, 2005). The project was implemented in the Pasłeka River Catchment in the northern part of Poland. The following main institutions participated in the project: Institute of Meteorology and Water Management (IMWM), Poland and DHI- Water and Environment, Denmark. The project has been sponsored by the Polish National Fund of Environmental Protection and Water Management (NFOSGW) and the Danish DANCEE.

The Pasłeka River (Fig. 1) discharges through the Vistula Lagoon into the Baltic Sea. In the Vistula Lagoon some eutrophication problems have been observed, among others, due to excess discharge of nutrients from the catchment (Fig. 2). Data and information were collected concerning soil types, land-use patterns and general

agricultural practice. In addition, monitoring of flow, water quality parameters and meteorological conditions was performed.

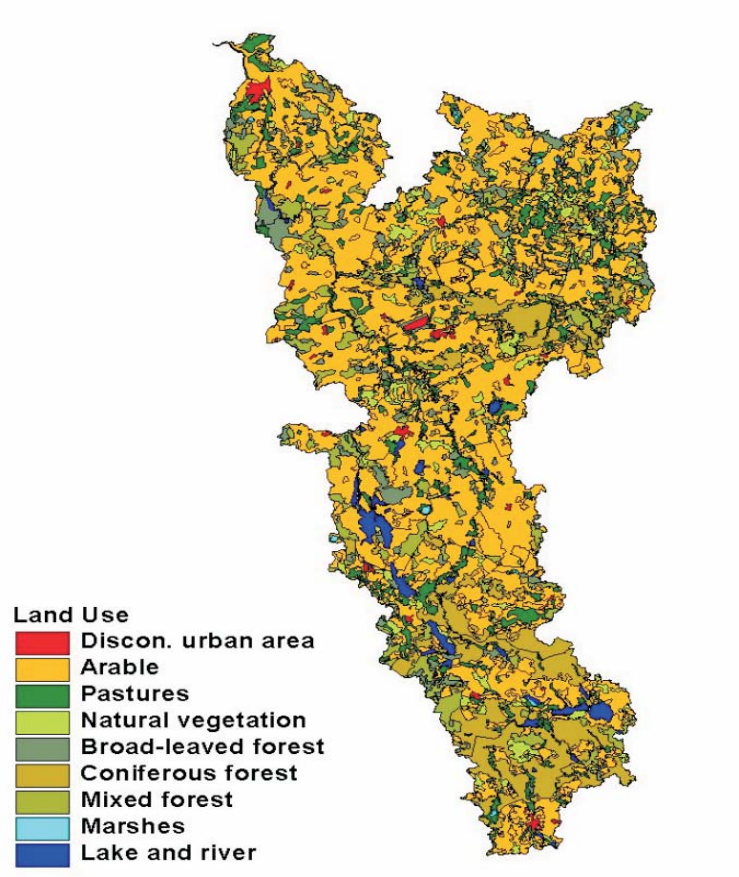

Figure 1. Land cover – Pasłeka River Catchment

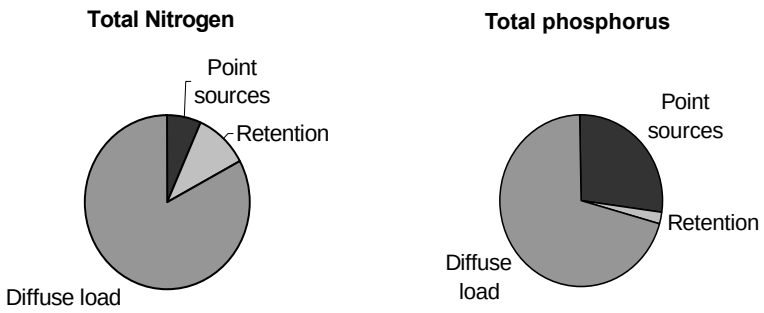

Figure 2. Load assessment - Pasleka River

The applied modeling tools (Fig. 3) were as follows:

- DAISY - an agricultural field model describing the relation between nutrient runoff from the root ozone and agricultural practice.
- MIKE BASIN - a catchment model suitable for describing the overall transport of water and nutrients through the river basin including different nutrient concentrations in different water compartments.
- MIKE 11HD, WQ and WET - a full dynamic hydrodynamic and water quality model system for rivers, lakes and wetland areas describing water flow, transport, and transformation of organic material and nutrient.

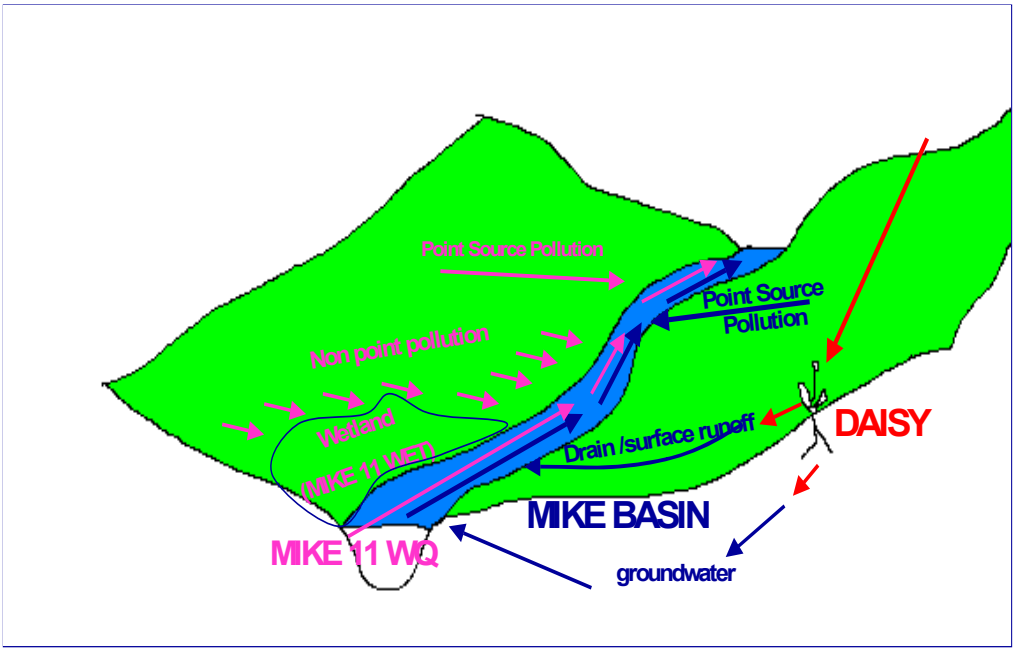

Figure 3. Outline of use modeling tools

Future load scenarios have been defined based on collected information on point sources, present agricultural practice and evaluation of potential development in agricultural activity. The following five scenarios were simulated by the model system covering the Pasłeka River catchment:

- Scenario 00 - Existing conditions.
- Scenario 01 - Existing management with increasing animal production
- Scenario 02 - Existing condition with grass in rotation instead of permanent grasslands
- Scenario 03 - Existing management and animal production at EU level (characterized as "worst case").
- Scenario 04 - Animal productivity of EU level plus optimal handling of slurry and crop rotation.
- Scenario 05 - animal productivity of EU level plus optimal handling of slurry and crop rotation plus catch crop.

Tables I and II summarize the DAISY and MIKE BASIN modeling results from the scenarios 0-5.

Table I. Summary of loss of $N0_3$-N per ha (leaving the root zone)

Scenario	Average loss kg $N0_3$ - N/ha	Difference in % of Existing Conditions
00	11	0
01	13	18
02	16	45
03	21	90
04	10	-9
05	6	-45

Table II. Summary of total nitrogen transport and area specific transport in Pasleka River system

Scenario	TN-Transport tons/year	Area specific transport kg/ha*
00	931	4.0
01	1016	4.4
02	1393	6.0
03	1654	7.1
04	841	3.6
05	567	2.4

- Numbers include point sources and retention of nitrogen in rivers, lakes and groundwater

The study shows in the case where animal productivity is increased to levels approaching 1.5 animal units (AU) per ha without changing the general agricultural management practice (scenario 3), a significant difference in the nitrogen loss can be expected. An increased loss of nitrate from the root zone of 90% is expected.

Furthermore, the study shows that through optimal agricultural management, using catch crops and extensive straw incorporation thus increasing animal productivity (scenario 5), it is even possible to achieve a reduction of nitrate loss compared to the existing situation. A 45 % reduction in nitrate loss from the catchment to the Baltic Sea has been simulated (scenario 05 compared to 00).

In addition, the study demonstrated that the existing riparian wetland zone along the Pasleka River has a high potential for protection against nitrogen to the river environment and thereby to the Baltic Sea. The planning and management tools (the modeling software) were installed at the Regional Boards of Water Management in Gdansk.

Conclusions

Eutrophication management should be based on controlling both point and non-point nutrients sources in a given watershed. Management of nutrients is only possible if all sources of the nutrients as well as their fate in the catchment are known.

As far as the nutrient point sources are concerned, there is presently emphasis on reaching very low levels of effluent nitrogen and phosphorus. However, successful strategies for nutrient management additionally need to be based also on economic considerations. Especially, the cost of removing the remaining soluble organic nitrogen from wastewaters must be justified by the environmental impact. Therefore, cost - effectiveness and sustainability of the existing technologies for nutrient removal should be evaluated.

In successful strategies for nutrient management it is also necessary to address non-point sources of nutrients, particularly from agriculture. The contributions of agriculture are dependent on many factors, such as type and volume of agricultural production, geologic, morphologic and climatic conditions, as well as agricultural practice (density of animals, intensity of crop production, etc.). The optimal application of scientific knowledge for nutrient management can be achieved by the use of modeling tools.

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Effects of ammonia on locomotor performance in fishes

by

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Ammonia, which is toxic to all vertebrates, has become a pervasive pollutant of aquatic habitats emanating from point sources such as sewage treatment plants, or diffuse sources such as agricultural and urban storm runoff (API, 1981). In aqueous solution, ammonia exists as two species, unionised ammonia gas (NH_3) and the ammonium ion (NH_4^+), with a pK_a of approximately 9.5 in freshwater. Here, the term "ammonia" refers to total ammonia, the sum of NH_3 and NH_4^+. In freshwater fishes, ammonia is thought to traverse the gill epithelium almost exclusively via passive diffusion of ammonia as a gas in solution (Randall and Wright, 1987; Ip *et al.,* 2001), so that water NH_3 concentration (partial pressure) determines the potential for toxicity. In seawater fish, gill permeability to the ammonium ion may also contribute to toxicity (Randall and Tsui, 2002). Ammonia is also a metabolite, produced in fish as an end product of protein and purine metabolism and then excreted predominantly by passive diffusion of NH_3 across the gill epithelium (Randall and Wright, 1987; Wright, 1995). In teleost fish ammonia can, therefore, accumulate to toxic levels, either as a consequence of exposure to elevated water ammonia concentrations or when excretion of the endogenous metabolite is inhibited. Within fish, the primary form of total body ammonia at physiological pH (7.0 to 8.0) is NH_4^+, and it is this chemical species that is responsible for toxic effects (Smart, 1976; Hillaby and Randall, 1979). The toxic effects of ammonia in teleosts have been the subject of recent reviews (Ip *et al.,* 2001; Randall and Tsui, 2002). In most completely aquatic teleosts, immersed in unpolluted water, plasma ammonia is typically between 150 and 300 μM (Walsh, 1998). The current paper will review briefly some studies of sub-lethal toxicological effects on locomotor performance of increased plasma ammonia concentrations.

Effects of ammonia on critical speed swimming

A number of investigators have demonstrated that sub-lethal increases in the concentration of ammonia in the plasma can impair the ability of freshwater salmonids to perform in a critical swimming speed (U_{crit}) test (Beaumont *et al.,* 1995a; Shingles *et al.* 2001; Wicks *et al.* 2002; McKenzie *et al.* 2003). This test involves exposing the fishes to sequential increments in current velocity in a swim tunnel until they fatigue (Brett, 1964). During this test, the fish will initially power swimming activity with slow-twitch oxidative "red" muscle but, as faster current velocities are imposed, it will eventually be obliged to recruit fast-twitch glycolytic "white" muscle to achieve the highest tailbeat

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frequencies and swimming speeds, and this will lead relatively rapidly to fatigue (Brett, 1964; Beamish, 1978). A negative linear relation has been described between plasma ammonia concentration and U_{crit}, as shown in Figure 1. In the first study to reveal this effect (Beaumont *et al.,* 1995a), an increase in plasma ammonia occurred in brown trout (*Salmo trutta*) as a consequence of exposure to sublethal concentrations of copper in soft acidic water (pH 5). Beaumont *et al.* (1995b; 2000a,b; 2003) attributed the impairment of swimming performance to plasma ammonia accumulation because there was no evidence of major disruptions to cardiorespiratory metabolism, particularly such as problems with O_2 uptake and cardiovascular convection. Figure 1 shows that exposure of brown trout to ammonia alone (Shingles, McKenzie and Taylor, unpublished data, reported in Shingles 2002 and McKenzie *et al*., 2003) impairs U_{crit} in a very similar fashion to the impairment observed by Beaumont *et al.* (1995a) in trout exposed to copper, thereby supporting these latter authors' conclusion that the effects of the heavy metal were mediated through ammonia accumulation (Beaumont, 1995b; 2000a,b; 2003; McKenzie *et al.,* 2003).

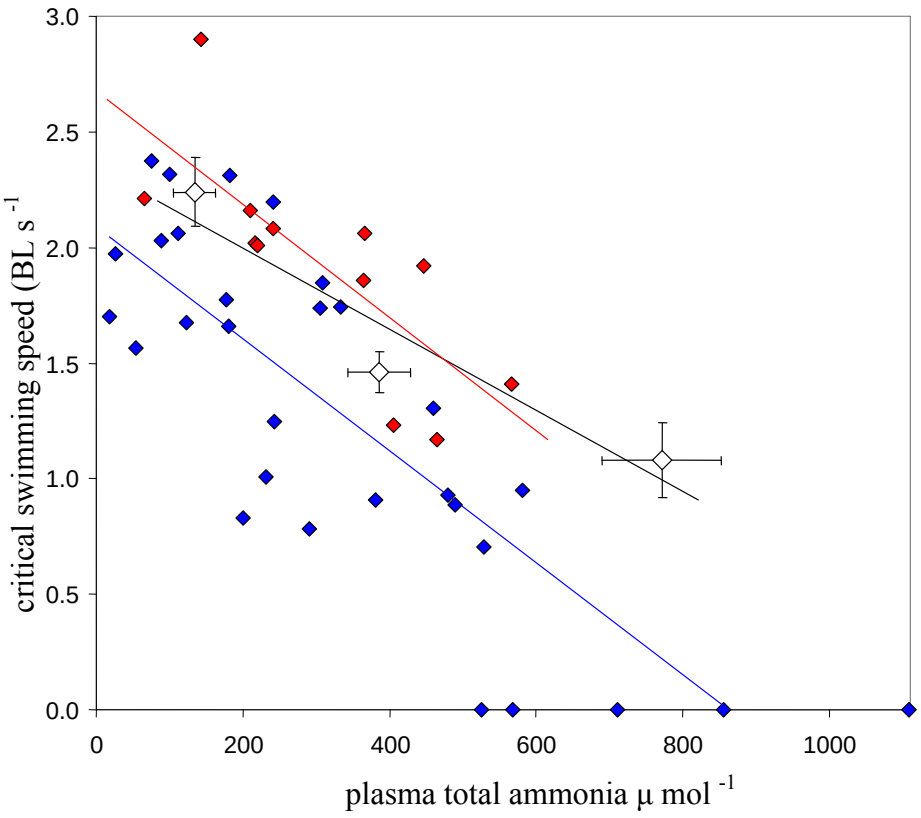

Figure 1. Linear relationships between plasma ammonia concentration and U_{crit} in brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*). The blue symbols are data for individual brown trout in which plasma ammonia accumulated following exposure to sub-lethal concentrations of copper in soft acidic water, replotted from Beaumont *et al.* (1995a). BL, bodylength. The blue line describes a least squares linear regression equation whereby U_{crit} = -0.0020 * [ammonia] + 2.089 (R^2 = 0.670, n =

30). The large black symbols are data for brown trout exposed to three water concentrations of ammonia, replotted from McKenzie *et al.* (2003). Plasma ammonia and U_{crit} were measured on separate groups of fish (n = 6 or 7), and the black line describes a least squares linear regression equation whereby mean U_{crit} = -0.0018 * mean[ammonia]) + 2.347 (R^2 = 0.903, n = 3). The red symbols are data for individual rainbow trout exposed to elevated water ammonia, plotted from data reported in Shingles *et al.* (2001). The red line describes a least squares linear regression equation whereby U_{crit} = -0.0024 * [ammonia] + 2.677 (R^2 = 0.590, n = 12).

One of the major toxic effects of ammonia is that, as the ammonium ion, it can substitute for potassium at vertebrate muscle and nerve membranes, thereby compromising their function (Raabe and Lin, 1985; Randall and Tsui, 2002). Wright *et al.* (1988) demonstrated that the distributions of NH_4^+ between extracellular and intracellular compartments could be used with the Nernst equation to calculate membrane potential (E_M) in fish. Beaumont *et al.* (1995b; 2000a,b) found that the relative plasma to white muscle (WM) ammonia distributions predicted a significant depolarisation of the tissue in brown trout that were hyperammonemic following exposure to copper in acidic water. Beaumont *et al.* (2000c) then made direct measurements of E_M in WM of hyperammonemic brown trout, and confirmed the depolarisation, presumably due to the replacement of K^+ with NH_4^+ (Table I). Since then, in all studies investigating effects of ammonia on U_{crit} performance in salmonids, application of the Nernst equation consistently predicts a significant depolarisation of WM (Table I). The reduced WM E_M (Beaumont *et al.*,1995b; 2000a,b; Shingles *et al.* 2001; Wicks *et al.* 2002 McKenzie *et al.* 2003) was judged to be sufficient to cause a complete loss of electrical excitability in that tissue (Jenerick, 1956), such that it could not be recruited to power anaerobic burst swimming at the highest speeds.

Table I. Plasma and white muscle ammonia concentrations, and membrane potential, in various salmonid species during exposure to sublethal concentrations of either copper in soft acidic water or water ammonia gas. Taken from McKenzie *et al.* (2003).

Species (live mass)	Exposure conditions	Pl. T_{amm}	Pl. NH_4^+	Pl. NH_3	WM T_{amm}	WM NH_4^+	WM NH_3	WM E_M (calc.)	WM E_M (meas.)
Salmo trutta[a] (300-600g)	Softwater (0.05 mM Ca^{++}) at 10°C, pH 7.0	109 ± 21	107 ± 21	1.4 ± 0.3	3168 ± 223	3162 ± 223	5.9 ± 0.4	-92.8 ± 3.4	-86.5 ± 2.9
	96h exposure to 0.08 μmol^{-1} Cu at pH 5.0	652 ± 95	645 ± 94	6.9 ± 1.0	4248 ± 616	4248 ± 616	6.9 ± 1.0	-54.5 ± 5.1	-52.2 ± 4.9
S. trutta[b] (370-740g)	Hardwater (280 mM Ca^{++}) at 15°C, pH 8.4	134 ± 29	132 ± 29	2.0 ± 0.4	2097 ± 544	2092 ± 543	4.2 ± 0.8	-71.5 ± 7.5	NA
	24h exposure to 7 µM NH_3	386 ± 42	380 ± 42	5.9 ± 0.5	2735 ± 612	2728 ± 611	7.8 ± 1.7	-49.9 ± 8.3	NA
	24h exposure to 14 µM NH_3	771 ± 92	757 ± 91	14.6 ± 1.6	4249 ± 315	4240 ± 314	9.3 ± 2.1	-43.4 ± 1.6	NA
Oncorhynchus mykiss[c] (380-790g)	Hardwater (280 mM Ca^{++}) at 14°C, pH 8.4	183 ± 30	180 ± 29	2.7 ± 0.6	1750 ± 360	1748 ± 360	2.2 ± 0.4	-60.3 ± 6.8	NA
	24h exposure to 20 µM NH_3	436 ± 34	429 ± 34	7.0 ± 0.5	1950 ± 240	1948 ± 240	2.3 ± 0.3	-34.9 ± 2.8	NA
O. kisutch[d] (350 ± 65g)	Softwater (0.12 mM Ca^{++}) at 9-12 °C, pH 6.0	5300 ± 700	5273 ± 696	26.7 ± 3.5	NA	NA	NA	-53.0 ± 3.0	NA
	4h exposure to 1.1 µM NH_3	6800 ± 900	6771 ± 896	28.6 ± 3.8	NA	NA	NA	-41.0 ± 4.0	NA
	4h exposure to 2.2 µM NH_3	8500 ± 1100	8466 ± 1096	34.1 ± 4.4	NA	NA	NA	-25.0 ± 3.0	NA
	4h exposure to 4.4 µM NH_3	9500 ± 1500	9453 ± 1493	46.8 ± 7.4	NA	NA	NA	-20.0 ± 4.0	NA

Pl., plasma; T_{amm}, total ammonia; WM, white muscle; E_M, membrane potential; calc., calculated with the Nernst equation; meas., measured directly; NA, not available.
All ammonia concentrations are µM, E_M is in mV.

[a] As reported by Beaumont *et al.*. (2000c). Sampled from fish at rest, arterial plasma obtained via chronic indwelling dorsal aortic cannula

[b] As reported by Shingles (2002) and McKenzie *et al.* (2003). Sampled from fish at rest, arterial plasma obtained via chronic indwelling dorsal aortic cannula

[c] As reported by Shingles *et al.* (2001). Sampled from fish following exercise to U_{crit}, arterial plasma obtained via chronic indwelling dorsal aortic cannula.

[d] As reported by Wicks *et al.* (2002). Sampled from fish following exercise to U_{crit}, venous plasma obtained by caudal puncture.

This depolarisation of WM may be the primary means by which ammonia impairs U_{crit} performance in salmonids. During a U_{crit} protocol, the incremental increases in aerobic red muscle (RM) work lead to an exponential increase in whole-animal oxygen demand, and this demand is met by increased cardiac work and internal oxygen convection (Jones and Randall, 1978). There is evidence that these processes function equally well in control and hyperammonemic brown trout. Beaumont *et al.* (2003) found no evidence that the reduced U_{crit} of hyperammonemic brown trout could be attributed to impaired cardiac performance. Exposure of brown trout for 24h to two sub-lethal concentrations of ammonia (100 μmol l^{-1} and 200 μmol l^{-1}) reduced U_{crit} but this was not linked to reduced maximum rates of oxygen uptake nor aerobic scope during the U_{crit} test (Table II, Shingles *et al.,* unpublished data). On the other hand, the hyperammonemic trout could not achieve the same maximum tailbeat frequencies and, at fatigue, WM of hyperammonemic trout did not exhibit the large lactate accumulation observed in control trout (Table II). Thus, these observations are consistent with the hypothesis that hyperammonemic brown trout have reduced U_{crit} because glycolytic WM is depolarised and therefore cannot be recruited to achieve the highest tailbeat frequencies and swimming speeds.

Table II. Mean (± SEM) values for selected exercise-related respiratory and performance variables in brown trout following exposure to one of three water ammonia concentrations: ambient (control); 98 ± 6 μmol l^{-1} NH_4Cl (low ammonia), or 210 ± 11 μmol l^{-1} NH_4Cl (high ammonia). $U_{crit,}$ critical swimming speed; MMR, maintenance metabolic rate; AMR, active metabolic rate as maximum measured M_{O2}; f_{TB} max., maximum tailbeat frequency; RM red muscle; WM, white muscle. N = 6 in all cases, *significantly different from all other groups, a different superscipt indicates a significant difference between the means for that variable, § indicates a significant difference between rest and fatigue (Tukey test *post-hoc* to two-way ANOVA, $p < 0.05$).

	control	low ammonia	high ammonia
U_{crit} (bodylengths. s^{-1})	2.24 ± 0.15*	1.46 ± 0.09	1.08 ± 0.16
MMR (mmol O_2 kg^{-1} h^{-1})	2.62 ± 0.39	2.43 ± 0.49	3.15 ± 0.90
AMR (mmol O_2 kg^{-1} h^{-1})	12.32 ± 2.50	10.82 ± 1.87	11.24 ± 1.98
Aerobic scope (mmol O_2 kg^{-1} h^{-1})	9.70 ± 1.00	8.39 ± 0.85	8.10 ± 0.87
f_{TB} max. (Hz)	4.90 ± 0.31*	2.85 ± 0.40	2.60 ± 0.25
WM lactate at rest (mmol g^{-1})	$10.4 \pm 2.1^{a,b}$	7.5 ± 1.7^{b}	14.6 ± 1.7^{a}
RM lactate at rest (mmol g^{-1})	4.6 ± 1.6	3.3 ± 1.0	5.0 ± 1.3
WM lactate at fatigue (mmol g^{-1})	$21.3 \pm 2.2^{a\S}$	11.2 ± 3.3^{b}	11.7 ± 3.1^{b}
RM lactate at fatigue (mmol g^{-1})	8.9 ± 1.4^{a}	3.1 ± 1.2^{b}	8.3 ± 2.1^{a}

Table III shows the ammonia accumulation in the tissues of the brown trout studied by Shingles *et al.* (unpublished data). Both WM and RM exhibited significant ammonia accumulation. As already stated, the accumulation in the WM was enough to cause a significant predicted depolarisation (Table I). Shingles *et al.* (unpublished data) did not find evidence for a significant depolarisation of RM, despite the ammonia accumulation in the tissue. The means by which RM might be able to maintain function while WM cannot is worthy of further investigation. The most striking data in Table III,

however, are the ammonia levels in the heart and brain. In the hyperammonemic groups, these tissues did not exhibit a significant increase in their ammonia content despite the large plasma accumulation. As shown in Figure 2, for the trout exposed to the highest concentration of ammonia, the distribution of ammonia between plasma and the intracellular compartment of these tissues was significantly less than would be predicted if it were distributed according either to E_M or pH. Thus, the heart and brain would appear to be protected from ammonia, and this must contribute to the ability of the trout to co-ordinate and perform prolonged aerobic exercise during the U_{crit} tests. Tsui *et al.* (2004) found that the performance of isolated ventricular trabeculae from rainbow trout was not significantly affected by extracellular ammonia concentrations exceeding 1 mM, significantly higher than the plasma levels that impaired U_{crit} performance in either rainbow trout (Shingles *et al.,* 2001) or brown trout (Beaumont *et al.,* 1995a,b; 2000a,b; 2003; Shingles *et al.,* unpublished data). Tsui *et al.* 2004 attributed this protective effect to background K^+ channels that were not as sensitive to NH_4^+ in the myocardium as they were in WM. The brain, on the other hand, may protect itself from accumulation through detoxification of ammonia to glutamine (Wicks and Randall, 2002; Randall and Tsui, 2002). Wicks and Randall (2002); Randall and Tsui (2002) and Tsui *et al.* (2004) suggest that these mechanisms for protecting heart and brain function may have evolved to cope with the surges of plasma ammonia that occur in salmonids after feeding. It is known that rainbow trout perform poorly in U_{crit} tests following feeding (Alsop and Wood, 1997) although the potential role of ammonia in this response remains to be explored.

Table III. Mean (± SEM) total ammonia concentrations in plasma (μmol l^{-1}) and selected tissues (μmol g^{-1}) of brown trout following 24h exposure to one of three water ammonia concentrations: ambient (control); 98 ± 6 μmol l^{-1} NH_4Cl (low ammonia), or 210 ± 11 μmol l^{-1} NH_4Cl (high ammonia). Data are shown for animals at rest (A) and at fatigue following sustained exercise (B). N = 6 in all cases, plasma measurements are not available for animals at fatigue. Where indicated, a different superscript indicates a significant difference between the means for that tissue (Tukey test *post-hoc* to two-way ANOVA, $p < 0.05$). *significant difference between animals at rest and at fatigue for a given ammonia exposure regime (Tukey test *post-hoc* to two-way ANOVA, $p < 0.05$).

	Control	Low ammonia	High ammonia
(A) At rest			
plasma	133.6 ± 29.2[a]	386.0 ± 41.5[b]	771.3 ± 92.2[c]
white muscle	1.2 ± 0.3[a]	2.0 ± 0.4[a]	3.1 ± 0.2[b]
red muscle	1.4 ± 0.4	2.1 ± 0.5	2.7 ± 0.2
heart	1.3 ± 0.5	1.8 ± 0.6	1.5 ± 0.5
brain	1.1 ± 0.3	1.1 ± 0.2	1.8 ± 0.5
(B) At fatigue			
white muscle	2.9 ± 0.5*	3.5 ± 0.8	3.7 ± 0.6
red muscle	2.8 ± 0.6[a,b]	2.1 ± 0.3[a]	4.7 ± 0.4[b]*
heart	1.4 ± 0.4	2.1 ± 0.7	2.7 ± 0.4
brain	1.9 ± 0.6[a]	1.6 ± 0.5[a]	2.8 ± 0.2[a]

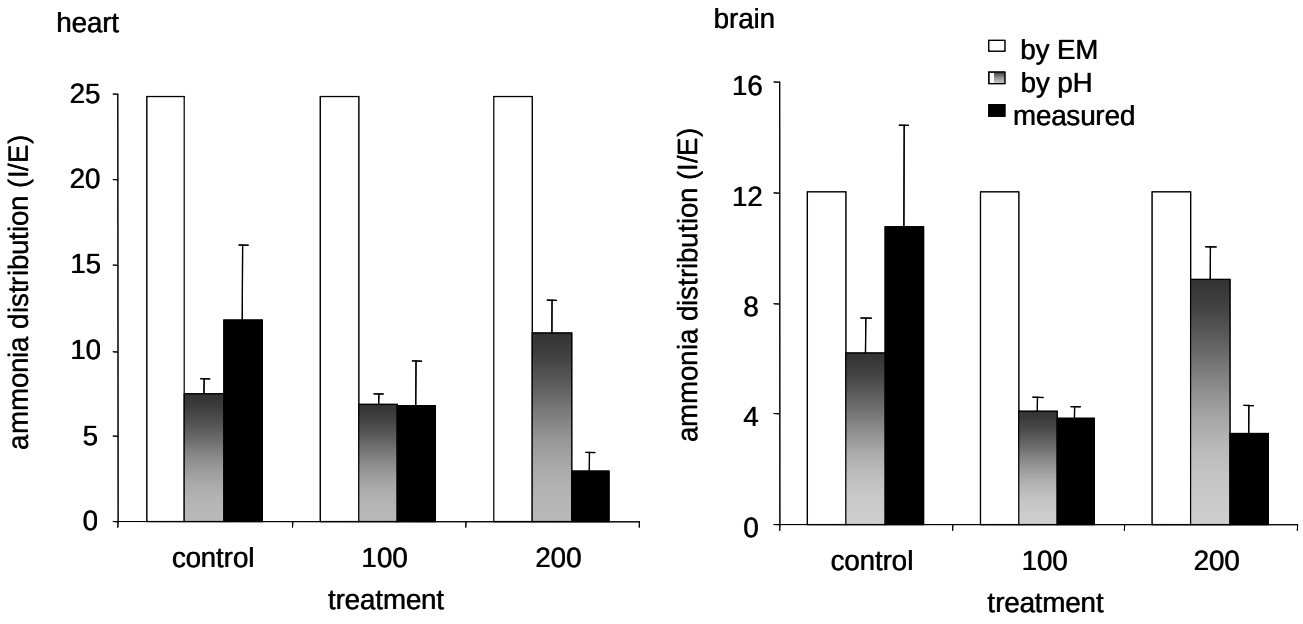

Figure 2. Ammonia distributions between the extracellular compartment (plasma) and the intracellular compartment of the heart and brain in brown trout (*Salmo trutta*) following 24h exposure to one of three water ammonia concentrations: ambient (control); 98 ± 6 μmol l^{-1} NH_4Cl ("100"), or 210 ± 11 μmol l^{-1} NH_4Cl ("200"). Distributions are given as the ratio of intra- to extra-cellular concentrations, when predicted from the plasma concentration and either the membrane potential (EM) or the pH of the two compartments, or as directly measured. N = 6 in all cases, for pH and measured distributions, values are mean ± SEM.

Effects of ammonia on performance of the escape response

Startle escape responses are used by many fish as the main defence against predator attacks, and their kinematics, performance, behaviour and physiology have been studied extensively (see Domenici and Blake, 1997 for a review). Escape responses comprise a sudden and brief acceleration, typically in a direction away from the startling stimulus (Domenici and Blake, 1993). They are usually triggered by one of a pair of Mauthner cells, giant neurons that permit a rapid response in the order of a few milliseconds (Eaton and Hackett, 1984). The response involves unilateral contraction of the axial musculature contralateral to the stimulus, so that the fish bend into a characteristic C-shape. This represents stage 1 and it can be followed by a second contraction, stage 2, on the opposite side of the body (Foreman and Eaton, 1993; Domenici and Blake, 1997). These movements are all exclusively dependant upon fast-twitch glycolytic WM function (Webb, 1998). Beyond stage 2, locomotor behaviour is

variable and can include either steady swimming or just coasting (Weihs, 1973). The success of an escape response for predator avoidance depends, therefore, on both sensory and locomotor performance, although little is known about how these might be affected by environmental factors (e.g. Webb, 1978; Webb and Zhang, 1994; Lefrançois *et al.,* 2005). There is no knowledge of how they might be affected by sub-lethal concentrations of pollutants such as ammonia.

A series of experiments were performed to investigate the effects of sub-lethal concentrations of ammonia upon performance of the startle reflex by a marine teleost, the golden grey mullet, *Liza aurata* (Shingles, McKenzie, Claireaux and Domenici, unpublished data). Figure 3 shows the effects of two water ammonia concentrations (nominally 400 μmol l^{-1} and 1600 μmol l^{-1}) on ammonia levels in the venous plasma, WM and brain following 24h exposure. The mullet were extremely tolerant of ammonia by comparison with salmonids. Initial 96h tests revealed that 2 mM total ammonia in seawater did not cause any fatalities. Both WM and brain exhibited large and significant increases in ammonia content, with little evidence that the brain was protected against ammonia accumulation in the manner observed in brown trout.

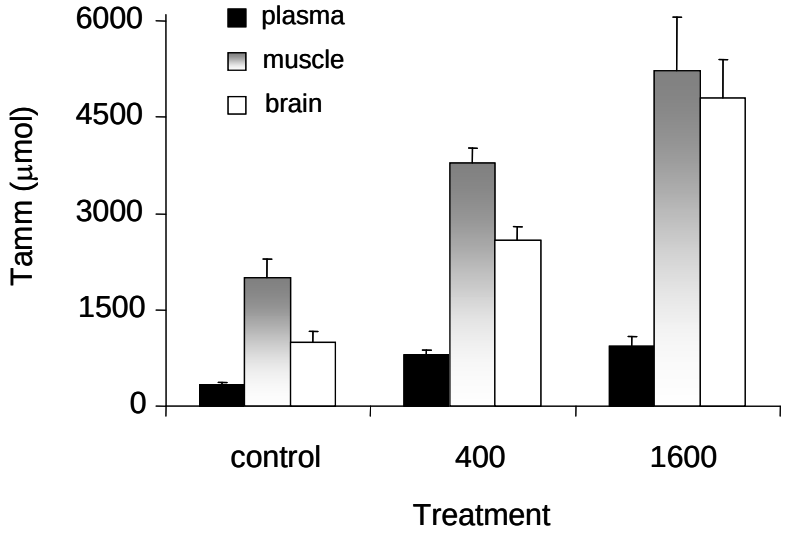

Figure 3. Mean (± SEM) total ammonia (Tamm) concentrations in the plasma (μmol l^{-1}), the white muscle and the brain (μmol g^{-1}) of golden grey mullet (*Liza aurata*) at 24h following exposure to one of three water ammonia concentrations: ambient (control); 400 μmol l^{-1} NH_4Cl, or 1600 μmol l^{-1} NH_4Cl. N = 8 in all cases.

Individual reflex responses by mullet to a mechanical stimulus, a small weight dropped into their tank, were filmed with a high speed camera (500 frames/sec). Digital video sequences were exported to tracking software for analysis and calculation of the various events and dependent variables that comprise the startle reflex. Detailed materials and methods are as reported in Le François *et al.* (2005) with the exception that fish were allowed 48h recovery from anaesthesia. Figure 4 shows the effects of ammonia upon a series of "non-locomotor" variables. There was no effect of ammonia on overall

responsiveness as shown by the percentage of animals that responded to the stimulus. This is an indicator of the fish's acoustic and/or visual sensitivity and its motivation to escape (Domenici and Blake, 1997). There was, however, a direct effect of ammonia upon response latency, the interval between stimulus onset to the first detectable movement leading to the escape of the animal (Fig. 4). Response latency will be a function of nervous performance throughout the reflex arc. Thus, these results indicate that the observed accumulation of ammonia in the brain (and presumably also peripheral nervous tissues) did not affect overall sensitivity and motivation to escape, but did have negative effects upon those elements of nervous performance which determine latency.

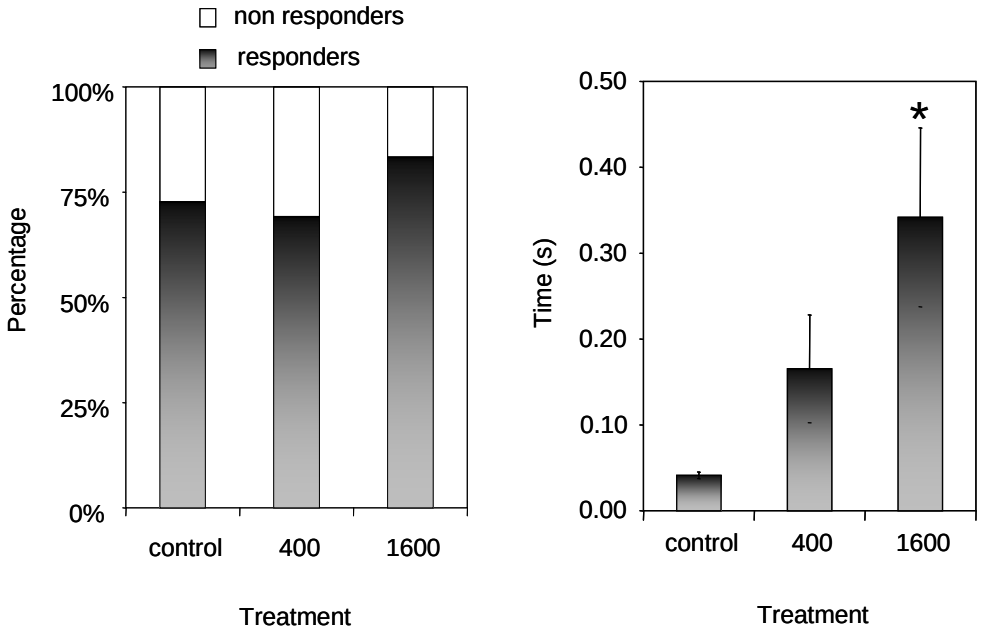

Figure 4. Responsiveness (percentage responding) and mean (± SEM) response latency to a startling stimulus by golden grey mullet (*Liza aurata*) at 24h following exposure to one of three water ammonia concentrations: ambient (control); 400 μmol l^{-1} NH_4Cl, or 1600 μmol l^{-1} NH_4Cl. N = 12 for responsiveness, N = at least 8 for latency. An asterisk indicates a significant difference from the control value (Tukey test *post-hoc* to one-way ANOVA, $p < 0.05$).

Once the escape response is triggered, "prey" locomotor performance can be evaluated with variables such as maximum turning rate during the C-bend, the maximum acceleration and maximum velocity achieved during the subsequent escape phase, and the total cumulative distance covered as a result (Domenici and Blake, 1993; 1997; Le François *et al.,* 2005). Figure 5 shows that maximum turning rate during the C-bend was significantly lower in mullet exposed to the highest concentration of ammonia. Maximum acceleration was not significantly different between ammonia-exposed animals and the controls, although there is some evidence of a trend that is consistent with the trends observed in the other locomotor performance variables. That is, the maximum velocity

achieved was significantly affected by both concentrations of ammonia, with an apparently direct relation between water (and therefore tissue) ammonia levels and performance. The cumulative distance covered was significantly reduced by exposure to the highest ammonia concentration. All of these locomotor variables are, of course, a direct result of glycolytic WM performance and, therefore, the results indicate that ammonia exposure has impaired WM function.

Further analysis of the data can provide a proximate reason for the impaired locomotor performance during the escape response. Figure 6 shows that the mullet exposed to ammonia typically only performed a single-bend reflex, hence an abbreviated and partial response by comparison to that observed in the control animals. A direct comparison of single versus double bend responses confirmed that the former generate lower acceleration, lower maximum velocity and, therefore, lower distance swum. This is another clear indication that WM function was impaired in the mullet exposed to ammonia. Unfortunately, it was not posible to calculate WM E_M in the hyperammonemic muscle because measurements of plasma and tissue pH were not made. However, it seems probable that the WM was depolarised in the hyperammonemic mullet, as has been observed in hyperammonemic salmonids (Beaumont *et al.,* 2000c).

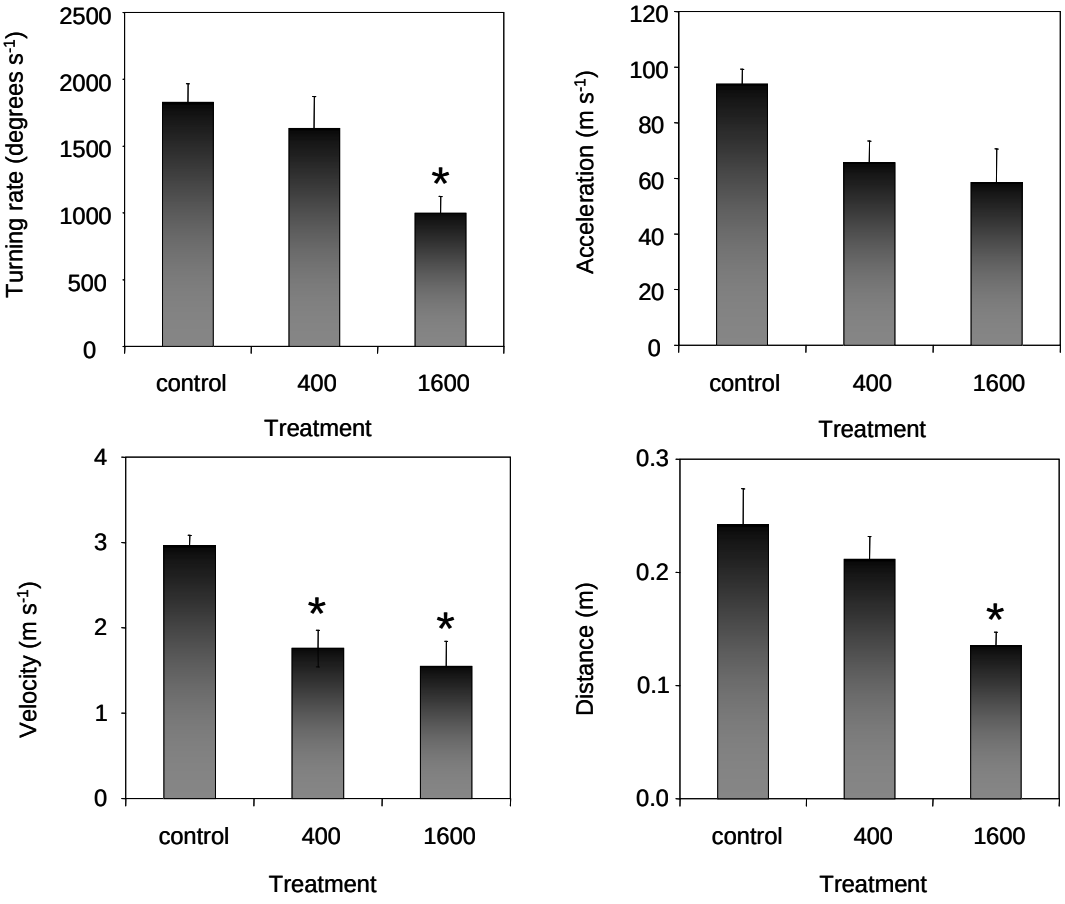

Figure 5. Mean (± SEM) turning rate, acceleration, escape velocity and distance swum in response to a startling stimulus by golden grey mullet (*Liza aurata*) at 24h following

exposure to one of three water ammonia concentrations: ambient (control); 400 μmol l^{-1} NH_4Cl, or 1600 μmol l^{-1} NH_4Cl. N = at least 8. An asterisk indicates a significant difference from the control value (Tukey test *post-hoc* to one-way ANOVA, $p < 0.05$).

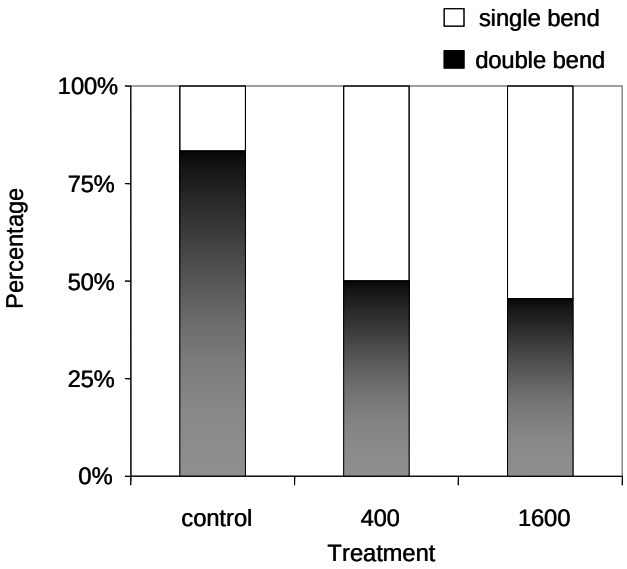

Figure 6. Percentage of either single or double bend responses to a startling stimulus by golden grey mullet (*Liza aurata*) at 24h following exposure to one of three water ammonia concentrations: ambient (control); 400 μmol l^{-1} NH_4Cl, or 1600 μmol l^{-1} NH_4Cl. N = at least 8.

Conclusions and perspectives

Their is now good evidence to indicate that exposure to sub-lethal concentrations of ammonia impairs the function of WM, such that fish cannot recruit the tissue to generate high tailbeat frequencies and high swimming speeds, or to power effective startle escape responses. This may have a number of implications for the animals in their natural environment. Fish recruit WM to negotiate velocity barriers (Peake and Farrell, 2004). This may be particularly important for salmonids during their spawning migration (Standen *et al.,* 2004), but also for all fish that might negotiate such barriers as part of their routine activities (Castro-Santos, 2005) or when seeking flow refuges during, for example, seasonal storms and floods. Thus, sub-lethal pollution by ammonia may not only impair the ability of salmonids to migrate (Wicks *et al.,* 2002), but also the ability of all fish species to successfully inhabit particular areas. The impairment of the startle response would presumably put the fishes at greater risk of predation, in particular, from aerial predators such a birds, that would not experience the toxic effects of increased water ammonia. The mullet was exceptionally tolerant of ammonia, but the fishes are known to colonise polluted habitats such as harbours. Salmonids might exhibit impaired escape responses at much lower ambient ammonia concentrations.

Acknowledgements

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Ammonia and salinity tolerance in the California Mozambique tilapia.

by

K. Suvajdzic[1], B. Sardella and C.J. Brauner

Introduction

The Salton Sea is a large inland lake (980 km^2) that was created in 1905, when water from the Colorado River was accidentally diverted, and flooded the Imperial and Coachella Valleys of SE California. The lake resides at 200 ft below sea level in a desert, and there is no outflow. However there is substantial evaporative water loss that approximately matches water input which is comprised largely of municipal and agricultural run-off. Consequently, in the last 100 years, the salinity of the Salton Sea has risen from freshwater to a current salinity of 43 g l^{-1}, and the salinity is currently increasing at a rate of about 0.3 g l^{-1} annually (Gonzalez *et al.,* 1998).

When the salinity of the Salton Sea reached that of seawater, a number of fish species were transplanted from the Sea of Cortez in the hopes of establishing a recreational fishery. Of those introduced, sargo (*Anisotremus davidsoni*), gulf croaker (*Bardiella icistius*), and orange mouth corvina (*Cynoscion xanthulus*), were very successful. Following this, the California Mozambique tilapia (*Oreochromis massambicus x O. urolepis hornorum*) was accidentally introduced, presumably from local fish farms, and more recently, has become the dominant fish species in the sea. Because of the ever increasing salinity, at some point an upper salinity limit will be reached beyond which sustainable fisheries for these species will not be possible. For this reason there has been interest in assessing the salinity tolerance of these species from a fisheries management perspective.

While salinities above that of seawater represent a considerable challenge to most fish, there are additional environmental factors that interact with salinity to affect a fishery, such as temperature, oxygen, ammonia, selenium, arsenic and sulfide (Watts *et al.,* 1991). We have been conducting experiments to investigate the salinity tolerance of the California Mozambique tilapia, and the influence that temperature and ammonia may have upon ionoregulation at salinities greater than seawater.

Salinity tolerance and the effect of temperature on California Mozambique tilapia

California Mozambique tilapia are remarkably tolerant of salinities greater than seawater. In fish that were acclimated to seawater (35ppt) at 25°C and then transferred every 5 days to a 10 g l^{-1} increase in salinity (at constant temperature), there were no changes in plasma Na^+, Cl^-, osmolality or gill Na^+,K^+-ATPase activity up to a salinity of

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65 g l^{-1}. These data indicate that these fish have a remarkable ability to tolerate high salinity at this temperature (Sardella *et al.,* 2004a). From 65 to 95 g l^{-1}, there was a progressive increase in all of these parameters. Despite an elevation in drinking rate and gill Na^+,K^+-ATPase activity (the predominant driving force for ion excretion), there was an elevation in plasma [Na^+], [Cl^-]and osmolality, at 75 g l^{-1} indicating that the tilapia were experiencing some degree of osmoregulatory stress at this temperature (Fig. 1). While fish in these experiments were only exposed to each salinity for 5 days before being subjected to the next salinity increase, longer duration exposure (28 days) to similar salinities at 25°C result in a qualitatively similar pattern (Sardella and Brauner, unpublished data).

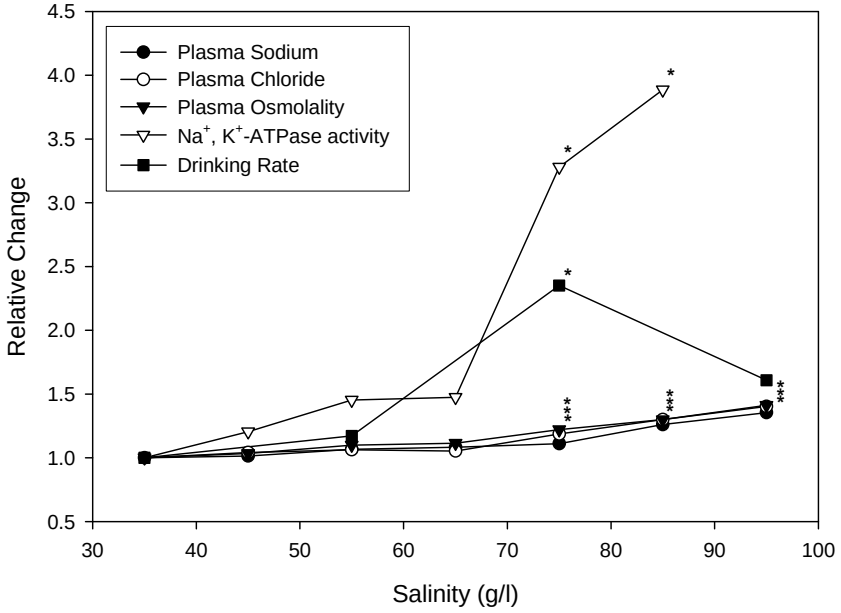

Figure 1. Indices of change relative to values measured in seawater-acclimated animals for Plasma osmolality, [Na^+], and [Cl^-], Na^+,K^+-ATPase activity and drinking rate (* absolute values statistically significant from seawater-acclimated fish, $p < 0.05$; modified from Sardella *et al.,* 2004a).

The Salton Sea experiences large seasonal fluctuations in temperature (13-35°C; Watts *et al.,* 2001) with little in the way of temperature refugia for fish, and thus it is very likely that fish in the Salton Sea will experience a great range of temperatures. When California Mozambique tilapia were acclimated to 35 g l^{-1} and then directly transferred to 15°C for 24 h, there were substantial increases in plasma Na^+, Cl^- and osmolarity, and a virtual elimination of gill Na^+,K^+-ATPase activity (Sardella *et al.,* 2004b). Simultaneous direct transfer from 35 g l^{-1} at 25°C to a salinity of 51 or 60 g l^{-1} at 15°C resulted in 100% mortality. At a constant temperature of 25°C, all tilapia survive direct transfer from 35 to 65 g l^{-1} with minimal osmoregulatory disturbances at 24 h and complete recovery by 5

days. Taken together, these data indicate that salinity tolerance is greatly reduced at 15 relative to 25°C. Thus, despite the tilapia's amazing ability to tolerate salinities greater than seawater at 25°C, they may be near to their salinity tolerance during the winter months when the water temperature drops below 15°C and large fish kills are observed in the Salton Sea (Sardella *et al.,* 2006).

Ammonia levels in the Salton Sea are also very high, and the following experiments were designed to assess ammonia tolerance of the California Mozambique tilapia and determine whether there are interactions between ammonia and salinity tolerance.

Ammonia toxicity and the effect of ammonia on salinity tolerance

Ammonia can exist in the ionized (NH_4^+) and unionized (NH_3) form, the latter being the most toxic to fish. Unionized ammonia levels have been reported to be as high as 1.2 mg l^{-1} in the Salton Sea, which exceeds the US EPA water quality criterion. To determine whether environmental ammonia levels could have an affect on survival of fish in the Salton Sea, we assessed the ammonia tolerance of California Mozambique tilapia, determined whether ammonia has an influence on salinity tolerance, and investigated the basis for ammonia tolerance; specifically whether this tilapia species is capable of producing urea during exposure to elevated water ammonia levels. It is known that some fish produce urea as a method of dealing with high ammonia levels.

California Mozambique tilapia hybrids (5-10 g) were exposed to a range of environmental ammonia levels for 96h at 25°C, at an average water pH of 8 following acclimation to either 35g l^{-1} or 44g l^{-1}salinity. Plasma ion levels and gill Na^+,K^+-ATPase activity were measured in surviving fish from these trials to determine whether ammonia exposure was associated with ionoregulatory disturbances. Total ammonia was measured and unionized NH_3 was calculated using the measured pH and a pKa of 9.354 in 35g l^{-1} and 9.374 in 44g l^{-1} seawater (Khoo *et al.*, 1977).

As seen in Figure 2, the 96hLC_{50} at 35 g l^{-1} was 9.75mM total ammonia or 0.48mM (8.26mg l^{-1}) unionized NH_3. In fish transferred to 44g l^{-1} during exposure to a range of environmental ammonia levels, the 96hLC_{50} was 0.49mM (8.27mg l^{-1}) NH_3 or 13.0 mM total ammonia, nearly identical to the 35g l^{-1} values. This is approximately seven fold greater than the unionized ammonia levels reported in the Salton Sea. In comparison with other fish, the California Mozambique tilapia are remarkably ammonia tolerant. Typical ammoniotelic teleosts have LC_{50} values for unionized ammonia less than 0.10 mM NH_3 (Walsh *et al*., 1993; Wang and Walsh, 2000; Person-Le Ruyet *et al*., 1995). High ammonia tolerance has been observed in a number of air-breathing fishes (Ip *et al*., 2004; Wood *et al.,* 2005, Randall *et al.,* 2004), including the oriental weatherloach (Tsui *et al*., 2004), the toadfish (Wand and Walsh, 2000), and others, and this is often associated with the ability to produce urea as discussed below.

In some marine fish, ionoregulatory disturbances have been observed during exposure to lethal ammonia concentrations (18.2 μmol l^{-1} NH_3 in Atlantic salmon), but

not at sub-lethal ammonia concentrations (Knoph and Thorud, 1996). Plasma from the California Mozambique tilapia that were transferred to 44g l^{-1} and survived the 96h ammonia exposures above did not exhibit any significant changes in plasma [Na^+] or [Cl^-] (Fig. 3). Minor elevations in gill Na^+,K^+-ATPase activity were observed at higher ammonia exposures (Fig. 4). In general, there appears to be little effect of ammonia on ionoregulation in California Mozambique tilapia, at least over this salinity range.

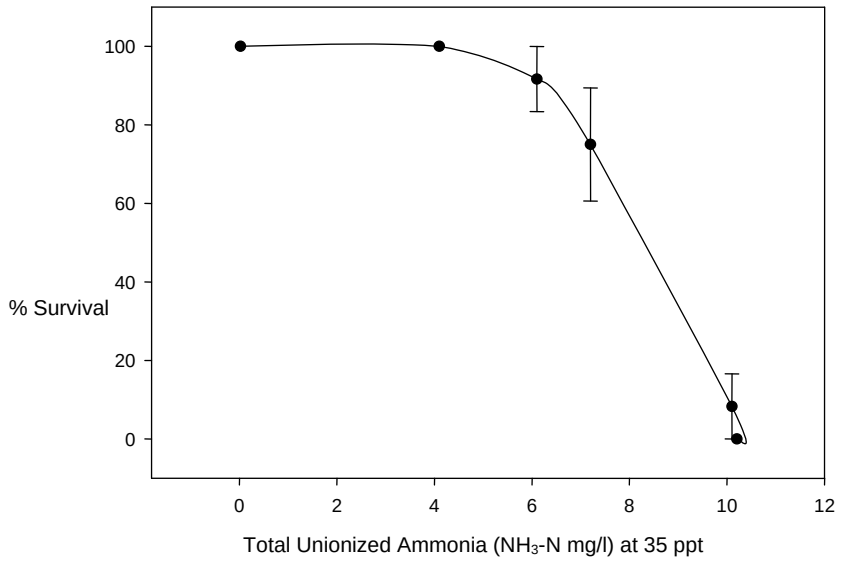

Figure 2. Percent survival of 35g l^{-1} acclimated fish following exposure to NH_3 (mg l^{-1}) for 96 hours.

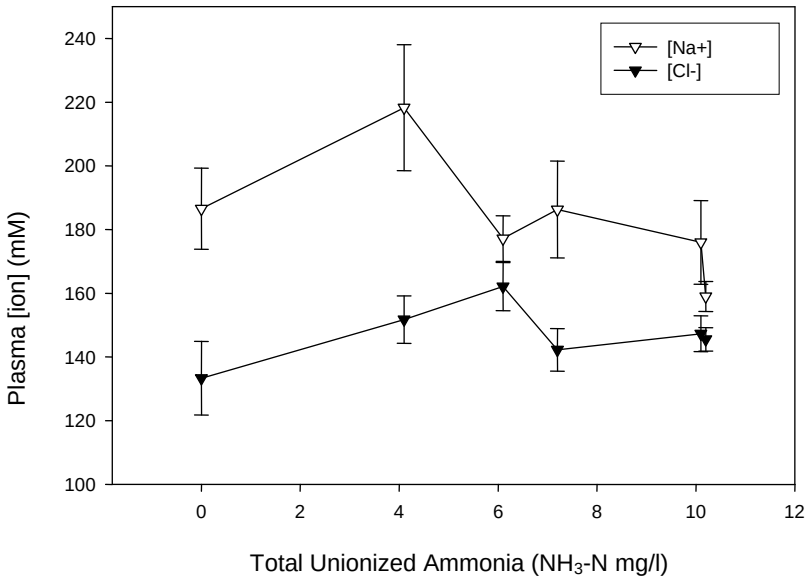

Figure 3. Plasma Na^+ and Cl^- concentrations (mM) in surviving fish following 96 h exposure to ammonia in 44g l^{-1} and 25°C.

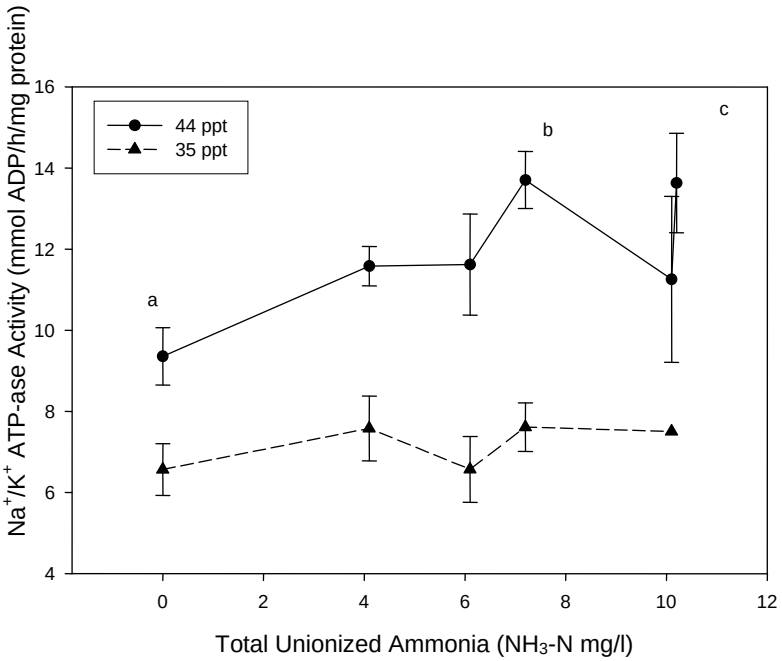

Figure 4. Branchial Na^+/K^+ ATPase activity in surviving fish following 96 h exposure to ammonia in 35g l^{-1} or 44g l^{-1} and 25°C.

Urea production

Fish that can survive exposure to high ammonia levels employ a range of strategies to survive what would otherwise be toxic conditions (Ip *et al.,* 2004; Randall *et al*., 2004; Wang and Walsh, 2000; Tsui *et al.,* 2004; Wilkie, 2002; Randall *et al.,* 1989; Wood *et al.,* 2005). The giant mudskipper *Periophthalmodon schlosseri* is able to decrease amino acid catabolism, or undergo partial amino acid catabolism thus decreasing ammonia production (Ip *et al.,* 2004; Randall *et al.,* 2004). Fish can also alter body surface pH to facilitate NH_3 volatilization as is seen in the Oriental weatherloach *Misgurnus anguillicaudatus* (Ip *et al.,* 2004; Tsui *et al.,* 2004). The slender African lungfish *Protopterus dolloi* expresses ornithine-urea cycle enzymes, and converts ammonia to urea (Ip *et al.,* 2004; Wood *et al.,* 2005), as does the Toadfish *Opsanus beta* and *O. tau* (Wand and Walsh, 2000). Of the mechanisms employed by fish to tolerate high internal ammonia levels, the most common is the production of urea (Wilkie, 2002), and this strategy has been observed in the Lake Magadi tilapia (*Oreochromis alcalicus grahami*) to tolerate high environmental pH (Randall *et al.,* 1989).

To determine whether the basis for California Mozambique tilapia to tolerate high environmental ammonia levels was associated with the ability to produce urea, fish were exposed to sub-lethal ammonia concentrations and urea appearance in the water was

measured. When tilapia hybrids were exposed to a sub-lethal ammonia concentration (8mM TAmm, or 4.4 mg l^{-1} unionized ammonia) minor changes in urea production were observed (Fig. 5). At no time did urea production ever exceed 15% of total nitrogenous waste excretion. Thus, although the California Mozambique tilapia appear to be extremely ammonia tolerant, they only produce limited amounts of urea and thus this is probably not their main mechanism of ammonia detoxification or basis for their exceptional ammonia tolerance.

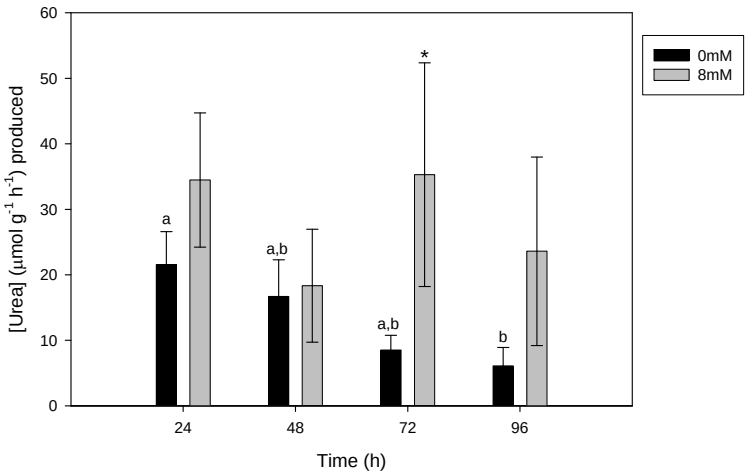

Figure 5. Urea production during a 96h exposure at 35 ppt to 0 and 8mM Total Ammonia. Letters (a, b, etc.) indicate significant difference within a treatment group. Asterisks (*) indicate significant difference at a given time between treatments.

In summary, California Mozambique tilapia hybrids are extremely ammonia tolerant with 96 h LC_{50}s of 0.48mM (8.26 mg l^{-1}) NH_3 at 35 g l^{-1}. This ability to withstand elevated ammonia levels is independent of salinity as the 96 h LC_{50} at 44g l^{-1} was nearly identical to that at 35 g l^{-1} (0.49mM/8.27 mg l^{-1} NH_3). Sublethal increases in environmental ammonia do not appear to have significant ionoregulatory consequences as there was no significant change in plasma [Na^+]or [Cl^-]concentration and only minor elevations in Na^+,K^+-ATPase. While some species of fish produce urea as a method of ammonia detoxification, this does not appear to be the case in these tilapia as urea production only increases slightly during sub-lethal ammonia exposure and never accounts for greater than 15% of total nitrogenous waste excretion. Although the tolerance of California Mozambique tilapia hybrids is far in excess of levels measured in the Salton Sea, how ammonia interacts with temperature and other abiotic factors has not been investigated and ammonia should not be discounted as a potential factor reducing survival in this species in the Salton Sea. Ammonia tolerance in other species of the Salton Sea is not known.

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Nitrite toxicity to fishes

by

R.C. Russo[1]

Introduction

Nitrite concentrations can build up in the aquatic environment from point sources such as fish culture systems with recirculated water; septic tanks; industrial effluents from metal, dye, and celluloid industries; and wastewater treatment plants if there is an imbalance among species of nitrifying bacteria. It can also enter aquatic systems from nonpoint sources such as fertilizer and animal wastes; feedlot discharges; nitric oxide and nitrite discharges from automobile exhausts; and leachates from waste disposal dumps. Nitrite is extremely toxic to many aquatic organisms, as shown by numerous studies of its toxicity and physiological effects.

Factors Affecting Nitrite Toxicity

There are differences in species sensitivities to nitrite, as shown in the toxicity data compilation in Table I. In general, saltwater fish species appear to be more tolerant of nitrite than are fresh water species. Salmonids are the most sensitive species, and centrarchids are the most resistant. Most reported toxicity tests were static bioassays. One research group (Tilak *et al*., 2002) performed both static and continuous flow tests and obtained very similar results; static values were consistently higher, but not significantly so. The U.S. Environmental Protection Agency EcoTox database has additional data records (U.S. EPA, 2002).

Several investigators have studied acute nitrite toxicity on fish of different sizes. Alcaraz and Espina (1995) reported that larger juvenile grass carp (*Ctenopharyngodon idella*) are more tolerant than smaller ones. Almendras (1987) found that smaller juvenile milkfish (*Chanos chanos* Farsskae) are more tolerant. Atwood *et al*. (2001b) reported that smaller Nile tilapia (*Oreochromis niloticus*) were significantly more tolerant of nitrite than larger specimens. Palachek and Tomasso (1984a) found that smaller fathead minnows (*Pimephales promelas*) were more tolerant than larger ones. Russo (1980), who tested rainbow trout (*Oncorhynchus mykiss*) of sizes 2-387 g, found no differences related to fish size; however, Lewis and Morris (1986), using a different statistical procedure on the same data, reported that small fish had significantly higher LC_{50}s than large fish. Hilmy *et al*. (1987) found a modest difference in susceptibility between 65- and 166-g *Clarias lazera*, with the larger fish somewhat more susceptible than the smaller ones. No significant difference due to fish size was found between channel catfish (*Ictalurus punctatus*) of 3.0 g and 80.2 g or between largemouth bass *(Micropterus salmoides*) of 2.8 g and 36.3 g (Palachek and Tomasso, 1984b; Tomasso, 1986).

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Water chemistry conditions affect nitrite toxicity. The aqueous nitrite equilibrium is pH-dependent, with relative concentrations of ionized nitrite (NO_2^-) and un-ionized nitrous acid (HNO_2) dependent on the pH of the system. The concentration of HNO_2 is 4-5 orders of magnitude less than the concentration of NO_2^- in the pH range 7.5-8.5 (Russo *et al*., 1981). It has been shown that over the pH range 6.4-9.0 the toxicity of total nitrite on rainbow trout (*Oncorhynchus mykiss*) decreases as pH increases. As pH increases, NO_2-N toxicity decreases and HNO_2-N toxicity increases (Russo *et al*., 1981). Furthermore, Bowser *et al*. (1983) found that dissolved oxygen affects the toxicity of nitrite: an oxygen concentration of 5 mg/L in the presence of nitrite was insufficient for channel catfish (*Ictalurus punctatus*), even though this species would normally tolerate lower oxygen concentrations.

In studies on juvenile grass carp (*Ctenopharyngodon idella*) at 3 temperatures, Alcaraz and Espina (1995) found lower toxicity on larger fish (7.60 g) at 29 ^oC than at 24 ^oC or 32 ^oC. A temperature of 29 ^oC had a "protective effect" for the larger fish; the authors suggested that this could be attributed to the fact that biochemical and enzymatic processes are sensitive to temperature, being more efficient in the temperature range corresponding to the preferred temperature of the species. Adult grass carp generally prefer 29 ^oC. At a temperature of 24 ^oC nitrite was less toxic to smaller (0.02 g) fish than at 29 ^oC or 32 ^oC. The authors suggested the possibility that smaller fish have a different interval of preferred temperature or perhaps the acclimation mechanisms in smaller fish have not developed completely.

Watenpaugh *et al*. (1985) investigated the temperature tolerance of channel catfish (*Ictalurus punctatus*) exposed to sublethal concentrations of nitrite for 24 hours. The critical thermal maximum (a measure of the upper limit of thermal tolerance) was inversely related to nitrite concentration. Percent methemoglobin was correlated with nitrite concentration and was inversely correlated with the critical thermal maximum. The authors concluded that nitrite exposure had the potential of adversely affecting the productivity of high density channel catfish aquaculture systems at higher temperatures.

Crawford and Allen (1977) studied the toxicity of nitrite on chinook salmon (*Oncorhynchus tshawytscha*) and found that the toxicity of nitrite in seawater was markedly less than that in fresh water and that increasing the calcium concentration in both fresh water and seawater decreased the toxicity of nitrite. However, Atwood *et al*. (2001a) tested southern flounder (*Paralichthys lethostigma*) in fresh and brackish water and found similar mortality at similar nitrite concentrations. In both fresh and brackish water, plasma nitrite concentrations were well below environmental concentrations. Plasma nitrite concentrations increased significantly with increasing environmental nitrite concentrations in both fresh and brackish water, but fish did not appear to concentrate nitrite in plasma. Grosell and Jensen (1999), in studies with European flounder (*Platichthys flesus*), reported concentration of nitrite in plasma. In studies with juvenile mullet (*Mugil platanus*) Sampaio *et al*. (2002) found that fish acclimated to fresh water were significantly more sensitive to nitrite than those held at higher salinities. Increased nitrite toxicity in fresh water vs. salt water has also been reported for European eel *Anguilla anguilla* (Saroglia *et al*., 1981) and for red drum *Sciaenops ocellatus* (Wise and Tomasso, 1989).

The presence of other chemicals affects nitrite toxicity. It is well known that chloride ions inhibit nitrite toxicity in fishes (Russo and Thurston, 1977; Perrone and Meade, 1977; Wedemeyer and Yasutake, 1978; Tomasso *et al*., 1979; Tomasso, 1986). Tomasso (1986), however, found no effect of chloride on toxicity of nitrite in largemouth bass (*Micropterus salmoides*). Atwood *et al*., (2001a) reported that environmental chloride in the form of salts had little effect on either survival or uptake of nitrite by southern flounder (*Paralichthys lethostigma*). The high serum chloride concentrations of saltwater species (e.g., pinfish) (Folmar *et al*., 1993) may help explain their greater tolerance to nitrite than freshwater species. Atwood *et al*. (2001b) tested Nile tilapia (*Oreochromis niloticus*) and found increased survival with added chloride. The plasma nitrite values in the tests with chloride added were well below the environmental concentrations. They also reported that there was no difference whether the chloride was added as calcium chloride or sodium chloride, whereas others (Wedemeyer and Yasutake, 1978 with rainbow trout (*Oncorhynchus mykiss*); and Mazik *et al*., 1991 with striped bass (*Morone saxatilis*)) reported that calcium chloride is a more effective nitrite toxicity inhibitor than sodium chloride. Chloride was more effective at inhibiting the uptake of environmental nitrite by shortnose sturgeon fingerlings (*Acipenser brevirostrum*) when chloride was added as calcium chloride rather than as sodium chloride (Fontenot *et al*., 1999). They found that shortnose sturgeon fingerlings previously exposed to nitrite (for 2 days) had significantly lowered plasma NO_2-N levels when calcium chloride was added to the water. They suggested calcium chloride could be an effective treatment for nitrite toxicity for shortnose sturgeon

Most channel catfish farmers in the southeastern United States routinely add sodium chloride to their ponds to prevent methemoglobinemia. In a survey of Alabama catfish farmers, Sipaúba Tavares and Boyd (2003) found most farmers maintain a chloride concentration of 50-100 mg/L through annual sodium chloride applications.

Other substances including bromide, sulfate, phosphate, nitrate, methylene blue, ascorbic acid, and uric acid have been reported to inhibit nitrite toxicity to different degrees.

Sublethal Effects

Clinical observations in fishes of stress induced by nitrite exposure reported by Das *et al*. (2004c) included: erratic swimming, frequent opercular movement, surfacing, resting on bottom of tank with irregular opercular movements, loss of equilibrium, and lying on their sides. Reduction in food consumption and growth rate have also been observed (Kumta and Gaikwad, 1997).

Stormer *et al*. (1996) exposed rainbow trout (*Oncorhynchus mykiss*) to nitrite concentrations of 1 mM for 8 days. They found nitrite accumulation in plasma to concentrations above the environmental level. Plasma nitrate concentration increased in parallel with the accumulation of nitrite. Hematocrit and blood hemoglobin decreased; the concentration of chloride and bromide in plasma decreased; K^+ concentration in caudal muscle tissue decreased significantly and water content of muscle tissue also decreased. Tested fish fell into two distinctly different groups: those that died between 1

and 2 days of exposure and those that survived up to day 4 but died before day 6. Nitrite accumulation was more rapid in the first group.

In experiments using isolated rainbow trout (*Oncorhynchus mykiss*) hepatocytes, Doblander and Lackner (1996) found that the hepatocytes detoxified nitrite through oxidation to nitrate. Detoxification was inhibited by bumetanide and furosemide, but was strongly accelerated by uric acid. Uric acid also enhanced detoxification activity in trout liver.

Das *et al*. (2004a) studied the effects of nitrite on mrigal (*Cirrhinus mrigala* (Ham.)), a fish species of major importance for aquaculture in India. Exposure of mrigal fingerlings to sublethal nitrite levels (1-8 mg/L NO_2-N) in static toxicity tests caused progressive reduction in total erythrocyte count, hemoglobin, and serum protein content. Both decrease and increase in total erythrocyte count were observed, depending on concentration and exposure period. Blood glucose decreased up to 24-h of exposure at all concentrations, but then increased until 96 h of exposure. Similar results were reported for catla (*Catla catla* (Ham.)) by Das *et al*. (2004b) and Das *et al.* (2004c).

In a 96-h study of the effects on enzymes of sublethal nitrite exposures to catla (*Catla catla*), rohu (*Labeo rohita*), and mrigal (*Cirrhinus mrigala*) Das *et al*. (2004c) observed similar responses in all three species. As nitrite concentration increased from 1 to 10.4 mg/L, reduction in activities was observed in acetylcholinesterase (AChE) in brain and liver; alkaline phosphatase (ALP) in serum, brain, and gill; and acid phosphatase (ACP) in gill. There was also a progressive increase in alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) activities in brain, gill, and serum; and ACP activity in serum and brain in response to increasing nitrite concentrations. Lactate dehydrogenase (LDH) activity increased in gill, liver, kidney, brain, and serum up to 8 mg/L nitrite with a reduction in activity at the highest (10.4 mg/L) concentration tested. AChE activity reduction was indicative of liver tissue damage. LDH activity increase was attributable to prevalence of anoxia. The increase in ALAT and ASAT activities in serum and the brain is an indicator of tissue damage. They suggested that the measurement of LDH activity in tissues (but not serum) of the Indian major carp fingerlings could be a biomarker for nitrite toxicity.

In 15-day exposures to sublethal nitrite levels with grass carp (*Ctenopharyngodon idella*) Alcaraz and Espina (1997) found that ingestion rates were not affected, but assimilation efficiency and assimilation were reduced at concentrations of 1.6 and 2.5 mg/L NO_2-N. Respiration rates decreased and nitrogen excretion rate increased at those levels. The sublethal nitrite exposures reduced the scope for growth of the fish.

Kumta and Gaikwad (1997) conducted sublethal exposures to 1.25 mg/L of $NaNO_2$ [0.2 mg/L NO_2-N] in mosquitofish (*Gambusia affinis*) for one month. Reported fecundity was significantly reduced in exposed fish. The gonadosomatic index was also reduced in exposed fish. These results demonstrate the adverse effect of nitrite on reproduction. Reductions in food consumption (67.3%) and growth rate (59.5%) were also observed.

In 5-d exposures of shortnose sturgeon (*Acipenser brevirostum*) fingerlings to nitrite Fontenot *et al*. (1999) found that plasma nitrite concentrations were more than 63 times the environmental concentration. Other species have also been reported to concentrate nitrite in plasma to concentrations greater than environmental exposure concentrations, including channel catfish *Ictalurus punctatus* (Tomasso *et al*., 1979; Palachek and Tomasso, 1984b; Tomasso, 1994); blue tilapia *Tilapia aurea* (Palachek and Tomasso, 1984b); and rainbow trout *Oncorhynchus mykiss* (Eddy *et al*., 1983).

In studies on sea bass (*Lates calcarifer*) exposed to 30 to 80 mg/L NO_2-N for 4 days, Woo and Chiu (1997) observed osmoregulatory dysfunction with elevated serum Na^+ and Cl^- levels and reduced branchial Na^+-K^+-ATPase activity. Serum lactate levels were significantly elevated and serum protein levels reduced at only the 80 mg/L exposure. Increased serum ammonia and urea and decreased serum glucose and liver glycogen levels were observed at lower nitrite levels. Significant decreases were found in activities of glycogen phosphorylase *a,* glutamate-oxaloacetate transaminase, and glutamate dehydrogenase in the liver. Exposure to 50 mg/L induced an increase in the rate of ammonia excretion.

Pinfish (*Lagodon rhomboides*) were exposed to nitrite for 96 h by Folmar *et al*. (1993), and 20 serum chemistry parameters were measured. They used concentrations of $NaNO_2$ from 1.2-11 mg/L [0.24-2.2 mg/L NO_2-N]. They analyzed for sodium, potassium, chloride, calcium, magnesium, iron, inorganic phosphorus, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, creatine kinase, blood urea nitrogen, creatinine, albumin, cholesterol, glucose, total protein, triglyceride, and uric acid. The only significant change observed was a decrease in uric acid at 48 h, while no difference was observed at 96 h. There were no other alterations in electrolyte balance or serum chemistries.

A recent review by Jensen (2003) provides a more comprehensive discussion of the physiological effects of nitrite on fishes and other aquatic animals, including ion regulatory, respiratory, cardiovascular, endocrine, and excretory processes.

Increase in susceptibility to infection can occur in fishes subjected to sublethal concentrations of some toxicants. Carballo and Muñoz (1991) investigated the susceptibility of rainbow trout (*Oncorhynchus mykiss*) to infection by fungus *Saprolegnia parasitica* (*sin. S. diclina* type 1). Only *Saprolegnia* spp. have been found on live salmonids, even though the fish come in contact with many fungal spores. Fish infected with these spores experience a breakdown in their osmoregulatory mechanism and, unless they can be treated, the condition is fatal. The authors tested the effects of ammonia, nitrite, copper, and cyanide on susceptibility to infection. In 10-d exposures to 0.05 mg/L NH_3-N or 0.12 mg/L NO_2-N at inoculum concentrations of $1.4x10^6$, $9.75x10^5$, and $5x10^5$ zoospores/L they found that 75% of ammonia-exposed fish tested at the highest inoculum concentration and 50% of the nitrite-exposed fish at the highest inoculum concentration developed infection. Infection occurred in 20% of ammonia-exposed fish at the medium fungal dose. No infection occurred in nitrite-exposed fish at the medium dose, and no infection occurred in either ammonia- or nitrite-exposed fish at the lowest fungal dose tested. No infection was found with copper and cyanide exposures. Their experiments confirmed that ammonia and nitrite predispose fish to saprolegniosis, and they concluded

that fish in fish farms that are subjected to elevated ammonia and nitrite levels are at increased risk of saprolegniosis.

Long-term nitrite toxicity

Wedemeyer and Yasutake (1978) tested 5- to 10-g steelhead trout (*Oncorhynchus mykiss*) at NO_2-N concentrations of 0, 0.015, 0.030, and 0.060 mg/L in a flow-through system over a 6-month rearing period. The exposed fish exhibited mild methemoglobinemia. There was no effect on growth, and transfer to 30% seawater over a 48-h period caused no mortalities. No hematological abnormalities were observed. At the 0.060 mg/L exposure concentration, there was minimal hypertrophy, hyperplasia and lamellar separation in gill epithelium. This decreased over time, and after 28 weeks of exposure most of the test fish had recovered.

Hilmy *et al*. (1987) carried out 6-month exposures on juvenile *Clarias lazera* at a NO_2-N concentration of 1/10 of the 96-h LC_{50} values (28 mg/L for 65-g fish and 32 mg/L for 166-g fish). Erythrocyte count, hemoglobin concentration, and hematocrit values were decreased from the beginning in nitrite-exposed fish, reaching minimum levels after 4 months exposure; after this, the values began to increase during the remainder of the exposure period. The nitrite exposure caused a mild but statistically significant methemoglobinemia. There was a significant decrease of serum total proteins in the first 4 months followed by an elevation during the remainder of the experiment; however, the values were still less than those of control fish. The authors surmised that this trend in the 5^{th} and 6^{th} months represents an adaptation response to nitrite. Histological effects of gill hypertrophy and hyperplasia were observed (Michael *et al*., 1987)

Joint toxicity of ammonia and nitrite

In exposures of rainbow trout (*Oncorhynchus mykiss*) to nitrite and ammonia simultaneously for 4 days Vedel *et al*. (1998) observed high mortality (68%) in fish exposed to 500 μM ammonia [7 mg/L NH_3-N] plus 600 μM nitrite [8.4 mg/L NO_2-N], the highest concentration combination tested. Nitrite and ammonia interactive effects (either synergism or antagonism) were not observed in the physiological parameters measured. Nitrite was accumulated in plasma to about twice the exposure concentration. Ammonia and nitrite exposure both caused a significant and additive increase in muscle potassium concentrations. There was also an increase in methemoglobin concentration by nitrite. Ammonia exposure decreased brain glutamate concentration while glutamine concentration increased. The authors suggested that ammonia was detoxified by reacting with glutamate to form glutamine.

Nitrite Toxicity to Invertebrates

Yildiz and Benli (2004) studied the effects of nitrite on narrow-clawed crayfish (*Astacus leptodactylus*) and found nitrite to be acutely toxic at a concentration range of 22-70 mg/L NO_2^- [6.7-21.3 mg/L NO_2-N] for 48-h exposures. The toxic concentration was increased to 31-80 mg/L NO_2^- [9.4-24.3 mg/L NO_2-N] when 100 mg/L chloride was added to test solutions. They measured hemolymph nitrite, total hemocyte counts (THC),

and hemolymph glucose at sublethal nitrite concentrations. They found accumulation of nitrite in hemolymph increased directly with water nitrite concentration. The hemolymph nitrite values were significantly higher than the exposure levels, up to 34 times the exposure level of 25 mg/L NO_2^- [7.6 mg/L NO_2-N]. After a 24-h recovery period in nitrite-free water, hemolymph nitrite decreased. Nitrite was completely eliminated from the hemolymph only when the lowest (9 mg/L NO_2^-, 2.7 mg/L NO_2-N) environmental concentration was used. THC decreased in nitrite-exposed crayfish and increased after 24-h recovery. Hemolymph glucose levels increased with increasing nitrite exposure concentrations. They also tested the effect of chloride addition and found the accumulation of nitrite in hemolymph to be low relative to the nitrite-only tests. THC increased in nitrite-plus-chloride tests and remained elevated after return to nitrite-free water. Hemolymph glucose levels did not change in nitrite-plus-chloride tests. The addition of environmental chloride itself caused a decrease in THC, indicating stress.

In studies where freshwater crayfish *Astacus astacus* were exposed to 0.8 mM nitrite [11.2 mg/L NO_2-N] for 7 days Jensen (1990) found a rapid accumulation of nitrite in hemolymph to concentrations much higher than the exposure concentration, plateauing at an internal/external ratio of about 10. There was essentially no mortality at the test concentration. Hemocyanin concentration of the hemolymph decreased significantly, and oxygen tension increased. Chloride and sodium concentrations in hemolymph decreased and plateaued after 2 days. Potassium, calcium, and magnesium concentrations of the hemolymph remained essentially constant throughout the 7-d test period.

A number of toxicity studies have been conducted on shrimp, and reported 96-h LC_{50} values for shrimp are summarized in Table II.

Shrimp *Litopenaeus vannamei* were exposed for 4 days to nitrite concentrations of <0.1 to 8.8 mg/L NO_2-N under different conditions of salinity, chloride and calcium levels (Sowers *et al*., 2004). Hemolymph nitrite increased significantly and was dose-dependent and higher than environmental nitrite concentrations. Hemolymph nitrite concentrations were inversely related to the level of dissolved solids and to the chloride concentrations. Nitrite uptake did not interfere with normal water and ion balance. 96-h LC_{50} values ranged from 8.4 to 30 mg/L NO_2-N under conditions of total dissolved solids of 2 to 10 g/L. Increasing total dissolved salt and chloride concentration resulted in reduced toxicity. They concluded that large quantities of salts would be required to manage water quality in shrimp ponds and suggested that a more economical approach would be to reduce pond inputs of nitrogen through stocking and feeding rates and feed formulations, and removal of pond wastes.

Lin and Chen (2003) conducted acute toxicity studies on *Litopenaeus vannamei* juveniles at salinity levels of 15, 25, and 35 ‰. The 96-h LC_{50} values were 76.5 mg/L NO_2-N at 15 ‰, 178.3 mg/L at 25 ‰, and 321.7 mg/L at 35 ‰. As the salinity decreased from 35 ‰ to 15 ‰, susceptibility to nitrite increased by 421% after 96 h exposure. The tests were continued to 144 h, with the LC_{50} values found at 144 h being 61.1, 152.4, 257.2 mg/L NO_2-N at 15, 25, and 35 ‰, respectively. The authors suggested that a "safe level" for culturing *L. Vannamei* juveniles would be 6.1, 15.2, and 25.7 mg/L NO_2-N in 15, 25, and 35 ‰ salinity.

The toxicity of nitrite on *Penaeus monodon* adolescents in 20 ppt seawater was reported by Chen *et al*. (1990a) to be 218, 193, 171, 140, 128, and 106 mg/L NO_2-N LC_{50} values at 24, 48, 96, 144, 192, and 240 h, respectively. The toxicity curve approached an asymptote at 240 h. The authors suggested that a safe value for culturing adolescent *P. monodon* at 20 ppt salinity, 7.57 pH, and 24.5 ^{o}C was 10.50 mg/L NO_2-N. In similar tests on *Penaeus chinensis* in 33 ‰ seawater (Chen *et al*., 1990b), the LC_{50} values at 24, 96, 120, 144, and 192 h were 339, 37.71, 29.18, 26.98, and 22.95 mg/L, respectively. The asymptotic LC_{50} was 22.95 mg/L NO_2-N at 192 h. The authors suggested that a "safe value" for *P. chinensis* juveniles was 2.30 mg/L NO_2-N.

Chen and Cheng (1995) studied sublethal effects of nitrite exposure in *Penaeus japonicus.* Nitrite exposures ranged from 5.12 to 50.86 mg/L NO_2-N in 30 ppt seawater for 24 h. Nitrite and urea accumulated in the hemolymph and excretion of ammonia increased with increasing nitrite over the entire concentration range tested. Nitrite exposure decreased the oxyhemocyanin and the ratio of oxyhemocyanin/protein. Cheng and Chen (2001) also investigated the time-course change of nitrogenous excretion in *Penaeus japonicus* after 48-h exposure to nitrite concentrations ranging from 0.076 to 1.433 mM at 30 ppt salinity. Hemolymph nitrite and urea increased with nitrite concentration and exposure time, whereas hemolymph ammonia was inversely related to nitrite concentration and exposure time. Excretion of total nitrogen, ammonia nitrogen, urea nitrogen, and organic nitrogen increased with nitrite concentration and exposure time. The authors found that exposure of *P. japonicus* for 24 h to nitrite concentrations as low as 0.076 mM increased ammonia nitrogen excretion by a factor of 1.9, urea nitrogen excretion by 200, and organic nitrogen excretion by 37 as compared to controls.

Sublethal effects of nitrite exposure to *Penaeus monodon* were studied by Chen and Cheng (2000). Exposure concentration was 0.72 mM nitrite at pH values of 6.8, 8.2, and 9.8, and exposure time was 48 h. Nitrite influx, hemolymph nitrite and osmotic differential increased with exposure time and were higher at pH 6.8. Water nitrite concentration, oxyhemocyanin, protein, the oxyhemocyanin/protein ratio, and hemolymph osmolality decreased with exposure time and were lower at pH 6.8. The same parameters were measured for shrimp placed in nitrite-free water after 3, 6, 12, and 24 h following 48-h exposure to nitrite. Water nitrite concentration increased with depuration time and was higher at pH 6.8, whereas hemolymph oxyhemocyanin, oxyhemocyanin/protein ratio, and hemolymph osmolality increased with depuration time and were higher at pH 9.8. Hemolymph nitrite decreased with depuration time and after 24 h, depuration was undetectable, with 72-88% recovery of oxyhemocyanin.

Penaeus setiferus postlarvae were exposed to NO_2-N concentrations of 25, 50, and 100 mg/L for 72 h in an investigation (Alcaraz *et al*., 1997) of effects of ammonia, nitrite, and their combination to thermal tolerance of the shrimp. Mortalities of 10, 20, and 35% occurred at nitrite concentrations of 25, 50, and 100 mg/L, respectively. The 72-h LC_{50} was 172.8 mg/L NO_2-N. The temperature tolerance of the tested shrimp postlarvae was unaffected by the 25 mg/L NO_2-N exposure. However, the temperature tolerance was reduced by 5.0 and 8.4% at exposures of 50 and 100 mg/L, respectively. No difference was observed in critical thermal maximum values between organisms exposed to 50 and 100 mg/L. Four different ammonia-nitrite combination exposures were tested, with concentrations being based on the individual toxicant's LC_{50} value. The researchers found no relationship between the mortality rate and the toxicity ratio of

ammonia and nitrite mixtures. The authors concluded that mortality rates were related to ammonia concentration and not to the toxicity ratios of ammonia and nitrite mixtures. However, the thermal tolerance of the shrimp exposed to ammonia-nitrite mixtures was related to the toxicity ratio of the mixtures. The critical thermal maximum did not decrease in organisms exposed to a joint concentration of 0.8 mg/L NH_3-N and 60 mg/L NO_2-N, but decreased significantly compared to controls in the higher joint concentrations tested. The authors concluded that the toxic contribution of both compounds determined the thermal response of the shrimp.

Koo *et al*. (2005) investigated the effect of nitrite on juvenile tiger crab (*Orithyia sinica*) after 30-d exposures to nitrite concentrations of 50, 100, 150, 200, and 250 mg/L NO_2-N. Survival rates decreased linearly with concentration and exposure time. The growth rate of the crabs decreased at 150, 200, and 250 mg/L nitrite. The intermolt period of the crabs was shortened between the first and second molt, and the numbers of moltings of crabs exposed to higher concentrations were significantly higher than that of controls.

Toxicity tests on seven species of aquatic invertebrates have been carried out by Russo and coworkers (unpublished data): two species of Ephemeroptera (mayfly), *Ephemerella doddsi* and *Ephemerella grandis*; three species of Plecoptera (stonefly), *Arcynopteryx parallela*, *Pteronarcella badia*, and *Isoperla fulva*; one species of Tricoptera (caddisfly), *Arctopsyche grandis*; and one species of Diptera (true fly), *Atherix variegata*. All of these species are common to cold water environments in the Western United States. The 96-h LC_{50} values for the mayflies ranged from 0.52-2.00 mg/L NO_2-N; for the stoneflies from 0.25-0.46 mg/L; and for the caddisfly 1.02-2.43 mg/L. No LC_{50} was obtainable for the true fly *Atherix variegata* because fewer than 50% of the test organisms died at the highest concentrations used in two tests; there was 38% mortality at 123 mg/L NO_2-N, the highest concentration tested. *E. grandis* and *P. badia* were also tested for nitrite toxicity with added sodium chloride to investigate whether added chloride reduced nitrite toxicity in these insect species, as is commonly observed with fishes. The addition of chloride ion greatly reduced nitrite toxicity in *E. grandis* and *P. badia.* The LC_{50} with added chloride was 3.5-10 times greater than without chloride for *E. grandis*; the LC_{50} was 30-50 times greater than without chloride for *P. badia*.

Table I. Toxicity of Nitrite in Different Fish Species.

Species	96-h LC_{50} (mg/L N)	Reference
Rainbow trout *Oncorhynchus mykiss*	0.2-0.3	Russo *et al*. (1974); Russo and Thurston (1977)
Cutthroat trout *Salmo clarkii*	0.5-0.6	Thurston *et al*. (1978)
Channel catfish *Ictalurus punctatus*	7.1	Tomasso (1986)
Walking catfish *Clarias lazera*	28-32	Hilmy *et al*. (1987)
Grass carp *Ctenopharyngodon idella*	6-13	Alcaraz and Espina (1995)
Mrigal *Cirrhinus mrigala* (Ham.)	10.4	Das *et al*. (2004a)
Indian major carp *Catla catla* Hamilton	120.84:24h static 17.43:24h flwth	Tilak *et al*. (2002)
Goldfish *Carassius auratus*	52	Tomasso (1986)
Fathead minnow *Pimephales promelas*	2.3-3.0	Russo and Thurston (1977)
Mosquitofish *Gambusia affinis*	1.5	Wallen *et al*. (1957)
Striped bass *Morone saxatilis*	50 (24 h)	Mazik *et al*. (1991)
Green sunfish *Lepomis cyanellus*	160	Tomasso (1986)
Bluegill *Lepomis macrochirus*	80	Tomasso (1986)
Largemouth bass *Micropterus salmoides*	140.2	Palachek and Tomasso (1984b)
Smallmouth bass *Micropterus dolomieui*	160	Tomasso (1986)
Guapote tigre *Cichlasoma managuense*	20.4	Chin and Shyong (1998)
Siberian sturgeon *Acipenser baeri,*Brandt	130 (72 h) (130.5 mg/L Cl^-)	Huertas *et al*. (2002)
Nile tilapia *Oreochromis niloticus*	8 to 81	Atwood *et al*. (2001b)
Blue tilapia *Oreochromis aureus*	16.2	Palachek and Tomasso (1984b)
Mullet *Mugil platanus*	35.9-36.2 SW 1.51 FW	Sampaio *et al*. (2002)
Southern flounder *Paralichthys lethostigma*	35.2	Atwood *et al*. (2001a)
Mottled sculpin *Cottus bairdi*	>67	Russo and Thurston (1977)

Table II. Nitrite Toxicity in Shrimp

Species	96-h LC_{50} (mg/L NO_2-N)	Salinity (g/L)	Reference
Litopenaeus vannamei	8.4-30 77-322	2-10 15-35	Sowers *et al*. (2004) Lin and Chen (2003)
Penaeus monodon	171	20	Chen *et al*. (1990a)
Penaeus chinensis	38	33	Chen *et al*. (1990b)
Penaeus penicillatus	39	25	Chen and Lin (1991)
Penaeus setiferus	173 (72-h)	25	Alcaraz *et al*. (1997)

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The formation of S-nitrosoglutathione in conditions mimicking hypoxia and acidosis

by

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Introduction

Nitric Oxide (NO) is synthesized from L-arginine by nitric oxide synthase (NOS) with the involvement, among other species, of molecular oxygen. Nitrite and nitrate are believed to be the waste forms of NO. During hypoxia/ischemia elevation of NO production occurs which, in principle, contrasts with the enzymatic mechanism which requires the presence of oxygen. A question arises from this situation; are the waste forms of NO produced in an irreversible process? It would seem more reasonable to think that NO metabolites can be recycled back to bioactive NO again. During renal vascular occlusion (Okamoto *et al*., 2005) as well as in an ischemic heart (Zweier *et al*., 1995), the pH rapidly decreases, and the production of NO increases sharply, independent of L-arginine administration. This shows that the direct reduction of nitrite (or its derivatives) to NO, under acidic conditions, has been invoked in a NOS-independent manner.

Discussion

The knowledge that in acidic conditions inorganic nitrites can dilate vessels is well known, and different concentrations of these species are present *in vivo* (100-500 □M). In these conditions, the *aci*-form of the nitrous acid, and consequently the amount of nitrous anhydride N_2O_3 increases (reactions [1] and [2]).

$$NO_2^- + H^+ \rightleftharpoons HNO_2 \qquad [1]$$

$$2\ HNO_2 \rightleftharpoons N_2O_3 + H_2O \qquad [2]$$

To account for the formation of NO, the reduction of HNO_2, as well as the spontaneous decomposition of N_2O_3 (Lundberg and Weitzberg, 2005), into NO and other NOx, has been invoked (reactions [3] and [4]).

$$HNO_2 \xrightleftharpoons{+e} NO \qquad [3]$$

$$N_2O_3 \rightleftharpoons NO + NO_2 \qquad [4]$$

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Concerning the latter mechanism, recently, Butler and Ridd (2004) have discussed the mechanism of formation of NO from nitrous acid under conditions of low pH. They concluded that the formation of N_2O_3 is the rate determining step and its homolytic cleavage, to form NO, is very slow. In particular, also at the lowest physiological pH (e.g. in ischemic situations), the amount of NO produced via such a process has been found to be lower than that produced *in vivo*. So, the extremely low rate of conversion of a nitrite derivative, via homolysis, into NO and NO_2, leads us to consider the key step in the NO production, a reduction process. In fact, it is reported that the amount of NO generated is dependent not only on the nitrite concentration and the pH, but also on the presence of reducing agents, such as vitamin C (Heller *et al*., 1999); proximity to heme groups, proteins and thiols; and oxygen tension.

Several processes involving NO which take place in hypoxic and/or acidotic conditions, could be due to the reaction of different reducing agents with N_2O_3 and supports the idea that the nitrite anion is a non enzymatic *reservoir* of NO. The *in vivo* increase of NO production with decreasing pH, reaches values similar to those seen during hypoxia. In this condition, an increase of the amount of N_2O_3 can be hypothesized and its interaction with Hb-Fe (reaction [5]) could lead to met-Hb, free NO and the nitrite anion (or HNO_2).

$$\text{Hb-Fe}^{++} \xrightarrow{N_2O_3} \text{Hb-Fe}^{+++} + \text{NO} + \text{NO}_2^- \qquad [5]$$

According to this mechanism, as hemoglobin deoxygenates, vacant hemes become nitrite reductase systems generating methemoglobin and NO (Henry, 1999). This reaction, in principle, can account for several findings. It reduces the amount of Hb, thereby increasing the amount of met-Hb and thus justifying the unequal amounts of MetHb-NO and Hb-NO experimentally detected. This leads to the hypothesis that significant concentrations of NO can avoid the hemoglobin-trap, accounting thus for the vasodilation (Doyle *et al*., 1981)

In this view, the mechanism of formation *in vivo* (Spencer *et al*., 2000) of Hb-SNO, and the functional consequences of this modification, could also be involved. The *S*-nitrosated Hb is a modified form of Hb that has been postulated to be implied in a dynamic cycle of intravascular NO uptake, transport, and delivery, and its potential role as an NO transporter is very attractive. However, the environment of β-93Cys is sensitive to the R ↔ T conformational equilibrium of Hb and studies have shown that the β-93Cys residues, at which NO can be bound and then released, are more accessible in the high-affinity conformation of oxy (R-state) Hb than in deoxy (T-state) Hb (Bonaventura *et al*., 2004). In particular, the normal deoxy conformation of Hb (T-state) cannot be assumed with NO on β-93Cys, even if proteins could in principle have sufficient conformational flexibility to accommodate a possible S-NO linkage. The hemoglobin is forced into the R-state when these thiols are modified, as evidenced by the easier formation of Hb-SNO with oxygenated Hb (arterial blood), than with deoxygenated Hb (venous blood).

The role of Hb-SNO, and other S-nitrosothiols, for example, as NO releasers *via* homolysis, or their ability to perform transnitrosation (Crawford, White and Patel, 2003),

would seem unusual in a non pathological situation. But, the primary area of debate is in trying to identify the extent to which the destabilized T-state of Hb-SNO enhances NO release and/or the transnitrosation process (McMahon, Gow and Stammler, 2000). In particular, it has been hypothesized that Hb-SNO elicits NO-dependent vasodilation in environments of low oxygen tension thereby stimulating blood flow to these regions. Deoxygenation of Hb-SNO is followed by the transfer of the NO group *via* a transnitrosation reaction to thiols such as glutathione (GSH).

The biochemical alterations induced by NO consumption show a possible pathogenesis of a variety of diseases via stimulation of a NO-derivative involved in blood flow regulation which is not susceptible to rapid reactions with oxyhemoglobin, and that can be readily converted back to NO when required (Webb *et al*., 2004). Thus, besides S-nitrosothiols (Gaston, 1999), could nitrite (Cosby *et al*., 2003; Gladwin and Schechter, 2004) also be considered a regulator of vasodilation in non-severe pathological conditions such as a slightly lower physiological pH? If, even a small to gentle fall in pH corresponds to an increase in the concentration of nitrous acid, and then nitrous anhydride, the latter could react with reducing species present in the medium such as vitamin C producing free NO. A possible indirect action of NO as a regulator could be carried out by thiol derivatives, which, reacting via an *in cage* process with the nitrous anhydride, lead to S-nitrosothiols thus avoiding the formation of free NO. This is confirmed from studies on NO-hemoglobin biochemistry which show that the proteins S-nitrosation in red blood cells occurs, if only at low levels and despite the presence of high affinity heme sinks for NO (Gladwin *et al*., 2003; Gladwin *et al*., 2002; Jia *et al*., 1996).

In a previous study (Grossi and Montevecchi, 2002) on the nitrosation of cysteine in acidic conditions, the final derived kinetic expression showed that the NO^+ nitrosating agent could be involved only in very acidic conditions (pH<3.5). At a higher pH (>3.5), the rate of nitrosation achieved depended on $[HNO_2]^2$ and was independent of the cysteine concentration which suggested that the formation of N_2O_3 was the rate determining step, and was the nitrosating species, not the nitrous acid itself (Williams, 1985, 2003). However, the detection of CySNO failed at pH > 5.3 and therefore no information about its possible formation at pHs closer to physiological pH could be obtained. In light of these results, we considered it of interest to verify the behavior of glutathione, probably one of the molecules responsible for the transport of NO as GSNO *in vivo*. GSNO is known to be a stable S-nitroso derivative, thus it was interesting to verify if it could be formed by direct nitrosation at a higher pH compared to those used for obtaining CySNO. To verify this hypothesis, experiments were conducted with glutathione and $NaNO_2$, in buffered deoxygenated aqueous solution, in a pH range from 4 to 6.86, mimicking physiological conditions of acidosis and hypoxia. The formation of GSNO was detectable throughout the entire pH range.

$$2NO_2^- + 2H^+ \rightleftharpoons 2\,HNO_2 \rightleftharpoons N_2O_3$$

$$N_2O_3 + GSH \xrightarrow{H^+} GSNO + HNO_2 \qquad [6]$$

But, the detection of GSNO at pH 6.86, a physiological pH, was unexpected and definitely a remarkable result (Fig. 1).

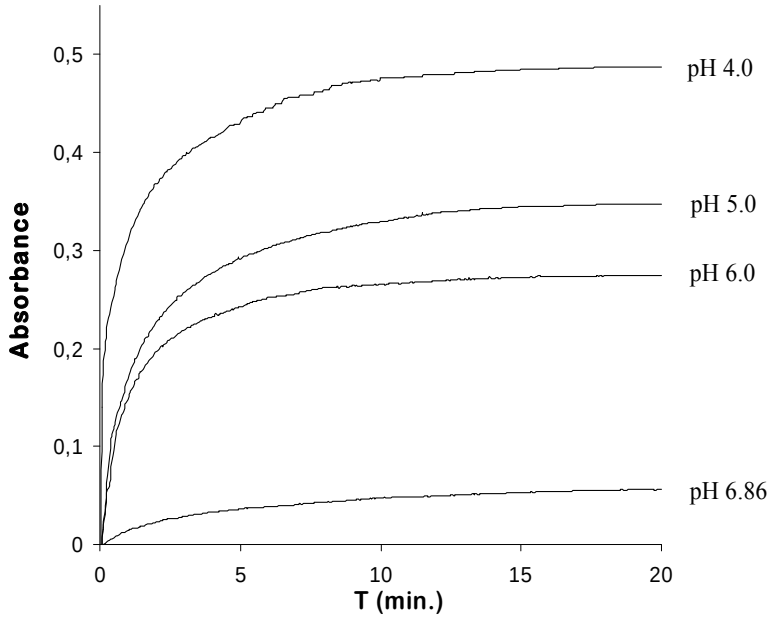

Figure 1. Plots of *A* vs. time (min) for reactions of 0.04 M GSH with 0.04 M $NaNO_2$ in buffered deoxygenated aqueous solution, pH 4.0 - 6.86, at 37°C.

The direct formation via nitrosation of an S-nitrosothiol at so high a pH has not been reported before, except to invoke transnitrosation. This result led us to formulate a reaction mechanism excluding the involvement of NO^+ (or H_2O-NO^+), because it can be formed only at very low pH, and NO, because it cannot act as a direct nitrosating agent. As for the reaction between cysteine and $NaNO_2$, in mildly acidic conditions an Electron Transfer process (SET) between the glutathione and N_2O_3 occurs. Via the formation of a radical ion pair *in cage* a S-nitroso derivative is formed followed by the loss of HNO_2 (reaction [6]), underlining that N_2O_3 can act efficiently as the nitrosating species at low concentration (Hughes, Ingold and Ridd, 1958). This result was also in agreement with the mechanism we hypothesized (Grossi, Montevecchi and Strazzari, 2001a and 2001b) for the nitrosation of alcohols in organic solvents, and for the nitrosation of *p*-cresol and styrene, in neutral or acidic conditions, by N_2O_3 (NO/O_2). In light of these findings, preliminary studies on the kinetics of this process show the linear dependence of the rate of formation of GSNO on pH (Fig. 2).

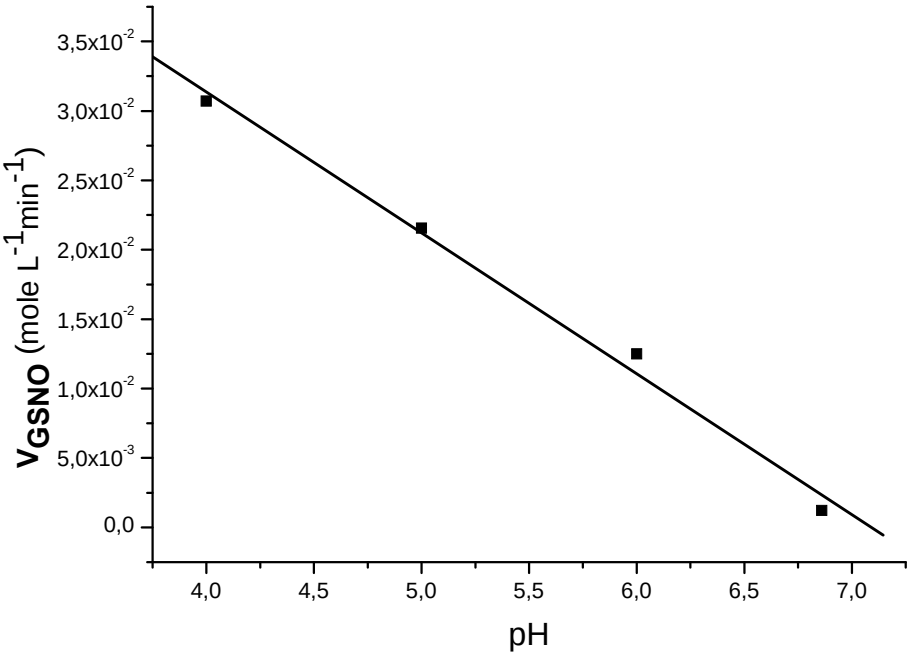

Figure 2**.** Plot of V_{GSNO} vs. pH for reactions of 0.04 M GSH with 0.04 M $NaNO_2$, at 37°C.

Conclusion

These results seem to stress that the reaction between GSH and $NaNO_2$ in anaerobic and aqueous buffer solutions can lead to the detection of the corresponding GSNO at pH mimicking physiological conditions. Preliminary results on the kinetic behavior of this reaction led to the hypothesis that N_2O_3 is the oxidant species, whose amount depends on its equilibrium with HNO_2. It also allows us to hypothesize that the interaction between N_2O_3 and haemoglobin is the process leading to the formation of free NO, responsible for vasodilation in hypoxia and/or acidotic conditions.

Experimental

The reactions were conducted in a thermostated bath at 37°C. The formation of GSNO was constantly monitored by measuring the absorbance at λ=543 nm (ε = 16.34 M^{-1} cm^{-1}), pumping the reacting solution using a peristaltic pump through a UV/vis spectrophotometer equipped with a flow-cell, and a computer for the continuous acquisition of data; the absorbance was detected every three seconds. The solutions were prepared using *buffer standard solution* carefully deoxygenated by bubbling with pure nitrogen.

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Nitrite regulates hypoxic responses and protects against ischemia/reperfusion injury

by

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Introduction

The elucidation of the nitric oxide (NO) vasodilatory pathway initiated the field of NO physiology which has since rapidly expanded. Conventionally, NO is thought to be a paracrine signaling molecule, with the enzyme nitric oxide synthase generating NO by the oxidation of arginine to citrulline, using tetrahydrobiopterin and oxygen as substrates. In the vasculature, NO produced in the endothelium diffuses to underlying smooth muscle cells where it binds the heme group of soluble guanylate cyclase to enable the enzyme to produce cGMP and initiate a signaling cascade that ultimately results in relaxation of the smooth muscle and vasodilation (Ignarro, Buga et al, 1987; Palmer, Ferrige et al, 1987). Beyond this classical cGMP mediated vasodilatory pathway, NO is now known to be an integral signaling molecule in a number of vascular responses including platelet activation, thrombosis, and angiogenesis.

Initially NO was thought to only mediate local paracrine signaling, and this idea was supported by studies measuring the halflife of NO in blood to be less than one second (Liu, Miller et al,1998). However, in the last decade, a number of studies have suggested that NO may not only be a paracrine signaling molecule as first thought, but may also be stabilized and transported in the circulation to mediate endocrine signaling at a later time and farther away from the site of NO production (Fox-Robichaud, Payne et al,1998; Cannon, Schechter et al, 2001; Rassaf, Preik et al, 2002; Ng, Jourd'heuil et al, 2004). For example, in the human forearm, inhaled NO (80 ppm) has been shown to increase blood flow in the human forearm when NOS is inhibited (Cannon, Schechter et al, 2001) and NO solution infused in one arm has been shown to increase blood flow in the other arm (Rassaf, Preik et al, 2002). While several molecules, including iron-nitrosyl hemoglobin (Gladwin, Ognibene et al, 2000a), S-nitrosoalbumin (Ng , Jourd'heuil et al, 2004), and S-nitrosohemoglobin (Stamler, Jia et al, 1997) have been proposed to be the endocrine transporters of NO, we have recently proposed that nitrite (NO_2^-) is the largest vascular storage form of NO (Cosby, Partovi et al, 2003). We have found that nitrite functions as a store of NO that can be bioactivated during to mediate hypoxic vasodilation, modulation of hypoxic mitochondrial respiration and cytoprotection from ischemia/ reperfusion (I/R) injury.

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Nitrite as a vasodilator

Nitrite, generated predominantly by the auto-oxidation of NO, has long been considered a physiologically inert product of NO metabolism in mammalian systems. Early in vitro experiments by Furchgott and colleagues demonstrated that nitrite may mediate vasodilation when applied to isolated aortic rings at high (100μM-1mM) concentrations (Furchgott and Bhadrakom, 1953). However, since the concentration of nitrite in blood was found to be less than one micromolar (Dejam, Hunter et al*,* 2005), this was thought to preclude a role for nitrite as a physiological vasodilator (Lauer, Preik et al*,* 2001). More recently, our lab has observed artery-to-vein gradients of nitrite in the human circulation and has measured increases in the consumption of nitrite during exercise (Gladwin, Shelhamer et al*,* 2000b; Cannon, Schechter et al*,* 2001). Furthermore, in healthy volunteers, we observed that inhaled NO gas mediated endocrine vasodilation, which was accompanied by a significant rise in plasma nitrite concentrations (Cannon, Schechter et al*,* 2001). Taken together, these data led to the hypothesis that nitrite may be an intravascular store of NO that contributes to blood flow regulation in vivo.

To test the vasoactivity of nitrite, near physiological levels of nitrite were infused into the human brachial artery while forearm blood flow was measured. Infusion of nitrite mediated vasodilation while subjects were at rest, in the presence of nitric oxide synthase inhibition by L-NMMA, as well as during exercise. This nitrite-dependent vasodilation, was accompanied by an increase in the formation of NO-modified hemoglobin (iron-nitrosyl and S-nitrosothiol) (Cosby, Partovi et al, 2003).

Several mechanisms of nitrite reduction to NO have been described, including acidic disproportionation (Zweier, Wang et al*,* 1995) and enzymatic reduction by xanthine oxidoreductase (Millar, Stevens et al*,* 1998). However, the correlation of vasodilation with the formation of modified hemoglobin led to the investigation of hemoglobin as a nitrite reductase, an idea that was supported by the previously characterized reaction of deoxyhemoglobin with nitrite, (equation 1):

$$\text{Nitrite} + \text{DeoxyHb} + \text{H+} \rightarrow \text{NO} + \text{MetHb} + \text{OH} \qquad (1)$$

By this reaction, deoxygenated hemoglobin (deoxyHb) reduces nitrite to NO while being oxidized to methemoglobin (metHb) (Doyle, Pickering et al*,* 1981). The NO generated from this reaction would then react with unreacted deoxyHb to form iron-nitrosyl hemoglobin.

The capability of this reaction to mediate vasodilation was tested in a series of aortic ring experiments in which rat aortic rings were treated with nitrite (0.1-1000μM) in the presence and absence of red blood cells (0.3% hematocrit) (Cosby, Partovi et al*,* 2003). While nitrite alone dilated vessels only at high concentrations, consistent with Furchgott's early experiments; in the presence of red blood cells physiological concentrations of nitrite mediated vasodilation, with 100nM nitrite mediating vasodilation when the oxygen tension of the vessel bath was dropped to 15mmHg. Indeed, in these experiments cyclic GMP was generated in the aortic rings, an effect that

was inhibited by the NO scavenger PTIO, confirming that the reduction of nitrite to bioavailable NO was responsible for cGMP dependent vasodilation (Cosby, Partovi et al, 2003; Crawford, Isbell et al, 2006).

To further understand the nitrite reductase activity of hemoglobin, biochemical aspects of the deoxyHb-nitrite reductase reaction previously characterized by Doyle and colleagues (Equation 1) was re-examined. Interestingly, kinetic analysis of this reaction showed that hemoglobin dependent nitrite reductase activity was regulated by the allosteric structural transition of hemoglobin from the deoxy (T) to oxy (R) state, with the maximal reductase activity and NO generation coinciding with the p50 of hemoglobin (Huang, Shiva et al, 2005b). This was found to be due to the differing characteristics of the two allosteric conformations of hemoglobin. Deoxy (T) state hemoglobin has free heme groups, and hence many available heme groups to react with nitrite. But as nitrite reacts with T state hemoglobin, the number of free hemes decreases, which decelerates the nitrite-hemoglobin reaction. However, this is countered by the reaction of nitrite with oxy (R) state hemoglobin which has a lower reduction potential (is a better electron donor) and hence accelerates the reaction. These two opposing processes reach an optimal balance at the p50 of hemoglobin. Consistent with this chemistry, the rate of the reaction of nitrite with purified hemoglobin with increasing oxygen saturation is sigmoidal, with maximum rate (and maximal NO production) occurring when hemoglobin oxygen saturation is between 40 and 60% (Huang, Shiva et al, 2005b).

In addition, as shown in Equation 1 above, hemoglobin-dependent nitrite reduction requires a proton to proceed; hence we investigated whether this reaction was regulated not only by hemoglobin allostery, but also by pH. Indeed, decreases in pH increased hemoglobing-dependent nitrite reduction rate (Huang, Shiva et al, 2005b). These data clearly demonstrate that hemoglobin is a nitrite reductase capable of producing bioavailable NO in a manner that is regulated by the allosteric transition of the hemoglobin molecule as well as pH. This regulation of nitrite reduction to NO by oxygen and pH suggested that this nitrite may be an important source of NO in an acidic environment where oxygen is limited, such as tissue ischemia.

Nitrite mediates cytoprotection during Iischemia/Rreperfusion

Recent studies utilizing several different models have now demonstrated that nitrite is cytoprotective against injury induced by ischemia/reperfusion (iI/R) (Webb, Bond et al, 2004; Duranski, Greer et al, 2005). Utilizing a Langendorff perfusion model of myocardial I/R, Webb and colleagues showed that low micromolar levels of nitrite protect the rat heart from injury (Webb, Bond et al, 2004). In vivo, Duranski et al, demonstrated that nanomolar increases (200nM) in the concentration of plasma nitrite reduced infarct size in a murine model of myocardial infarction at a dose as low as 1.2nmoles. A dose of 48 nmoles increased plasma nitrite levels by 200nM and reduced infarct size maximally (over 71% versus control). Nitrite has also been shown to have similar effects in a murine model of hepatic I/R, in which levels of circulating liver enzymes, ALT and AST, were reduced by 67% (as compared to the nitrate control) with nitrite (48 nmoles) treatment (Duranski, Greer et al, 2005). Interestingly, in both murine

and Langendorff models of I/R, nitrite-dependent cytoprotection was inhibited by the NO scavenger PTIO, suggesting that cytoprotection occurs secondary to nitrite reduction.

The mechanism by which nitrite is reduced to NO during ischemia/reperfusion remains unclear. Several potential nitrite reductases in tissue may be responsible. Webb and colleagues show that in heart homogenates, addition of nitrite produces NO, an effect that is partially blocked in the presence of allopurinol, an inhibitor of xanthine oxidase (Webb, Bond et al, 2004). The mitochondrion has been implicated in nitrite conversion to NO (Kozlov, Staniek et al, 1999; Castello, David et al, 2006), and in the heart, myoglobin may play this role (Huang, Shiva et al, 2005b). It is also unknown whether cytoprotection is dependent solely on NO production or whether nitrite may mediate effects independent of NO formation.

Previous studies have shown that nitrite is able to mediate post-translational modification of proteins (Bryan, Rassaf et al, 2004; Duranski, Greer et al, 2005), metal centers (Huang, Keszler et al, 2005a,; Huang, Shiva et al, 2005b) and lipids (O'Donnell, Eiserich et al, 1999), as well as regulate gene expression (Bryan, Fernandez et al, 2005). Several of these modifications, including nitrosylation of heme and S-nitrosation of thiols, have been measured and associated with nitrite-dependent cytoprotection in the murine models of liver and heart I/R (Duranski, Greer et al, 2005). The specific identities of these modified proteins are yet to be identified, although several potential target proteins exist. S-nitrosation of key regulatory proteins involved in apoptosis has been shown to be cytoprotective in several models of NO-mediated cytoprotection from I/R injury. One such example is the downstream apoptotic mediator caspase-3, which is inhibited by S-nitrosation, subsequently inhibiting apoptosis (Rossig, Fichtlscherer et al, 1999; Maejima, Adachi et al, 2005).

Another possible mechanism of cytoprotection involves regulation of reactive oxygen species (ROS) generation, particularly from the mitochondrion. Small amounts of ROS generation have been determined to be a necessary component of mitochondrial signaling in cytoprotection (Chandel, Maltepe et al, 1998; Vanden Hoek, Becker et al, 1998). However, the large burst of ROS generated after reperfusion from ischemia is believed to be one of the mechanisms whereby cellular injury and necrosis occurs (Saikumar, Dong et al, 1998; Di Lisa, 2001; Sorescu and Griendling, 2002). NO is a known regulator of mitochondrial function, with low concentrations reversibly inhibiting complex IV (Shiva, Oh et al, 2005) and higher concentrations mediating S-nitrosation of complex I (Clementi, Brown et al, 1998; Burwell, Nadtochiy et al, 2006). Regulation of electron transport by nitrite in this manner may decrease electron transport through the chain during ischemia, preventing the formation of damaging ROS production by the mitochondrion upon reperfusion.

Although the specific molecular mechanism of nitrite dependent cytoprotection remains unknown, it is clear that nitrite is an important hypoxic signaling molecule which, in addition to mediating physiological effects, could potentially be used therapeutically for the prevention of I/R induced injury (Figure. 1). Current research

focuses on further characterizing the effects of nitrite in I/R and other pathological models as well as determining the molecular mechanisms of its actions.

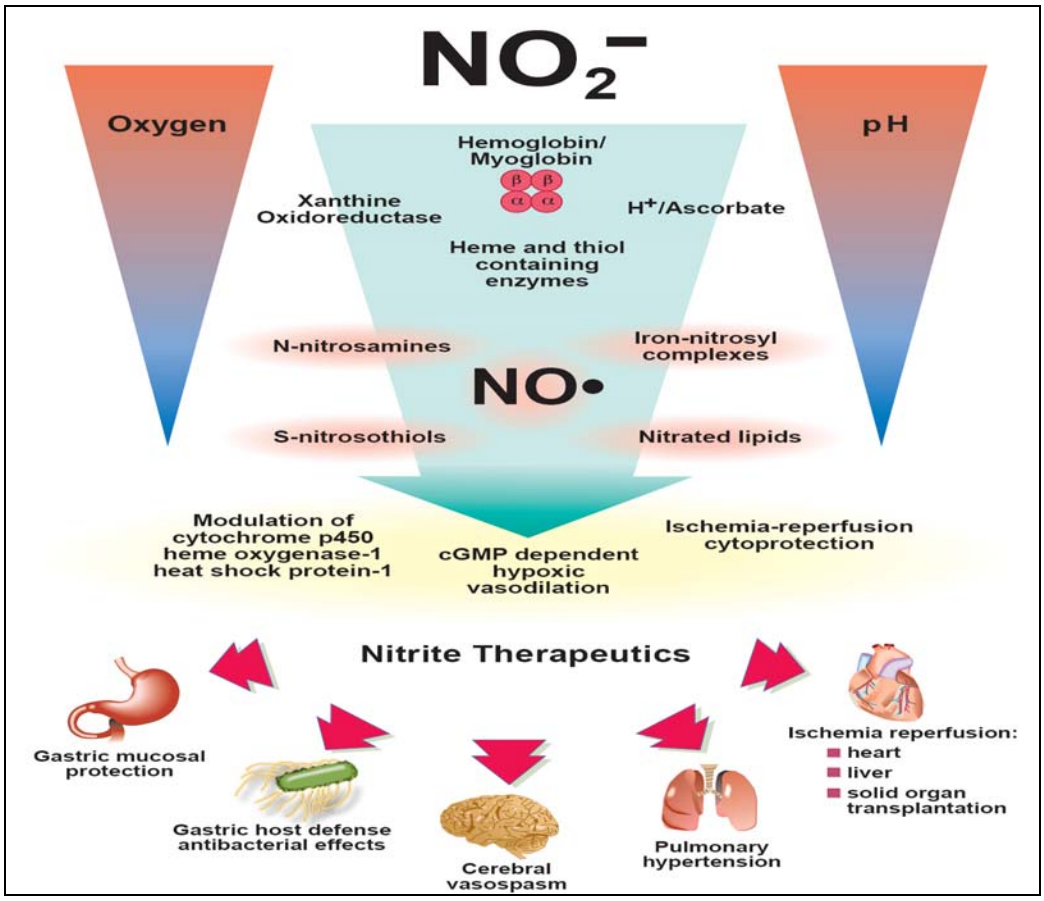

Figure 1. Nitrite is a hypoxic signaling molecule. Nitrite is reduced to nitric oxide along a physiological oxygen and pH gradient by a number of mechanisms. The NO results in modification of iron centers, thiols, amines and lipids to mediate biological responses that may be utilized therapeutically. (Taken with permission from Nature Chemical Biology (2006)).

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Influence of nitrite on the mechanical performance of fish and mammalian hearts

by

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The free radical nitric oxide (NO) is an important signalling molecule involved in many physiological and pathological processes in biological systems. The high membrane permeability and short life make this molecule ideal as a short distance intercellular and intracellular messenger. In particular, NO represents one of the most important regulators of the cardiovascular system.

It is well acknowledged that NO is generated in biological tissues by specific nitric oxide synthases (NOSs) which metabolize arginine to citrulline with the formation of NO. However, over the last years, a growing body of evidence suggests that the nitrite anion (NO_2^-) may represent the largest form of intravascular and tissue storage of NO (Gladwin *et al*., 2004). Nitrite anion is relatively abundant in blood and tissues: it is found in human plasma *in vivo*, arteries (540±74 nM) and veins (466±79 nM) (Gladwin *et al*., 2000); as well as in rat plasma *in vivo* (150 to 1.000 nM) and aorta (>10 microM) (Rodriguez *et al*., 2003).

Mechanisms for the *in vivo* conversion of nitrite to NO may involve either enzymatic reduction or non-enzymatic reduction (Cosby *et al*., 2003 and references therein). Some proteins show nitrite reductase capacity, i.e. glutathione-S-transferases, xanthine oxidoreductase, deoxy-hemoglobin, cytochrome P-450 enzymes, and, recently, also eNOS (Gautier *et al*., 2006 and references therein). Each mechanism would occur preferentially during pathological hypoxia and acidosis (Gladwin *et al*., 2005), which occur in disease states, such as ischemia (Duranski *et al*., 2005). Because the generation of NO from L-arginine by NOS enzymes depends on oxygen, this alternative method of NO production represents an important protective mechanism in ischemic conditions where oxygen is rapidly depleted (Duranski *et al*., 2005).

Recently, some authors have reported that nitrite has a distinct and important signalling role under normal physiological conditions. Nitrite is capable of modulating many important signalling pathways, including soluble guanylyl cyclase (sGC) stimulation, cytochrome P-450 activity and the expression of two archetypical proteins, heat shock protein 70 (Hsp 70) and heme oxygenase-1 (HO-1) (Bryan *et al*., 2005).

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The aim of this research was to explore the biological activity of nitrite as a putative signaling molecule under basal conditions in both mammalian (rat) and non-mammalian (temperate and Antarctic teleost and frog) heart models. Using isolated and perfused heart preparations of fish (*Anguilla anguilla* and the hemoglobinless Antarctic icefish *Chionodraco hamatus*), frog (*Rana esculenta*) and rat (*Langendorff* technique), we have assessed the effects of nitrite on myocardial contractility. The use of hearts without coronary vessels (icefish and frog) or poorly vascularized hearts (eel), allowed the evaluation of direct myocardial effects of nitrite, independent from effects on vascular reactivity. The working heart preparations, standardized for these cold-blooded animals, mimic the physiological conditions of the heart *in vivo* (Gattuso *et al*., 1999; Imbrogno *et al*., 2001). At the same time, these preparations permit the analysis of the mechanical performance free from extrinsic neuronal and endocrine influences. The advantages of the classic Langendorff rat heart preparation have been illustrated by Legssyer *et al*. (1997). The nitrite dose-response curves (nanomolar- micromolar range) revealed a remarkable negative inotropism of nitrite in eel, frog and rat hearts. In contrast, nitrite induced significant positive inotropic effect in the icefish (Fig. 1). Notably, the nitrite-dependent inotropic effect in these species correlates with the effect observed with NO donors, i.e. negative inotropism in eel and rat, positive inotropism in icefish (Fig. 2).

To analyze if the nitrite effects involved enzymatic NO generation, nitrite dose-response curves in the presence of NOS inhibitors (L-NMMA and L-NIO) and sGC inhibitor (ODQ) were tested in the hearts of icefish and rat. The two NOS inhibitors completely blocked nitrite-dependent positive inotropism in icefish, while in the rat heart they did not modify nitrite-dependent negative inotropism (data not shown). In both rat and icefish, ODQ pretreatment completely blocked nitrite-dependent inotropism. These results indicate that in icefish the nitrite positive inotropic effect needs a functional NOS system while in rat the nitrite inotropic effects were NOS-independent; in both species, nitrite effect is cGMP-dependent.

Our data support the hypothesis that the biological activity of nitrite, which is already significant at nanomolar concentrations, may be of relevance in modulating the "normal" function of the vertebrate heart. At the same time, the comparative analysis emphasizes the importance of the species-specific cardiac biochemical conditions, which underlies the myocardial action of nitrite.

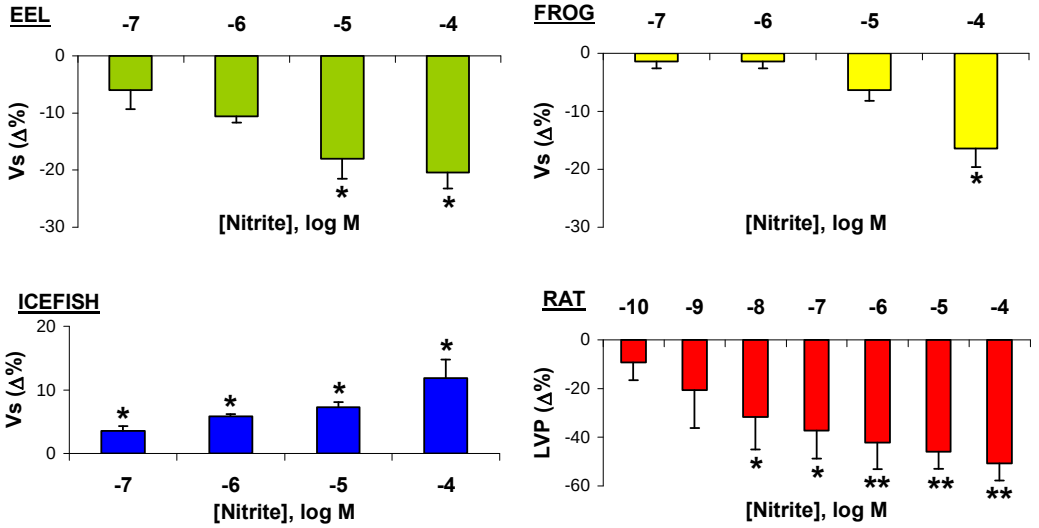

Figure 1. Dose-response curves for nitrite on stroke volume (Vs) and left ventricular pressure (LVP) in isolated and perfused hearts of eel, frog, icefish and rat. Percentage changes were evaluated as means ± S.E.M. (n= 3-5; *p<0.05, **p<0.025).

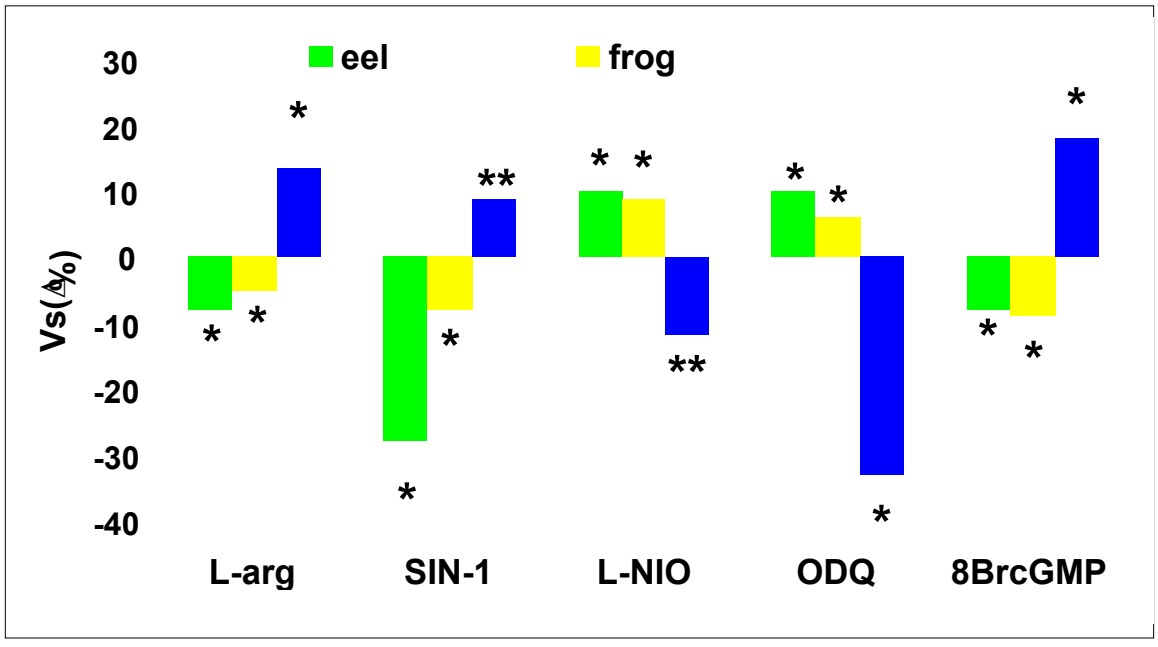

Figure 2. Effect of NO-cGMP in eel (Imbrogno *et al*., 2001), frog (Sys *et al*., 1997) and icefish (Pellegrino *et al*., 2004).

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Potential NO-routes in the piscine circulation

by

C. Agnisola[1]

Nitric oxide (NO) is a well sutdied and ubiquitous regulatory biological compound that acts as a specific mediator in various physiological and patho-physiological processes in cardiovascular, nervous and immunological systems. Being a highly diffusible, membrane-permeant and short living gas molecule, it is ideal for short distance intercellular and intracellular signalling. In particular, NO represents one of the most important regulators of vascular resistance, by acting mainly via soluble guanylate-cyclase (Dattilo *et al.,* 1997; Shah *et al.,* 2000).

Nitric oxide appeared as a signalling molecule before the radiation of metazoans. Accumulating evidence reveals that NO is used as a signalling molecule in a wide variety of invertebrate and vertebrate animals. Despite the fact that fish constitute a group of animals with high diversity, and they have colonized a wide variety of environments, information on the role of NO in the regulation of fish circulation is relatively scant.

Nitric oxide is synthesized by nitric oxide synthase (NOS) isoforms, eNOS (endothelial, calcium-dependent), nNOS (neuronal, calcium-dependent) and iNOS (inducible, calcium-independent) (Alderton *et al.,* 2001), which utilize L-arginine, oxygen, and NADPH as substrates, and require FAD, FMN, calmodulin, and tetrahydrobiopterin as cofactors (Mayer, 1995). Plasma NO is mainly derived from endothelial NOS (Dattilo *et al.,* 1997; Bredt, 1999). Endothelial NO production is stimulated by mechanical forces (shear stress and cyclic strain) and a variety of humoral factors, including acetylcholine, vascular endothelial growth factor (VEGF), bradykinin, estrogen, sphingosine 1-phosphate, H_2O_2, angiotensin II, and ATP (Arnal *et al.,* 1999; Boo *et al.,* 2003; Cai *et al.,* 2003).

Vascular endothelium is continuously exposed to shear stress (a frictional force exerted on the vessel surface per unit area by blood, as it flows at a constant flow rate in the vessel). It is well known that, in mammals, shear stress controls vascular tone and diameter, vessel wall remodeling, hemostasis, and inflammatory responses (Davies 1995). Laminar shear stress has antiatherogenic effects (Boo *et al.,* 2003). NO produced by eNOS is involved in this role of shear stress. NO-mediated responses to shear stress include vessel relaxation, inhibition of apoptosis, and platelet and monocyte adhesion triggered by a variety of pro-atherogenic factors (Boo *et al.,* 2003). eNOS stimulation by a step increase in shear stress occurs in two phases: an initial burst phase (lasting from seconds up to 30 min), which is Ca^{2+}/calmodulin dependent, followed by a Ca^{2+}-independent phase, in which NO production rate is maintained at a lower level, that involves protein kinases and eNOS phosphorylation (Boo *et al.,* 2003). The exact mechanism is still under debate, and several phosphorylation sites have been identified.

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Chronic changes in shear stress may also modify eNOS expression level by both transcriptional induction and stabilization of mRNA (Davis *et al.,* 2001). It is possible that the acute, robust NO production due to a step increase in shear stress may play a critical role in vessel relaxation, whereas the chronic, low level of NO production due to the steady laminar shear stress may play a critical role as an antiatherogenic and anti-inflammatory molecule in cardiovascular biology and pathobiology.

Information on the role of shear stress in the regulation of blood flow in fish is lacking. A putative role of shear stress in trout coronary circulation has been proposed by Mustafa and Agnisola (1998), where a flow-dependent NO release was involved in the vasodilatory response to adenosine. This stretch-dependent effect has been recently confirmed in the trout coronary circulation (Agnisola and Mustafa, unpublished), where a significant inhibition of adenosine vasodilatory response by gadolinium (Gd^{3+}), a known inhibitor of stretch-activated ion channels (Hamill *et al.,* 1996), was observed. Moreover, using a constant-flow preparation of the isolated and perfused trout heart, it has been possible to demonstrate that NO release from the preparation is directly related to perfusion flow, independent from the presence of adenosine (Fig. 1, left panel). At a constant flow, NO release in the presence of adenosine is half that of the control (Fig. 1, right panel). These results can be explained assuming that in trout coronaries there is a shear-stress dependent nitric oxide release from the endothelium.

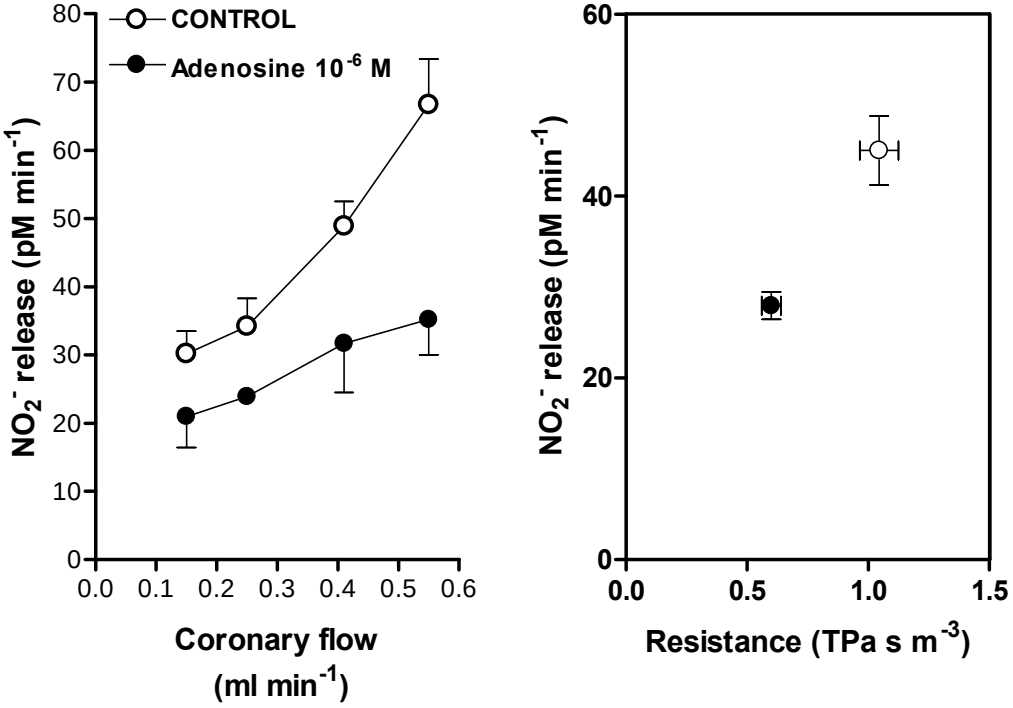

Figure 1. NO production (measured as NO_2^- in the effluent from the preparation) in the coronary system of the isolated trout (*Oncorhynchus mykiss*) heart under constant flow perfusion conditions. *Left*: relationship between NO release and coronary flow in the absence and presence of adenosine, a known vasodilator. *Right*: effect of the reduction of coronary resistance induced by adenosine 10^{-6}

M on NO release under constant flow perfusion conditions; flow = 0.34 ± 0.04 ml min^{-1}. Data are means ± SE of 5 determinations.

Some information is available on the endothelial NO release induced by neurohumoral factors in fish. NO signalling has been reported to be involved in the angiotensin II effect on the eel heart (Imbrogno *et al.,* 2003). Oxytocin induced, NO mediated vasodilation has been reported in rainbow trout (Haraldsen *et al.,* 2002). NO has also been involved in vasodilation induced by serotonin and acetylcholine on the coronary vasculature of the trout heart (Mustafa *et al.,* 1997). A NO-dependent mechanism for acetylcholine-induced vasodilation has been reported in crucian carp (Hylland *et al.,* 1995) and trout (Soderstrom *et al.,* 1995).

NO appears to also be involved in the role of red blood cells (RBC) as O_2 sensors. One main pathway through which RBCs contribute to the regulation of blood flow and O_2 delivery is via ATP release, depending on the oxygenation state of haemoglobin. Erythrocytes release ATP when haemoglobin is deoxygenated (Sprague *et al.,* 2003). ATP, acting via endothelial P_{2Y} receptors, stimulates vasodilatation through the release of nitric oxide (NO) (Takemura *et al.,* 1998), prostaglandins, and endothelium-derived hyperpolarizing factor (EDHF) (Wang *et al.,* 2005). Preliminary experiments on the isolated and perfused trout heart have shown that ATP exerts vasodilatory effects on coronary resistance in the concentration range of 10^{-9}-10^{-6} M; however, this effect appears weak if compared with adenosine vasodilation, and disappears at higher concentrations (Fig. 2). Apparently, there is little aim in exploiting the putative erythrocyte and NO-dependent role of plasma ATP in this fish model.

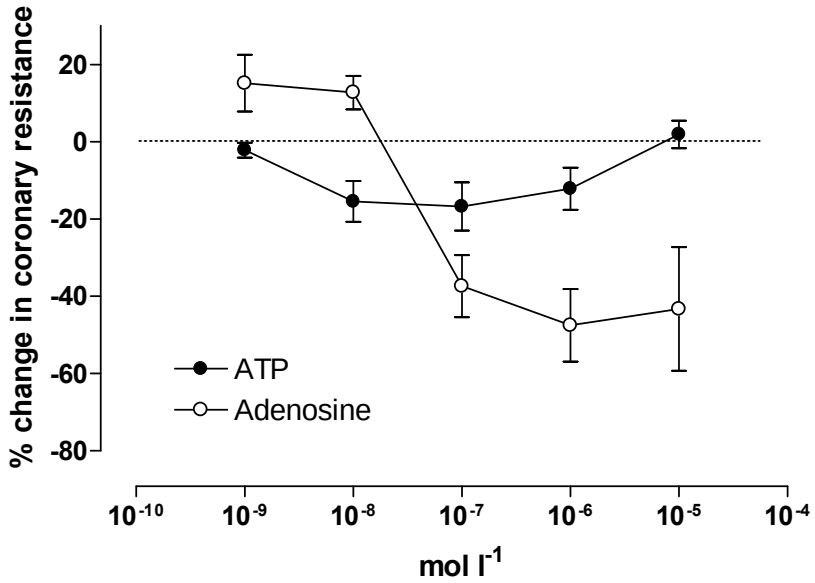

Figure 2. Dose response curve of ATP and adenosine in the perfused coronary system of the isolated, non-working trout (*Oncorhynchus mykiss*) heart. Data are means ± SE of 5 determinations.

Another possible mechanism for the role of RBCs as O_2 sensors involves a NOS-independent pathway for NO release. Nitrite anion (NO_2^-), which can be relatively abundant in blood and tissues (up to μM levels in mammals, Gladwin *et al.,* 2000; Rodriguez *et al.,* 2003), has been proposed as the largest intravascular and tissue storage form of NO. Mechanisms for the *in vivo* conversion of nitrite to NO may involve either enzymatic, non-enzymatic, or acidic reduction (Cosby *et al.,* 2003 and references therein). Several proteins show nitrite reductase capacity: glutathione-S-transferases, xanthine oxidoreductase, deoxy-hemoglobin, cytochrome P-450 enzymes, and, recently, also eNOS (Gautier *et al.,* 2006 and references therein). This mechanism would occur preferentially during pathological hypoxia and acidosis, when NOS is inactive (Webb *et al.,* 2004; Duranski *et al.,* 2005), but recently some authors reported that nitrite is a signalling molecule also under physiological conditions (Bryan *et al.,* 2005).

This NOS-independent role of NO may also occur in fish. Jensen and Agnisola (2005) have recently demonstrated in the coronary circulation of the isolated trout heart perfused with erythrocyte suspensions, that nitrite is converted to NO in a process that is not inhibited by the NOS inhibitor L-NA. This supports the possibility of deoxyHb-mediated reduction of nitrite to NO, a mechanism that would be significant under hypoxic conditions to help maintain oxygen supply to tissues.

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Physiological effects of nitrite: Balancing the knife's edge between toxic disruption of functions and potential beneficial effects

by

F.B. Jensen[1]

In relation to vertebrate biology, nitrite has mainly been known for its toxic effects. In human health, main concerns has been with the potential formation of possible carcinogenic nitrosamines (an issue still not settled) and methemoglobin (metHb) formation following excess intake of nitrite or nitrate (that can be reduced to nitrite in the digestive system) in the diet. Aquatic animals, like fish, are more prone to experience high nitrite concentrations than terrestrial animals. Freshwater fish actively take up nitrite across the gills, resulting in well-documented toxicity (Lewis and Morris, 1986). Several physiological disturbances have been discovered in aquatic animals that add to the standard methemoglobinemia caused by nitrite, and the picture is emerging that toxicity results from the impact of multiple nitrite-induced physiological disturbances (Jensen, 2003).

In recent years the conception that nitrite is exclusively a toxicant has been refined, and important biological functions of nitrite are beginning to emerge (Gladwin *et al*., 2005). Nitrite is naturally present at low concentrations (0.15-0.6 μM in plasma of mammals), which is due to the endogenous production of nitrite as an oxidative metabolite of the important messenger molecule nitric oxide (Kleinbongard *et al*., 2003). Additional sources are intake of nitrite via the diet, and reduction of nitrate to nitrite by bacteria in the oral cavity (Lundberg and Weitzberg, 2005). At the low natural concentration, nitrite is far from biologically inert. Nitric oxide can be re-generated from nitrite, whereby nitrite may function as a vascular storage pool of NO activity that participates in blood flow regulation and other functions (Cosby *et al*., 2003). Nitrite may also act as a signaling molecule on its own and regulate gene expression (Bryan *et al*., 2005). Thus, nitrite appears to have vital functions that have been carried through in evolution. There seems to be a schism between beneficial effects of nitrite at low concentrations and harmful effects at high concentrations.

The physiological effects of nitrite in aquatic animals was recently comprehensively reviewed (Jensen, 2003). This presentation gives an overview of physiological disturbances in nitrite-exposed fish and additionally describes recent developments that suggest nitrite to be an important compound in animal biology at its natural low endogenous concentration.

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Nitrite in the environment

Nitrite is part of the nitrogen cycle in ecosystems, where it is an intermediate in bacterial nitrification and denitrification processes. The concentration of nitrite in unpolluted waters is normally low (well below 1 μM), but an imbalance in either nitrification or denitrification can lead to a build up of nitrite. Elevated nitrite levels pertain to habitats receiving nitrogenous waste and various hypoxic habitats (Eddy and Williams, 1987), including the oxygen minimum zone of the oceans (Anderson *et al*., 1982). Apart from its environmental relevance, nitrite is a matter of concern in aquaculture industry, where episodic nitrite poisoning can cause extensive fish death (Svobodová *et al*., 2005). Aquaculture facilities have a high density of fish and a large production of waste products (including ammonia excreted by the fish). Discharge of ammonia and establishment of nitrification is likely to cause a transient increase in nitrite because *Nitrosomonas* (that oxidize ammonia to nitrite) tend to grow faster than *Nitrobacter* (that oxidize nitrite to nitrate), and because *Nitrobacter* is inhibited by elevated free NH_3 levels (Balmelle *et al*., 1992). Nitrite concentrations of 1 mM or more can be reached in the water in such circumstances (Jensen, 2003).

Nitrite uptake in fish

When exposed to raised environmental [NO_2^-], freshwater fish accumulate nitrite in blood plasma to much higher concentrations than in the ambient water (Bath and Eddy, 1980; Margiocco *et al*., 1983; Jensen *et al*., 1987). To understand why, a brief look at the ion uptake mechanism in the freshwater fish gill is useful (Fig. 1). Freshwater fish need an active uptake of ions to compensate for passive ion losses. The current view is that a proton pump in the apical membrane creates the driving force for Na^+ uptake through sodium channels (Perry, 1997; Randall and Brauner, 1998; Marshall, 2002; Evans *et al*., 2005). The protons come from hydration of CO_2 (catalyzed by epithelial carbonic anhydrase), and the bicarbonate formed subsequently serves as counter ion for Cl^- uptake via an anion exchange mechanism. Nitrite has an affinity for this uptake mechanism, so whenever nitrite is present in the water, part of the Cl^- uptake will be shifted to nitrite uptake (Fig. 1), and internal concentrations in the millimolar range can eventually develop.

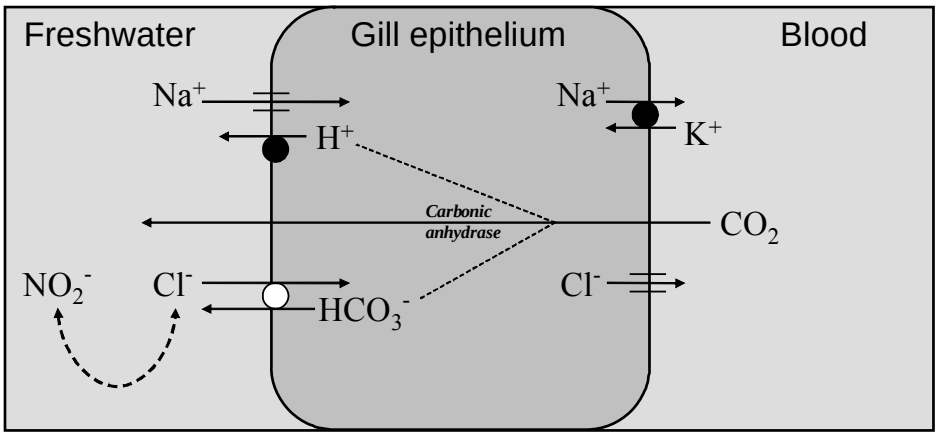

Figure 1. Current view on mechanisms of ion uptake in the freshwater fish gill, illustrating the ability of nitrite to compete with chloride for the Cl^- uptake mechanism. An apical membrane H^+-ATPase creates the driving force for Na^+ uptake via a sodium channel. Na^+ subsequently exits the basolateral membrane via the Na^+-K^+-ATPase. Hydration of CO_2, catalyzed by epithelial carbonic anhydrase, supplies H^+ for the proton pump and delivers HCO_3^- for Cl^- uptake via an apical membrane Cl^-/HCO_3^- exchanger. The proton pump energizes Cl^- uptake by raising cytosol $[HCO_3^-]$ and by acidifying the boundary layer, lowering external $[HCO_3^-]$. Cl^- (or nitrite) presumably exits the basolateral membrane via a chloride channel. Further details on gill ion transport and involved epithelial cells can be found in Perry (1997), Marshall (2002) and Evans *et al*. (2005).

Several observations are in agreement with nitrite being transported by the branchial Cl^- uptake mechanism. Nitrite influx and Cl^- influx show saturation kinetics (supporting carrier-mediated uptake), and NO_2^- is a competitive inhibitor of Cl^- uptake and vice versa (Williams and Eddy, 1986; Tomasso and Grosell, 2005). Elevation of ambient $[Cl^-]$ is known to effectively protect against nitrite uptake (Bath and Eddy, 1980) and nitrite toxicity (Perrone and Meade, 1977; Russo *et al*., 1981). The branchial Cl^- uptake rate varies between species, and species with low uptake rates (e.g. eel, tench and carp) are less sensitive to nitrite than species with high uptake rates (rainbow trout, perch, pike) (Williams and Eddy, 1986). Most freshwater fishes accumulate nitrite in blood plasma when exposed to this anion, but a few species (e.g. bluegill, largemouth bass and striped bass) do not concentrate nitrite in plasma (Tomasso, 1986; Palacheck and Tomasso, 1984; Mazik *et al*., 1991). This puzzling observation appears to be explained by exceptionally low (barely detectable) branchial Cl^- influx rates, as reported in eel (Hyde and Perry, 1989) and bluegill (Tomasso and Grosell, 2005).

In addition to the variation in nitrite uptake between species, some species may show intraspecific variation, as reported in rainbow trout (Margiocco *et al*., 1983; Williams and Eddy, 1988; Stormer *et al*., 1996; Aggergaard and Jensen, 2001). Two studies that exposed rainbow trout to 1 mM ambient nitrite in hard freshwater of the same

composition, suggest that the splitting of rainbow trout into groups with different sensitivity to nitrite is highly reproducible (Stormer *et al*., 1996; Aggergaard and Jensen, 2001). In both studies the rainbow trout could be divided into two distinct groups. Sensitive trout showed fast accumulation and died between 1 and 2 days of exposure, whereas less sensitive trout accumulated nitrite slower and survived more than 4 days. The nitrite accumulation pattern and the nitrite levels reached in the two groups were very similar in both studies, as was the physiological disturbances (Stormer *et al*., 1996; Aggergaard and Jensen, 2001). The separation into two groups raised the idea that this might be gender-related. To test this hypothesis, individual rainbow trout were exposed to 1 mM nitrite for 18 hours (allowing the splitting up into two groups without causing mortality) and then returned to nitrite-free water (to study recovery from nitrite exposure). The fish indeed could be divided into two groups based on their degree of nitrite accumulation; but males and females were present in both groups (Fig. 2).

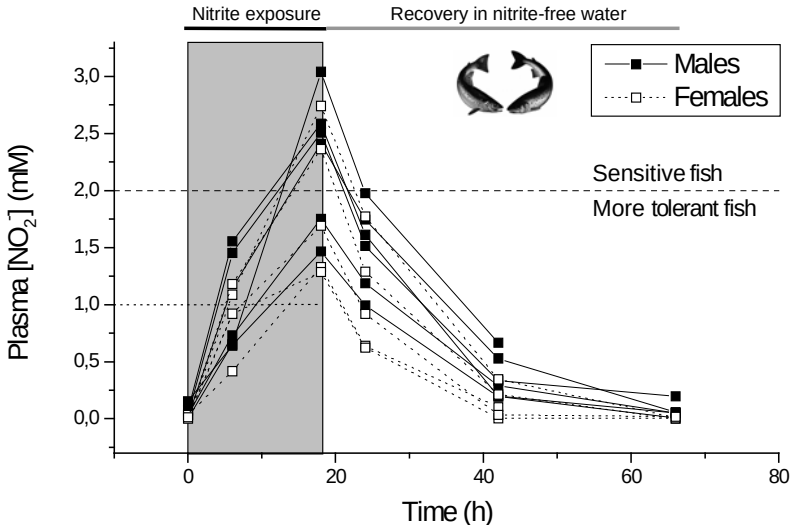

Figure 2. Time-dependent changes in plasma nitrite concentration in six male and five female rainbow trout during 18 h exposure to 1 mM nitrite and subsequent recovery in nitrite-free water. Successive blood samples were drawn through indwelling dorsal aortic catheters, and results are depicted for each individual fish. Sensitive fish reached plasma [NO_2^-] values well above 2 mM during nitrite exposure, whereas more tolerant fish attained significantly lower values. Males and females were present in both groups. When the fish were returned to nitrite-free water the nitrite build-up was reversed (F. Zachariasen and F.B. Jensen, unpublished data).

Thus, the intraspecific variation was not gender related. The most sensitive fish were shown to have a significantly higher branchial nitrite influx than the less sensitive fish (F. Zachariasen and F.B. Jensen, unpublished). Chloride cells are the likely site of Cl^- uptake (Perry, 1997) and are additionally known to proliferate upon nitrite exposure (Williams and Eddy, 1988). Thus, a relevant hypothesis for future testing is that chloride cell

number and/or the degree of chloride cell proliferation differ between the two groups of rainbow trout.

Seawater fish are hypo-osmotic regulators and secrete NaCl across the gills. Thus, the problem of nitrite uptake via the active Cl^- uptake route that prevails in freshwater fish is eliminated. However, nitrite can still be taken up passively across the gills. Further, marine fish drink seawater taking up ions and water across their intestine. The European flounder has been shown to take up nitrite across the gut epithelium if nitrite is present in the lumen. The transport is inhibited by bumetanide, suggesting that part of the uptake is mediated via an affinity of nitrite for the $Na^+/K^+/2Cl^-$ cotransporter that is involved in intestinal ion uptake (Grosell and Jensen, 1999). When exposed to waterborne nitrite it appears that some 66% of whole-body NO_2^- uptake is via the intestinal route (Grosell and Jensen, 2000). Nitrite uptake is, however, lower in seawater than in freshwater fish. In the European flounder plasma $[NO_2^-]$ stays below the ambient concentration (Grosell and Jensen, 2000).

The transport of nitrite from the extracellular space into intracellular compartments has been studied in some detail for red blood cells (Jensen, 2003), whereas little is know about uptake in other cell types. Nitrite is, however, known to concentrate in liver, brain, gill and muscle tissue of fish (Margiocco *et al*., 1983).

Nitrite-induced physiological disturbances

When active branchial Cl^- uptake partly shifts to nitrite uptake and passive Cl^- efflux persists, extracellular $[Cl^-]$ can be predicted to decrease, as is also observed in carp and rainbow trout (Jensen *et al*., 1987; Stormer *et al*., 1996). Proliferation of chloride cells (e.g. Williams and Eddy, 1988) may be considered a response aimed at restoring chloride balance, but in this particular circumstance it appears maladaptive, because an increased capacity for Cl^- uptake also increases nitrite uptake. While nitrite is accumulated in plasma, plasma nitrate increases in parallel, because some of the nitrite is endogenously converted (detoxified) to nitrate (Stormer *et al*., 1996; Doblander and Lackner, 1997). Plasma lactate also increases, as tissue O_2 delivery becomes compromised by high methemoglobin levels (Jensen *et al*., 1987). The sum of extracellular anion, however, stays constant during nitrite exposure (Jensen *et al*., 1987; Stormer *et al*., 1996).

Among the cations, the most prominent effect concerns potassium. Extracellular $[K^+]$ increases (Jensen *et al*.,1987) whilst the intracellular K^+ content decreases in red blood cells (Jensen, 1990) and skeletal muscle (Stormer *et al*., 1996; Knudsen and Jensen, 1997). The K^+ efflux from red blood cells results from activation of K^+/Cl^- cotransport and leads to erythrocyte shrinkage in carp (Jensen, 1990, 1992). The K^+ loss from skeletal muscle is substantial and could increase extracellular $[K^+]$ to much higher levels than observed, suggesting further transport of K^+ to the ambient water with the development of an overall potassium deficit (Knudsen and Jensen, 1997). Interference with K^+ balance has been observed in a number of fish species and also in invertebrates (freshwater crayfish), suggesting that it is a general effect of nitrite (Jensen, 2003).

Elevated extracellular [K^+] is unfavorable for heart and nerve function by causing depolarization, and the reduced intracellular K^+ content may critically influence muscular metabolism.

Nitrite reacts with both oxygenated hemoglobin (oxyHb) and deoxygenated hemoglobin (deoxyHb) to form metHb, but the reaction mechanisms, reaction kinetics and reaction products differ. The stoichiometry for the reaction between oxyHb and nitrite is (Kosaka and Tyuma, 1987):

$$4Hb(Fe^{2+})O_2 + 4NO_2^- + 4H^+ \rightarrow 4Hb(Fe^{3+}) + 4NO_3^- + O_2 + 2H_2O \quad (1)$$

Thus, in parallel with the oxidation of heme iron ($Fe^{2+} \rightarrow Fe^{3+}$) to form metHb, nitrite is oxidized to nitrate (see Jensen, 2003 for further details). In the reaction with deoxyHb, nitrite is reduced to nitric oxide, whereby deoxyHb functions as a nitrite reductase (Cosby *et al*., 2003):

$$Hb(Fe^{2+}) + NO_2^- + H^+ \rightarrow Hb(Fe^{3+}) + NO + OH^- \quad (2)$$

The formation of metHb, which is non-functional in regards to O_2 transport, can be countered by metHb reductase activity inside the erythrocytes. At low nitrite concentrations, metHb levels need not increase significantly, but at elevated nitrite concentrations metHb levels increase. At a given nitrite load, a balance is established between nitrite-induced oxidation of hemoglobin and reduction to functional Hb via metHb reductase systems (Jensen, 1990, 1992). The continuous increase in nitrite load in nitrite-exposed fish, however, forces metHb levels upwards, eventually reaching levels of 70-85 % of the total Hb (Eddy and Williams, 1987; Jensen *et al*., 1987; Aggergaard and Jensen, 2001). Such high metHb levels drastically decreases the arterial O_2 content and result in a severe tissue O_2 shortage that becomes reflected in elevated plasma lactate levels (Jensen *et al*., 1987). Methemoglobinemia is the prime reason for the disruption of O_2 transport, but additional contributions may come from decreases in blood O_2 affinity (related to erythrocyte shrinkage in carp), total Hb content (possibly via increased removal of damaged erythrocytes), and interaction of nitrite with cellular heme proteins such as myoglobin and cytochromes (cf. Jensen, 2003). Fish can tolerate relatively high blood metHb levels at rest, but their swimming performance is impaired (Brauner *et al*., 1993) as is their ability to handle environmental hypoxia. An increased ventilatory activity (Aggergaard and Jensen, 2001) can be seen as an attempt to ameliorate O_2 conditions, but during continued nitrite exposure the decline in blood O_2 capacitance becomes critical and ultimately lethal. However, if the fish are returned to nitrite-free water the metHb build-up is reversed (Jensen, 2003) in parallel with the elimination of nitrite from the blood (Fig. 2).

Nitrite exposure is associated with a rapid and lasting increase in heart rate in rainbow trout (Aggergaard and Jensen, 2001). It is interesting that this increase in heart rate occurs very early during the exposure, before any significant increases in metHb and plasma [K^+] have developed. The effect on heart rate appears related to the mere appearance of nitrite in the blood. It was suggested that nitric oxide was generated from

nitrite, and that the vasodilation caused by this was countered by an increased cardiac pumping to re-establish blood pressure (Aggergaard and Jensen, 2001). Indeed, NO can be generated from nitrite in various ways, including non-enzymatic acidic reduction and enzymatic reduction by xanthine oxidoreductase or by deoxygenated Hb (Gladwin *et al*., 2005). Such nitric oxide generation from nitrite may play a physiological role in nitric oxide homeostasis at the low nitrite levels seen under normal (non-toxic) circumstances (cf. below). During nitrite exposure in fish, however, where nitrite concentrations can rise to the millimolar level, an excess production of NO may ensue. As NO is an important signal molecule that is involved in processes ranging from blood flow regulation to neurotransmission, such abnormal NO levels may have a number of critical effects.

The putative physiological role of nitrite in nitric oxide homeostasis

Nitric oxide produced in vascular endothelial cells exerts its function by diffusing into underlying vascular smooth muscle, causing its relaxation, which results in local vasodilation and increased blood flow. Nitric oxide is a free radical with a short life time in blood. NO can be inactivated inside erythrocytes (e.g. by reacting with oxyHb to form metHb and nitrate), and in plasma its reaction with O_2 forms nitrite. Nitrite and nitrate were long considered relatively inert metabolites of NO. However, the possibility that NO can be re-generated from nitrite has recently attracted considerable attention. The formation of NO from nitrite by enzymatic and non-enzymatic means is favored by low pH and/or low Po_2 and is believed to be of physiological importance in humans, particular in hypoxia and during ischemia (Gladwin *et al*., 2005).

The idea has emerged that nitrite at its natural low concentration in mammalian blood functions as a vascular storage pool of nitric oxide that can be activated in a physiological appropriate way (Cosby *et al*., 2003). The reaction of nitrite with deoxyHb (equation 2 above) has attracted particular interest, because this leads to an NO production that is linked to the degree of Hb deoxygenation, which may supply a mechanism for matching blood flow to O_2 conditions (Cosby *et al*., 2003; Nagababu *et al*., 2003). Thus, increased deoxygenation of Hb in the microcirculation of hypoxic tissues can be predicted to elevate erythrocyte NO formation from nitrite. The formed NO can react with unoxidized heme groups to form iron-nitrosyl-hemoglobin, $Hb(Fe^{2+})$-NO, but escape of some of the NO from the erythrocytes should suffice to cause vasodilation and elevate blood flow and tissue oxygenation. The mechanism is supported by nitrite infusion-caused vasodilation in the human forearm at near-physiological nitrite concentrations and by hypoxic vasodilation experiments using aortic rings (Cosby *et al*., 2003; Crawford *et al*., 2006). The function of Hb as a nitrite reductase is modulated by both heme deoxygenation and heme redox potential and shows maximal activity around 50 % Hb oxygen saturation (Huang *et al*., 2005; Crawford *et al*., 2006).

Nitrite entry into red blood cells is an essential first step of the proposed mechanism. In carp, nitrite transport across the red blood cell membrane is strongly oxygenation-dependent. At physiological pH, nitrite extensively enters deoxygenated red

blood cells, whereas it hardly permeates oxygenated cells (Jensen, 1990, 1992). When pH is lowered, nitrite starts to enter oxygenated red blood cells, but at much lower rates than in deoxygenated cells (Jensen, 1992). A similar oxygenation dependency pertains to tench and whitefish erythrocytes (Jensen, 2003). Thus, in these fish, nitrite preferentially permeates erythrocytes with low oxygen saturation, thus apparently supplying nitrite for deoxyHb-mediated NO generation in an appropriate manner. Further, in carp, as nitrite enters erythrocytes with low O_2 saturation, a subsequent full oxygenation stops nitrite influx (Knudsen and Jensen, 1997), showing that oxygenation functions as a switch that obliterates further nitrite entry.

There is limited information on the oxygenation dependency of nitrite transport in mammalian erythrocytes. When nitrite is added to a suspension of pig erythrocytes at physiological pH, nitrite quickly permeates and equilibrates across the membrane, and then continues to enter the cells as result of intracellular nitrite removal (via its reactions with hemoglobin), but the membrane permeation shows little oxygenation dependency (Jensen, 2005). There is an extensive entry of nitrite into both oxygenated and deoxygenated pig erythrocytes, which contrasts sharply with the oxygenation-dependency of nitrite fluxes in carp, tench and whitefish. This difference is not just some simple difference between non-nucleated mammalian erythrocytes and nucleated erythrocytes of lower vertebrates, because in rainbow trout (like in pig) there is no significant oxygenation dependency of erythrocyte nitrite entry (Jensen and Agnisola, 2005). It appears that in carp, tench and whitefish, erythrocyte nitrite transport is governed by a major oxygenation-dependent change in the membrane permeability to nitrite (high P at low O_2 saturation and low P at high O_2 saturation). In pig and rainbow trout nitrite permeation is similar in oxygenated and deoxygenated erythrocytes, and nitrite quickly equilibrates across the membrane, after which further entry is governed by the chemical reactions that remove nitrite inside the cells (Jensen, 2005).

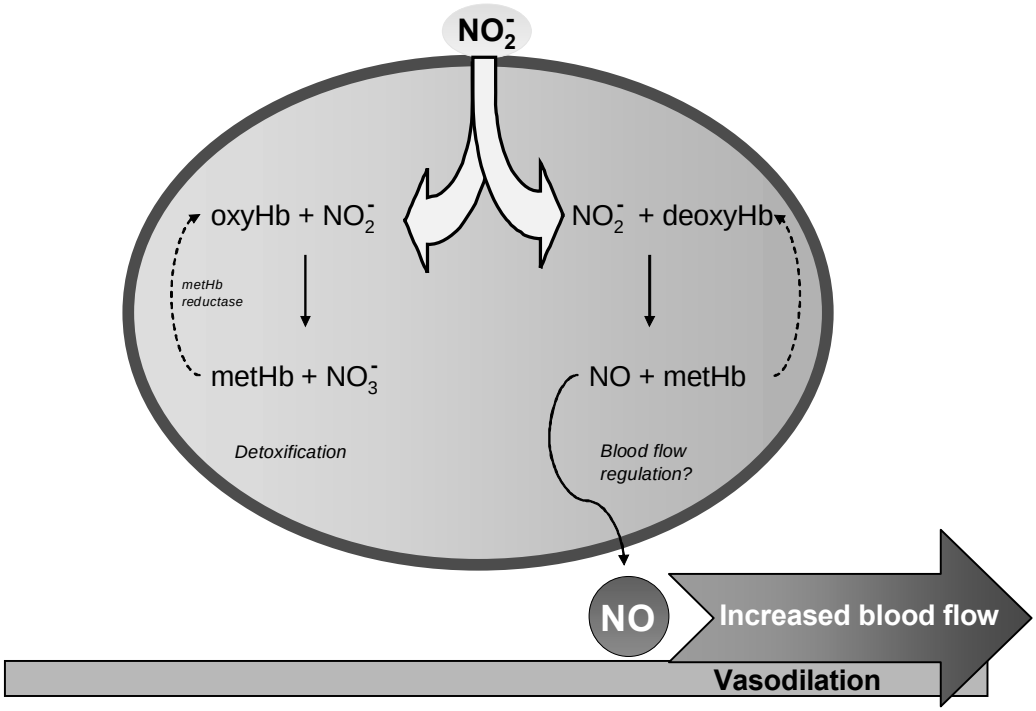

Figure 3. Nitrite entering erythrocytes can react with deoxygenated heme groups to form nitric oxide that may escape from the cells and partake in local blood flow regulation. The reaction of nitrite with oxygenated heme groups can be considered a nitrite detoxification mechanism, because it converts nitrite to non-toxic nitrate, while the oxidized heme groups (metHb) can be reduced to functional Hb via metHb reductase. See text for further details.

A preferential entry of nitrite into erythrocytes at low O_2 saturation would seem ideal for its subsequent reaction with deoxyHb to form NO that via its release from the RBCs could participate in blood flow regulation (Fig. 3, right). The reaction with oxyHb is, however, also important, as it may form a defense against inappropriate high levels of nitrite (Fig. 3, left). Thus, nitrite reacting with oxyHb is detoxified to non-toxic nitrate (Doblander and Lackner, 1997; Jensen, 2003). Of course, the concomitant formation of metHb is a toxic effect of nitrite, but this can be countered by metHb reductase activity that regenerates functional Hb. In this way each Hb molecule can participate in several oxidation-reduction cycles that eliminate excess nitrite, and blood metHb levels need not increase significantly. A dynamic balance may accordingly exist between the need for NO release from partly deoxygenated erythrocytes to promote blood flow at low O_2 tension, and the need for detoxification of nitrite in oxygenated erythrocytes at inappropriate high nitrite concentrations (Jensen, 2005). The entry of nitrite into both oxygenated and deoxygenated red blood cells may support both these functions. The situation with the endogenous levels of both NO and nitrite is like balancing on a knife's edge between potential beneficial effects at low levels and toxic effects at high levels.

The idea that nitrite is converted to NO by deoxyHb has mainly been examined in mammalian models. A recent study tested the hypothesis that nitrite is converted to vasoactive NO in the coronary circulation of the isolated trout heart. Perfusion of the

coronary vessels with hypoxic saline elicited NO production. The nitric oxide synthase inhibitor L-NA inhibited this NO production and decreased coronary flow, showing that NO produced in the endothelium is vasoactive (Jensen and Agnisola, 2005). A switch to perfusion with an erythrocyte suspension caused an increased NO signal that was also inhibited by L-NA. The change in NO production upon subsequent nitrite addition, in contrast, was not inhibited by L-NA, suggesting that it may have occurred via deoxyHb-mediated reduction of nitrite to NO. All prerequisites for a conversion of nitrite to NO inside the red blood cells appeared fulfilled: (1) nitrite rapidly permeated the erythrocyte membrane, (2) there was a significant decrease in HbO_2 saturation in the coronary circulation, (3) there was a gradient in $[NO_2^-]$ and a rise in metHb between the input and output of the coronary circulation, and (4) a nitric oxide signal was registered (Jensen and Agnisola, 2005). Thus, the study supports the idea that NO can be produced from nitrite in the erythrocytes, but it cannot be excluded that the heart itself may generate NO from nitrite by means of cellular heme proteins or xanthine oxidoreductase activity (Jensen and Agnisola, 2005). The NO formation associated with nitrite was without effect on coronary flow. So, apparently NO was produced from nitrite without causing vasodilation. This may reflect that the NO was produced in the capillaries after the resistance vessels, and that the signal was not conducted to upstream arterioles (Jensen and Agnisola, 2005). Further research is required to fully uncover the role of nitrite-erythrocyte interactions in blood flow regulation.

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Hypoxia in fish

by

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Introduction

Vertebrates try to maintain oxygen delivery in the face of reductions in water oxygen levels. If oxygen delivery is compromised and tissue oxygen levels fall then energy expenditure is reduced and anaerobic metabolism is up-regulated (Boutilier *et al*., 1987). Fish reduce energy expenditure during hypoxia by inhibiting feeding and reproduction, moving to a lower temperature and reducing swimming activity (see Randall, 2004, for review). These energy savings are considerable and genes associated with aerobic metabolism are down-regulated, probably in response to the reduction in aerobic energy expenditure (Hung, 2005). Anaerobic metabolism is up-regulated to maintain function in the face of limitations in aerobic energy production. The liver plays a central role in these responses, but studies of the effects of hypoxia on liver cellular changes in vivo are rare compared with in vitro studies. Hypoxia causes DNA damage and apoptosis in mammalian cell lines (Thompson, 1998; Bras *et al*., 2005), however, in vivo responses to DNA damage are known for only a few mammals and nothing is known of in vivo responses of fish liver to hypoxic DNA damage.

In studies of the in vivo responses of the liver of common carp, Cyprinus carpio L, to hypoxia we observed extensive DNA damage during the first days of hypoxic exposure, as indicated by Terminal transferase mediated dutp Nick End Labelling (TUNEL). TUNEL labeling was very high (found in around 60% of the liver cells) during hypoxia, especially after four days of exposure to aquatic hypoxia at 0.5 mg O2. L-1 (Poon and Randall, 2003). The level of TUNEL staining was reduced after about a week of hypoxic exposure. Such extensive DNA damage will often lead to programmed cell death or apoptosis. In fact TUNEL is often used to indicate apoptosis.

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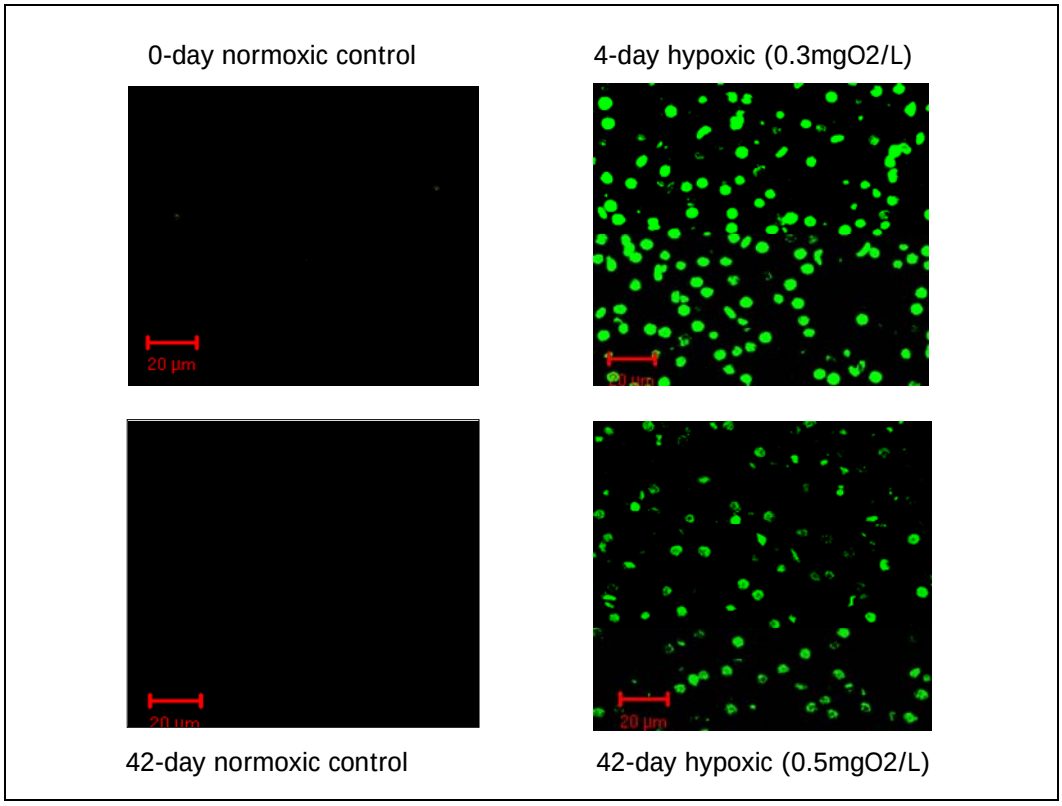

Figure 1. Sections of normoxic and hypoxic carp liver. Cells stained by TUNEL and examined with confocal microscopy are shown in fluorescent green. The 4-day hypoxic liver was exposed to 0.3mgo2/L whereas the 42-day hypoxic liver was exposed to 0.5mgO2/L. Scale bar 20µm.

If the TUNEL signal was indicative of rates of apoptosis in the in vivo hypoxic carp liver then, in the face of low rates of cell proliferation, the carp liver should have been reduced in size after six weeks of hypoxia (0.5 mg O2. L-1), however, both the size of the liver and the number of liver cells did not change significantly during these 42 days of hypoxia. Cell-proliferation rates were always low, as indicated by the protein expression level of a cell mitosis indicator, proliferating cell nuclear antigen (PCNA), and by flow cytometry. Thus rates of apoptosis, which were low in the normoxic liver, appeared not to increase during hypoxia. The absence of any increase in apoptosis during hypoxia was also supported by results using a single strand DNA (ssdna) antibody to assay for single strands of DNA, and a DNA fragmentation assay, as well as flow cytometric analysis of normoxic and hypoxic liver cells. We also measured the activities of caspase-3, an enzyme involved in one of the pathways of programmed cell death, and found no change in activity. A TEM investigation of carp liver cells indicated no inflammation, and no necrosis or apoptosis during hypoxia. Thus liver cells were stained by TUNEL during the first days of hypoxia, indicating DNA damage, but there was no apoptosis. As the level of TUNEL staining decreased during prolonged hypoxia, DNA

nick ends must have been repaired during hypoxia, probably after the initial stress had been ameliorated by various cellular changes.

Table I. There is no increase in apoptosis in carp liver, *in vivo*, when exposed to hypoxia. Various methods were used to determine the extent of apoptosis and the results are summaries in the text (from Poon and Randall, 2006).

Experiment	Result
Anti-ssdna staining	No observable difference at 0-, 4-, 8-, 16-, 28-, and 42-day hypoxia (0.5mgo_2/L)
Caspase-3 activity	No significant increase at 4-day hypoxia (0.3mgo_2/L)
DNA fragmentation	No DNA laddering observed at 42-day hypoxia (0.5mgo_2/L)
Flow cytometry	No significant increase percentage of cells under sub-G1 peak at 7-day hypoxia (0.5mgo_2/L)
Liver size	No significant decrease at 42-day hypoxia (0.5mgo_2/L)
Mrna level of Bcl-2	A significant increase at 4-day hypoxia (0.5mgo_2/L)
Number of cells per mm^2 (cell size)	No significant change at 0-, 4-, 8-, 16-, 28-, and 42-day hypoxia (0.5mgo_2/L)
PCNA	No significant change at 42-day hypoxia (0.5mgo_2/L)
TEM	No significant increase number of irregular shaped nuclei at 2-day hypoxia (0.5mgo_2/L)
TUNEL	More than 5-fold increase was observed at 4-day hypoxia (0.3mgo_2/L) and 4-, 16-, 28-, and 42-day hypoxia (0.5mgo_2/L)

In order to repair DNA, cell cycle arrest must be maintained. During hypoxia there was an initial increase in protein levels of the cell cycle inhibitor, p27, and the increase in this protein was associated with the increased levels of DNA damage. In addition, there were increases in the levels of a number of anti-apoptotic genes, including Bcl2 and erythropoietin (EPO), and the down regulation of pro-apoptotic genes such as Tetraspanin 5 and Cell death activator in the in vivo carp liver. The anti-apoptotic factor, heat shock protein 70 (HSP70) decreased during hypoxia (Poon *et al*., 2006, unpublished data). Double immunological staining of liver cells for TUNEL and HSP70 indicated that only cells with no TUNEL staining showed high levels of HSP70. Thus HSP70 was associated with undamaged cells.

We conclude that the carp liver, in vivo, is maintained in a quiescent state during hypoxia with no change in apoptotic rate and little or no cell division, maintained by a variety of mechanisms, including p27, and the up-regulation of a number of anti-apoptotic genes and the down regulation of a number of pro-apoptotic genes. The cellular mechanisms seem to be directed towards preventing apoptosis in the face of DNA damage and promoting DNA repair.

When common carp were exposed to hypoxia of 0.5 mg O2 L-1 for six weeks, most of the changes in gene expression occurred during the first few day after the onset of hypoxia (Hung, 2005). These changes were not related to starvation as starvation alone

is associated with only minor changes in gene expression during the first week. The changes in expression appear to be a response to, rather than a cause of, the reduction in metabolism. Hypoxia inducing factor 1 (HIF1) expression increased early and was then reduced during long term exposure to hypoxia. HIF2 and HIF4 expression also increased. The relative role of these various hifs in fish is not clear.

There was also a large increase in the gene expression and level of uncoupling proteins (ucps) in carp liver, in vivo, during hypoxia. In mammals it is clear that ucps are inserted into the inner mitochondrial membrane and will short circuit the proton gradient generated by NADH oxidation (see Krauss *et al*., 2005 for review). In the presence of oxygen there will be a futile cycle of protons across the mitochondrial membrane the consequence of which is the production of heat. This is important in regulating heat production in mammals, but why are there uncoupling proteins in fish, when clearly fish are not homeothermic endotherms like mammals? Increases in heat production in fish would indeed be futile. The rate of production of reactive oxygen species (ROS) is related to mitochondrial membrane potential, at least in the rat (Korshunov *et al*., 1998). The production of reactive oxygen species decreases with mitochondrial membrane potential and UCP lowers mitochondrial membrane potential and, therefore, ROS production.

UCP2 mrna was up-regulated more than six fold in carp liver during hypoxia exposure and, using isolated mitochondrial preparations, we were able to show palmitate stimulation of UCP activity. In addition, palmitate reduces H2O2 production in isolated fish mitochondria, presumably by activating UCP. Free fatty acid levels increase during hypoxia in the carp liver and this may stimulate UCP2 to decrease mitochondrial membrane potential and, thus, decrease ROS production. UCP appears not to be inhibited by ATP in carp and so the control of UCP may be somewhat different from that of mammals, which is known to be inhibited by ATP (Hagen *et al*., 2000). In summary it is possible that ucps can rapidly decrease the production of ROS in fish liver during hypoxia.

ROS levels in tissues can be regulated either by reducing production or increasing rates of removal by increasing antioxidant capacity. We found large increases in both UCP1 and UCP2 mrna levels in carp liver during hypoxia. There were much smaller but significant increases in UCP1 mrna levels in brain tissue but there was only a small initial increase and then a decrease in UCP gene expression in the kidney during hypoxia. Increasing rates of ROS removal during hypoxia do not seem to be an option as several antioxidant genes were suppressed in both liver and kidney during hypoxia. The kidney has a high antioxidant capacity normally and perhaps this suffices during hypoxia and up regulation of kidney ucps is not required. The liver, on the other hand, appears to control production rather than removal during hypoxia. The kidney presumably attempts to maintain aerobic capacity as most of the changes in gene expression returned to levels close to control levels during hypoxia whereas those in the liver do not. Thus responses to hypoxia at the tissue level are complex and are probably tissue specific.

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Gene expression profiles of common carp, *Cyprinus carpio*, during prolonged starvation and hypoxia reflect differences in hypometabolism

by

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Introduction: Metabolic Depression

Metabolic depression in response to environmental stress has been reported in both invertebrates and vertebrates. A diverse range of environmental stressors that result in metabolic depression in animals have been studied, including anoxia / hypoxia (e.g. crucian carp and coral-reef shark; Nilsson and Renshaw, 2004), food deprivation (torpor in salamander; Hervant *et al*., 2001), dehydration (e.g. aestivation in snails and lungfish; Guppy *et al.*, 2000; Chew *et al*., 2004) and diapause (embryos of annual killifish; Podrabsky and Hand, 1999). Much work has been devoted to understanding metabolic depression by means of suppressing energy production (e.g. oxidative phosphorylation) and energy consuming processes (e.g. ion pumping, protein synthesis etc) in a coordinated fashion, and many reviews have been published on this subject (Hochachka *et al*., 1996; Hand, 1996; Guppy, 2004; Storey and Storey, 1990, 2004). These reviews tend to concentrate on biochemical mechanisms of metabolic depression and ignore behavioural and physiological strategies such as moving to a lower temperature, reduced activity, and inhibition of feeding and reproduction. Several studies investigating the responses to environmental stresses by means of global gene expression screening have been carried out in the last decade (O'Hara *et al*., 1999; Gracey *et al*., 2001, 2004). However, our knowledge of molecular adaptation to hypoxia is still very limited and the molecular mechanisms associated with physiological adaptations have yet to be delineated.

Survival of animals subjected to environmental stress depends on several factors centred on matching energy expenditure to reduced energy availability through energy cost saving. Common carp, although not an anoxia tolerant species, are able to survive a substantial period of hypoxia exposure and starvation. There are many similarities in the responses of animals to hypoxia and starvation such as depressed metabolism, reduced locomotion and impaired reproductive ability. Nevertheless, not all responses are the same. During hypoxia, ATP supply is limited and therefore a quick response to maintain ATP balance is followed by regulated hypometabolism (Boutilier, 2001). During

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starvation, however, the rate of energy store utilization becomes the deciding factor in the survival of fish. The mechanisms of gene expression by which cells respond and adapt to hypoxic conditions have recently been studied (Semenza, 2001; Gracey *et al*., 2001), but less is known about cellular responses to starvation in fish. The processes of coordinated gene expression that mediate the whole cellular response towards energy conservation are virtually unknown. Therefore, we hypothesized that there are similarities in the gene expression profiles during starvation and hypoxia, in both cases directed towards energy conservation. In this study, we explored the underlying mechanism of hypoxia tolerance in common carp and their survival during starvation at the molecular level, and to relate the changes of gene expression with changes in stored substrates.

Materials and Methods

Carp were exposed to prolonged (six weeks) exposure to hypoxia or starvation. All fish survived these treatments. Water temperature was maintained between 19±1°C with a constant photoperiod 12L:12D. The dissolved oxygen level (DO) was maintained at approximately 7.0mgO_2/L for the starved group and between 0.5-0.65mgO_2/L for the hypoxic fish. Hypoxic fish were fed daily but starved fish were deprived of food completely. Liver and kidney samples were collected at day 0 (control), 4, 8, 16, 28, 42 and frozen immediately in liquid nitrogen until processing. Total RNA was extracted using Trizol and further purified using QIAgen RNeasy mini kit. Total RNA from all time points were reverse-transcribed, labeled and hybridized to carp microarray (Gracey *et al*., 2004). Data analysis and statistics were done using the software GeneSpring. Hepatic glycogen, protein and lipid levels were also measured.

Results and Discussion

Metabolic depression during food deprivation and hypoxia, although not measured in this study, has been reported in several fish species including starved perch (Mehner and Weiser, 1994) and rainbow trout (Lauff and Wood, 1996), as well as hypoxic carp (Van Ginneken *et al*., 1998). Liver and kidney gene expression profiles reflected the metabolic depression observed in both starved and hypoxic carp; however, the responses were somewhat different, with a different time frame between starvation and hypoxia and between tissues. *Table I* shows the general gene expression patterns of common carp during hypoxia and starvation. In general, carp kidney genes respond to starvation much faster than liver. The expressions of liver genes remain relatively unchanged until day 16 during starvation, indicating that this period of starvation does not have any impact on the hepatic metabolism in carp, and this animal might be employing a "wait and see" strategy during this time. Unlike starvation, carp liver and kidney responded to hypoxia similarly and more acutely (i.e. within the first few days of exposure).

Glycolysis and oxidative phosphorylation

During starvation, hepatic glycogen dropped rapidly at day 4 and this level was maintained until day 42 when another significant decrease was detected (Fig. 1). At the molecular level, induction of expression of numerous glycolytic genes including pyruvate kinase (PK), 6-phosphofructokinase (PFK), L-lactate dehydrogenase A (LDH-A), β-enolase and fructose-bisphosphate aldolase B were detected after 42 days of starvation. Similar temporal induction was also observed in tricarboxylic cycle (TCA) genes (citrate synthase and isocitrate dehydrogenase) and gluconeogenic genes (G6Pase, F-1,6-BP, phenylalanine catabolic genes, 4-hydroxyphenylpyruvate dehydrogenase and homogentisate1,2-deoxygenase). This induction of glycolytic genes coincides with a decrease in hepatic glycogen content at day 42 (Fig. 1). Carp kidney, on the contrary, sustained suppression of genes encoding enzymes for glycolysis, gluconeogenesis, and TCA cycle.

Reduction in resting metabolism may have occurred prior to day 4, and pre-existing glycolytic enzyme activity was sufficient to drive the usage of glycogen without having to increase the expression of glycolytic or glycogenolysis genes. The early decline in glycogen concentrations seen in this study might be glucagon-driven as suggested by Moon and Foster (1995), and the late-mobilization of hepatic glycogen also suggests that glycogen is either not the preferred fuel or it is reserved during starvation in fish. One probable explanation is that hypoxia is a common environmental stress, and during oxygen limited periods, anaerobic respiration is the only means of ATP production. Therefore, it is crucial for carp to reserve glycogen which aids its survival during episodes of oxygen shortage.

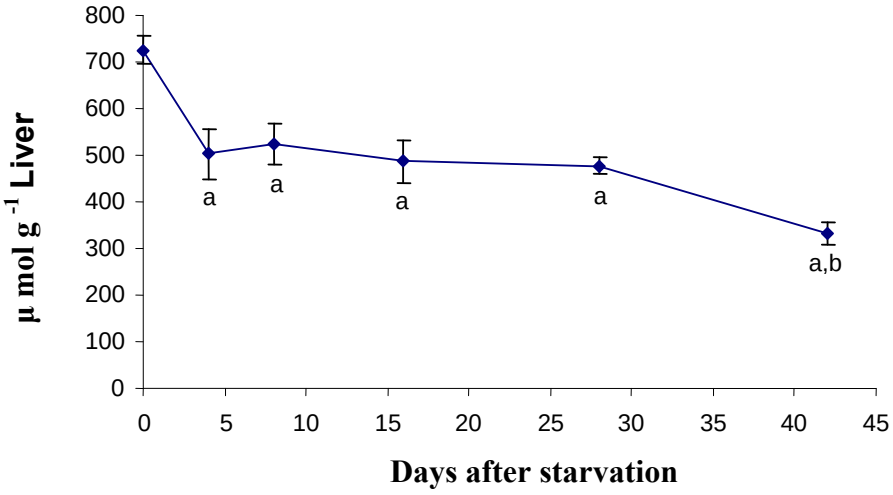

Figure 1. Changes of glycogen content of carp liver during starvation.
Data are presented as Mean ± SE (One-Way ANOVA, $p<0.05$).
a: significantly different from Day
b: significantly different from Day 0, 4, 8, 16, 28.

On the other hand, when carp were exposed to hypoxia, hepatic glycogen declined rapidly at day 4 and then gradually returned to control levels from day 16 onwards (Fig. 2). The initial decline of hepatic glycogen is probably related to the induction of numerous glycolytic genes (fructose-bisphosphate aldolases, hexokinase, glyceraldehydes 3-phosphate dehydrogenase, phosphoglycerate mutases, beta-enolase) during early hypoxia exposure. Similar patterns of glycolytic gene induction are seen in the liver of *G. mirabilis* exposed to hypoxia (Gracey *et al*., 2001). The up-regulation of glycolytic genes, in turn, was probably mediated by hypoxia-inducible factor 1a (HIF1a) which showed a significant 1.2-fold ($p<0.05$) increase at day 4 of hypoxia. Congruent with glycolytic gene expression, gluconeogenic genes (fructose-1, 6-bisphosphatase and fructose-1,6-bisphosphatase isozyme 2) and phenylalanine catabolism genes, whose pathway is solely cytosolic, were also induced. A similar induction of gene expression was also observed in the carp kidney during hypoxia. The degree of induction of the above genes gradually decreased as hypoxia continued. Changes in gene expression and in hepatic glycogen levels indicate that common carp were probably well adjusted to the hypoxic environment after 2 weeks of exposure and able to replenish glycogen stores from consumed food even though they ingested only half of the normoxic food intake. Rapid restoration of hepatic glycogen further indicates that glycogen is important as an energy source and necessary for carp to survive hypoxia.

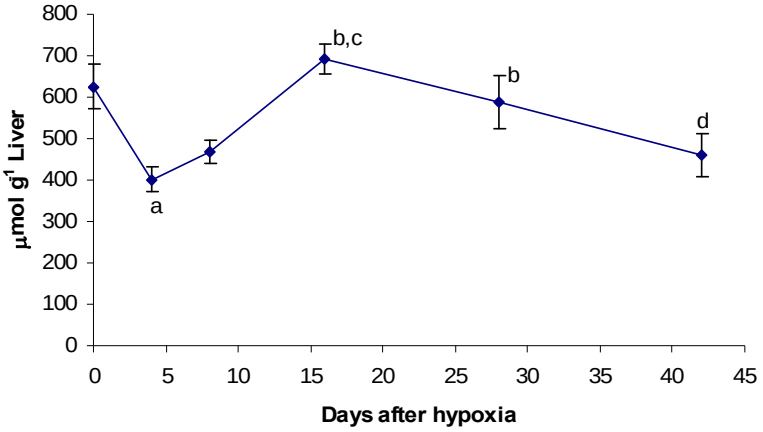

Figure 2. Change of hepatic glycogen of carp during hypoxia exposure. Data are presented as Mean ± SE (One-Way ANOVA, $p<0.05$).
a: significantly different from Day 0
b: significantly different from Day 4
c: significantly different from Day 8
d: significantly different from Day 16.

Oxidative phosphorylation and ATP production

Starvation did not induce congruent patterns of differential expression of genes involved in aerobic respiration and the electron transport chain in the carp liver. At the

same time, expression of beta-oxidation genes remained unaltered, which implies that mitochondrial function was not impaired during food withdrawal. In the carp kidney, however, sustained suppression of genes encoding enzymes for TCA cycle, ETC, beta-oxidation and ATP synthases was observed. Because oxygen is not limited during food withdrawal, mitochondria can remain functional and supply energy via aerobic respiration. The general suppression of genes involved in ATP production in carp kidney indicates that energy demand during starvation has decreased substantially.

During hypoxia, suppression of various ETC genes (cytochrome P450 3A40 and 2J2, NADH-ubiquinone oxidoreductase subunit B14.5b) and ATP synthases (ATP synthase beta, delta chains and ATP synthase lipid-binding protein) was detected in the carp liver and a greater number of ETC and ATP synthase genes were found suppressed in the carp kidney. Carp are oxyconformers meaning that their energy demands decline quickly with decreasing external oxygen supply resulting in their entering a state of hypometabolism. The lack of oxygen resulted in an inhibition of ATP supply and probably a depletion of ATP stores; hence the animal is forced to enter hypometabolism. This forced hypometabolism probably occurred at the onset of hypoxia exposure. With prolonged hypoxia, hypometabolism was associated with suppression of a large number of genes involved in ATP consumption and turnover.

In addition to controlling ATP production by ETC, production of adenosine has been proposed to contribute to the regulation of depressed metabolism and its elevation has been reported during times of energy deficiency in fish, including hypoxia. The actions of adenosine include 1) stimulation of glycogenolysis to fuel glycolysis with glucose (Magistretti *et al*., 1986); 2) inhibition of ATP uptake during protein synthesis (Tinton and Buc-Calderon, 1995); 3) a relative reduction in anaerobic respiration (Bernier *et al*., 1996); 4) impairment of protein synthesis (Krumschnabel *et al*., 2000); 5) depression of cardiac activity (MacCormack and Driedzic, 2004); as well as 6) channel arrest in the brain of anoxic turtle (Buck, 2004). In other words, adenosine aids in hypoxia and anoxia survival by reducing ATP consumption to match production and hence results in metabolic depression (Nilsson and Renshaw, 2004). In this study, sustained induction of hepatic S-adenosylhomocysteinase 2 during 6 weeks of hypoxia exposure might be related to adenosine production (including the net breakdown of phosphorylated adenylates ATP, ADP and AMP). S-adenosylhomocysteinase 2 catalyzes the reversible hydrolysis of S-adenosylhomocysteine to homocysteine and adenosine without the requirement of cofactors (Palmer and Abeles, 1979). At the same time, suppression of genes encoding S-adenosylmethionine synthetase alpha and beta forms was detected in the kidney. This enzyme prevents the coupling of methionine and adenosine, hence sparing one molecule of ATP and adenosine. The overall reduction in gene expression during prolonged hypoxia in both liver and kidney in this study in hypoxic carp was probably in response to metabolic depression. No differential expression of S-adenosylhomocysteinase and S-adenosylmethionine synthetase alpha and beta forms was found in the starved carp tissues, indicating that different control mechanisms were involved in metabolic depression during hypoxia and starvation.

Lipid and Protein Metabolism

Synthesis of macromolecules requires ATP, and not surprisingly, lipid synthesis was suppressed during both starvation and hypoxia in carp. Suppression of lipogenesis genes was observed at day 4 in liver and kidney of hypoxic carp and at day 8 and day 16 in starved carp kidney and liver, respectively. It was interesting to note that genes encoding ribosomal proteins were suppressed only in carp kidney during starvation but remained relatively unchanged in the liver. This indicates the important role of carp liver as the main metabolic organ during starvation.

Translation machinery, on the other hand, remained fairly active during hypoxia in both liver and kidney, which might be related to the requirement for rapid production of glycolytic enzymes in the two organs studied. Interestingly, the magnitude of the increase in expression of translational factors and ribosomal proteins declined in the kidney as hypoxia persisted, however induction remained high in the liver, indicating that the liver is the main ATP supplier as the fish adjusted to hypoxic stress. Likewise, suppression of ribosomal gene expression was detected only in skeletal and cardiac muscles, but not liver, of hypoxic goby fish (Gracey *et al*., 2001). In extreme cases of anoxia, half-lives of proteins increased by 40% to 50%, respectively (Land and Hochachka, 1994). The changes in expression of numerous signaling molecules and transcriptional/translational modulators during starvation and hypoxia also reflect rapid re-organization of cellular functions during these two stresses in carp.

In addition to synthesis, degradation of macromolecules is also energy-expensive. For example, protein degradation by ubiquitin-proteasome pathway is ATP-dependent. Therefore, it becomes economical to have proteins preserved rather than degraded during energetically stressed period such as hypoxia and starvation. During starvation, ubiquitin-proteasome genes remained unchanged during the first 2 weeks of starvation but showed congruent induction at day 28, but no change in total hepatic protein was detected. This could be due to either the amount of protein change being too little to be detected, or the action of protein degradation being counteracted by the up-regulation of ubiquitin carboxyl-terminal hydrolase isoenzyme L1, a deubiquitining enzyme that prevents protein degradation from taking place.

In the case of oxygen shortage, it has been demonstrated that proteolysis is inhibited in isolated, anoxic turtle hepatocytes (Land and Hochachka, 1994), as well as anoxic *A. franciscana* embryos (Anchordoguy and Hand, 1994) and hibernating ground squirrels (van Breukelen and Carey, 2002). Suppression of a large number of ubiquitin-proteasome genes at day 4 in the carp kidney and thereafter, was subsequently ameliorated. This is probably due to the adaptive strategy of carp to depress metabolism during hypoxic conditions. Hence, proteolysis continued to maintain basal cellular protein turnover. On the other hand, hypoxia had no effect on ubiquitin-proteasome gene expression in the carp liver, indicating that this ATP-dependent pathway is still functioning during hypoxia in the carp liver, probably related to the re-organisation of cellular proteins. However, as observed during starvation, no change in total hepatic

protein content was observed during hypoxia. In the case of hypoxia, genes involved in lipid and protein metabolic pathways were generally down-regulated. On the other hand, no change in hepatic lipid content was observed in carp (data not shown) after 6 weeks of starvation. Lipid metabolic genes showed varied changes in expression in both starved carp liver and kidney.

Interactions between Starvation and Hypoxia in Common Carp

During the first 2 days of hypoxia, common carp were reluctant to take food pellets, but appetite resumed after 2 days, probably when the fish had adjusted to the hypoxic condition. However, the amount of food intake during hypoxia was approximately half the usual daily intake. Reduced food intake may lead to slower growth rates of common carp under hypoxic conditions. Reduction of food intake and retardation of growth is indeed commonly observed in fish during hypoxia exposure (Secor and Gunderson, 1998; Pichavant *et al*., 2000; Taylor and Miller, 2001; Foss *et al*., 2002). Although hepatosomatic index (HSI) of common carp after 6 weeks hypoxia was not different from day 0 (data not shown), HSI of common carp might have dropped (but no measurements were made) during the initial phase of hypoxia exposure due to more than 30% reduction of hepatic glycogen stores at day 4 and day 8. HSI of the parallel control fish was significantly higher than the starved fish indicating that hepatic storage has probably increased under fed condition. Hepatic glycogen stores of the hypoxia fish, however, returned to levels that were close to the time 0 level from day 16 onwards, indicating that common carp were probably well adjusted to the hypoxic environment after about 2 weeks of exposure and were able to replenish the glycogen store from consumed food, even though ingestion was about half of the normoxic food intake. Although there was a reduction of food intake by carp during the first couple days of hypoxia exposure, its effect on gene expression was minimal, as changes in gene expression were evident only after 16 days of starvation in the carp liver. Furthermore, feeding is an energy-expensive process. Fish eat when energy is available to process food. Therefore, some changes in gene expression late in hypoxia probably relate to the restoration of feeding.

Conclusion

Many physiological activities such as reproduction and locomotion are suppressed during both hypoxia and starvation, and energy utilization is reduced. Responses to hypoxia can occur rapidly, within hours, whereas changes associated with starvation are much slower. During hypoxia, oxygen limitations resulted in a decrease in oxidative phosphorylation. This led to a reduction of the ATP pool, which in turn resulted in suppression of gene expression. Changes in gene expression appear to follow, rather than direct, the changes in metabolism during hypoxia. In the case of starvation, oxygen is readily available, and it seems that the liver continues as normal and other tissues contribute to the general reduction of metabolism. One major group of genes that were suppressed in liver was digestive and lipogenesis enzymes; whereas severe suppression of ATP production pathways, protein biosynthesis and many other cellular functions were detected in kidney. With decreased ATP turnover during food deprivation, the liver was

able to supply ATP required for the animal, and only minimum alterations were seen in the gene expression patterns. During prolonged hypoxia, in contrast, liver metabolism had to be reorganized. Anaerobic respiration, mediated by HIF, was enhanced to sustain the hypoxia-driven, reduced ATP turnover. The liver, with strong induction of anaerobic respiration, appeared to play a more prominent role as an ATP contributor than kidney. Gene expression during both starvation and hypoxia in carp is directed towards metabolic depression. In the liver, genes were greatly suppressed in response to hypoxia, but not many changes were seen in response to starvation. In the kidney, common suppression of genes was seen during early hypoxia and starvation. However, suppression (or further suppression) of genes was observed in prolonged starvation; whereas during hypoxia, initial suppression was gradually lessened, probably reflecting the carp's ability to adjust to the surrounding reduced oxygen level.

In addition, the changes of gene expression did not appear to be substrate-driven in both cases. Although hepatic glycogen was depleted during early exposure to hypoxia, it was later replenished as carp settled well into prolonged hypoxia exposire. During starvation, carp appeared to prolong the period of substrate availability by conserving stored substrates and consuming them at very slow rates. Hence, expressions of substrate mobilizing-associated genes was maintained in the liver but suppressed in the carp kidney. Nonetheless, both hypoxia and starvation resulted in reduction in reproduction-associated gene expression. Energy was channeled to self-survival during stressed periods rather than to gamete production. During starvation, energy was probably conserved to ensure survival, but in the case of hypoxia, inhibition of reproduction may be initially via the effects of cortisol, followed by suppression of reproduction-associated gene expression. Clearly, in this study, metabolic depression in response to starvation is greater in the carp kidney than liver. In fact, other less vital and energy-demanding organs may show even more drastic reduction in energy use.

The gene expression pattern of carp in response to starvation and hypoxia are clearly different with little overlap. However, how metabolic depression is initiated during exposure to hypoxia and starvation awaits elucidation.

Table I. General Gene Expression Patterns of Common Carp in Response to Prolonged Starvation and Hypoxia.

	Liver		Kidney	
Metabolism / ATP production	**Starvation**	**Hypoxia**	**Starvation**	**Hypoxia**
Glycolysis	Induced after 28 days	Induced	Suppressed	Induced
Gluconeogenesis	Suppressed and then returned to control levels at day 42	Cytosolic gluconeogenic genes: induced	Suppressed	Induced (cytosolic gluconeogenic genes)
TCA cycle	Induced after 28 days	Suppressed at day 4 & some genes gradually returned to near time 0 levels by day 42	Suppressed	Suppressed and returned to control level by 42 days
Oxidative phosphorylation and ATP synthases	No change	Suppressed	Suppressed	Suppressed and returned to control levels by day 42
β-oxidation	No change	Suppressed	Suppressed or no change initially, increased at day 28	Suppressed and returned to control levels by day 42
Lipid transport & metabolism	Varied expression	Suppressed	Induced from day 8 onwards	Varied expression
Proteasome-ubiquitin pathway	Induced after 28 days	Varied expression	Varied expression	Suppressed and gradually returned to control levels by day 28
Other proteases and peptidases	Suppressed from day 16 onwards (Digestive enzymes)	Varied expression	Varied expression	Suppressed
Macromolecules Biosynthesis				
Fatty acid / lipid	Suppressed from day 16 onwards	Suppressed	Suppressed from day 8 onwards	Suppressed
Protein: Transcriptional factors	Varied expression	Poly(A)-binding protein: induced	Increased expression	Varied expression
Protein: Translation	Translational factors: varied expression; Ribosomes: no change	Ribosome genes: no change	Translational factors: varied expression; Ribosomes: suppressed from day 8 onwards	Varied expression
Others				
Transporters	Varied expression	O_2 transporters-associated genes: varied expression; intra-/intercellular transporters: suppressed;	O_2 transporters: suppressed; Others transporters: varied expressions	Suppressed in general; myoglobin induced strongly
Oxidative stress response	UCP1: suppressed Antioxidant genes: varied expression	UCP1: induced Antioxidant genes: suppressed	UCP1: induced; Antioxidant genes: suppressed	UCP1: induced at day 4 only; Antioxidant genes: suppressed & returned to time 0 levels
Immuno-related	Varied expression	Varied expression	MHC / blood coagulation factor: induced; Cytokines / inflammatory response: suppressed	Immuno-related: varied expression
Steroidogenesis and reproduction related	Suppressed	Suppressed	Not detected	Not detected

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Studies of gene expression in brain of anoxic crucian carp

by

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Introduction

Vertebrates are obligated oxygen-consumers, and without proper oxygenation ATP demands rapidly exceed ATP production, leading to cell death. However, there are exceptions to this rule, and the crucian carp, *Carassius carassius*, survives months of anoxia at temperatures close to 0 °C. This implies that it manages to cope with the encountered energetic problems, through an increase in the rate of anaerobic ATP production and/or a depression of the metabolic rate (hypometabolism). Indeed, both processes appear to play a role in the anoxic survival of crucian carp (reviewed by Nilsson, 2001).

The vertebrate brain has a high rate of ATP use, most of which is associated with the ion pumping needed to sustain ion gradients across the neurolemma. Thus, lowering of ion-fluxes through ion-channels represent a potential way of reducing neuronal ATP needs. Indeed, "channel-arrest" has been hypothesized to be an important mechanism for energy conservation in brain tissue of anoxia-tolerant vertebrates such as crucian carp and several species of freshwater turtles (Hochachka, 1988; Lutz *et al.,* 1985). Channel arrest has been demonstrated in the anoxic turtle brain, which, among other adaptations, show reduced NMDA receptor function (reviewed by Bickler and Buck, 1998). In this study we assessed the role of channel arrest in anoxic crucian carp brain tissue by investigating the mRNA expression of the glutamatergic AMPA- and NMDA receptors.

AMPA receptors (AMPARs) are excitatory, ionotropic glutamate receptors that usually have low Ca^{2+} permeability. They are important mediators of excitatory neurotransmission and play vital roles in synaptogenesis and synaptic plasticity (Tanaka *et al.,* 2000). Moreover, AMPARs are central in ischemic neuronal cell death, being partially responsible for the excitotoxic events that follow ATP depletion (Arundine and Tymianski, 2003). Until now, four AMPAR subunits have been characterized in mammals, GluR1-4, while eight subunits have been characterized in *Danio rerio* (zebrafish), GluR1a,b-GluR4a,b. Functional AMPARs are tetrameric complexes combining the different subunits, and the subunit composition is a major determinant of receptor function. Numerous studies have reported changes in AMPAR function mediated by changes in subunit composition, and such alterations are known to occur in response to ischemic insults (review by Tanaka *et al.,* 2000).

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NMDA receptors (NMDARs) are excitatory, ionotropic glutamate receptors that have high Ca^{2+}permeability. They are key players of synaptic plasticity (Collingridge *et al.,* 1983; Wenthold *et al.,* 2003) and they are the main mediators of neuronal cell death in ischemia (Arundine and Tymianski, 2003). So far, seven NMDAR subunits have been characterized in mammals; NR1, NR2A-D and NR3A-B. Functional NMDARs are believed to consist of two NR1 subunits and two NR2 subunits, and receptor diversity is mainly decided by the NR2 subunit composition (Dingledine *et al.,* 1999). One way of studying properties of NMDARs in an organism would therefore be to study NR2 subunit composition.

We hypothesized that the properties of AMPA- and NMDA receptors change in crucian carp brain tissue upon anoxia-exposure, and that these changes are caused by alterations in the expression of the various receptor subunits. Here we report the cloning of subunits of AMPA- (GluR1a,b-GluR4a,b) and NMDA (NR1 and NR2A-NR2D) receptors in crucian carp, and show their expression in normoxic vs. anoxic brain tissue. Four experimental groups were investigated; normoxia 7 days, anoxia 1 day, anoxia 7 days and anoxia 7 days/normoxia 7 days.

Results

Judging from their expression, the composition of AMPARs and NMDARs seem to be fairly stable during anoxia. Only GluR3a, NR1 and NR2C mRNA levels were significantly changed, all being decreased (Table I).

Table I**.** Anoxic mRNA levels of AMPAR subunits (GluR1a,b-GluR4a,b) and NMDAR subunits (NR1, NR2A-NR2D) in crucian carp brain at 10 °C, standardized to normoxic levels. Significant changes are indicated by arrows, and were found for GluR3a (A7), NR1 (A1, A7 and A7R7) and NR2C (A1).

mRNA	Anoxia 1 day (A1)	Anoxia 7 days (A7)	Anoxia 7 days; Reox 7 days (A7R7)
GluR1a	–	–	–
GluR1b	–	–	–
GluR2a	–	–	–
GluR2b	–	–	–
GluR3a	–	↓	–
GluR3b	–	–	–
GluR4a	–	–	–
GluR4b	–	–	–
NR1	↓	↓	↓
NR2A	–	–	–
NR2B	–	–	–
NR2C	↓	–	–
NR2D	–	–	–

Discussion

The subunit expression of AMPARs and NMDARs in crucian carp brain tissue was strikingly well preserved during such a drastic insult as one week of anoxia. Thus, the anoxic crucian carp appears to keep its glutamatergic system in a steady-state, retaining its functionality. This tentative conclusion goes well with the suggestion that crucian carp needs to retain its brain tissue ion conductance to be able to survive anoxia in an active state (Nilsson, 2001). Thus, after finding no effects of anoxia on K^+ and Ca^{2+} permeability in crucian carp brain slices it was postulated that channel arrest is not an important component of the anoxic survival strategy utilized by the crucian carp (Nilsson, 2001). Having said this, based on the current knowledge it is not possible to fully exclude channel-arrest as a mean of lowering neuronal energy expenditure in anoxic crucian carp brain. There are at least two reasons for this. Firstly, proteins such as AMPARs and NMDARs may be altered at the post-translational level rather than at the transcriptional level (e.g. by controlling the state of phosphorylation). Such post-translational changes have been shown to occur in NMDARs of anoxic turtle brain tissue (Bickler *et al.,* 2000). Secondly, electric activity and the formation of action potentials are influenced by numerous factors other than properties of AMPARs and NMDARs. Major sites for ion fluxes in brain are sodium channels, and how they are influenced by anoxia has so far has not been examined in crucian carp brain.

Still, the glutamate receptors did show some changes in expression during anoxia. Interestingly, the mRNA level of the GluR3a AMPAR subunit was significantly lowered after 7 days of anoxia (Table I). In mammals, AMPARs containing GluR3 subunits are thought to be important in long-term depression (LTD) events of mammalian hippocampal neurons, and they are thought to target and stabilize AMPARs to synapses (Meng *et al.,* 2003). Moreover, in 2006 Satake *et al.* ascribed a role for GluR2/GluR3-containing AMPARs in modulation of presynaptic function. They found such AMPARs in presynaptic membranes of GABAergic interneurons, and by activating them they managed to inhibit GABA-release into corresponding synapses. Speculatively, a lowering of GluR3a in anoxic crucian carp may serve to lower the presence of GluR2/GluR3a AMPARs in presynaptic membranes. Eventually this may lead to increased GABAergic activity, which in turn will have inhibitory effects on the electric activity of the brain. Increased levels of extracellular GABA is seen in the anoxic crucian carp brain, and is thought to function as a metabolic depressant by reducing electric activity (Hylland and Nilsson, 1999).

We found lowered mRNA levels of the NR1 NMDAR subunit in the anoxic groups (Table I). Intuitively, this could indicate a general lowering of functional NMDARs in anoxic crucian carp brain, since NR1 is thought to be an essential part of all NMDAR complexes. However, NR1 is known to be expressed in a surplus stock, and the level of functional NMDARs has been suggested to be decided by the availability of NR2 subunits (Wenthold *et al.,* 2003). Thus, the lowering of NR1 expression seen in anoxic crucian carp brain does not necessary mean a reduced number of functional NMDARs.

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Doing the impossible: Anoxic cell division in the crucian carp (*Carassius carassius*)

By

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Vertebrates depend on an uninterrupted supply of oxygen to maintain energy production. Some species, however, are able to survive severe hypoxia for several hours, including many fishes of the Amazon, like the oscar cichlid (*Astronotus ocellatus*) (Muuscze *et al.,* 1998), and several coral reef fishes (Wise *et al.,* 1998; Nilsson and Nilsson, 2004). However, the true master of hypoxic and anoxic survival appear to be the crucian carp (*Carassius carassius*). It lives in lakes and ponds in northern Europe and Asia, where it is able to survive under the ice cover in the winter with little or no oxygen for several months (Holopainen, 1986).

The crucian carp has solved the problem of living without oxygen in a very exotic manner. When oxygen levels drop, the crucian carp up-regulates glycolysis (Storey, 1987), but in order to avoid self-pollution by increased lactate levels, lactate is converted to ethanol, which leaves the fish over the gills (Johnston and Bernard, 1983). In this way the crucian carp is able to maintain energy production in the absence of oxygen as long as there is glycogen present in the liver (Nilsson, 1990). Still, glycolysis is much less effective than aerobic respiration and releasing an energy-rich compound like ethanol is energetically wasteful. It is therefore advantageous for the crucian carp to minimize the energy consumption while in anoxia, something it does by reducing brain activity and locomotor activity (Nilsson, 1992, Nilsson *et al.,* 1993).

It is also beneficial for the crucian carp to postpone the onset of anaerobic respiration for as long as possible. The crucian carp does this by being very efficient at taking up oxygen from its environment: its haemoglobin has an extremely high oxygen affinity (Sollid *et al.,* 2005a), allowing it to take up oxygen at normal rates even if ambient oxygen falls to 10% of air saturation (Sollid *et al.,* 2003).

The high oxygen affinity of the haemoglobin might be partially responsible for bringing about a peculiar gill morphology in the crucian carp: under normoxic conditions, its gills completely lack protruding lamellae (Fig. 1a) (Sollid *et al.,* 2003). This is exceptional as the lamellae are the primary site of gas exchange, making up most of the respiratory surface area of fish gills. However, due to the high oxygen affinity of the crucian carp haemoglobin, the fish is still able to respire normally with only a fraction of the respiratory surface area of other fish. Exposing a minimal surface area to the exterior

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milieu has the potential for limiting the uptake of pathogens and toxic substances, and reducing energy costs associated with excessive water influx and ion loss, a challenge faced by all freshwater fish (Sollid *et al.,* 2003). Thus, in normoxic water this gill morphology has probably several advantages.

However, under hypoxic conditions, the crucian carp gills change morphology, and protruding lamellae appear (Fig. 1b-e), increasing the respiratory surface of the gills. Interestingly, this change of morphology is completely reversible, and the lamellae disappear again on re-exposure to normoxic conditions (Fig.1f).

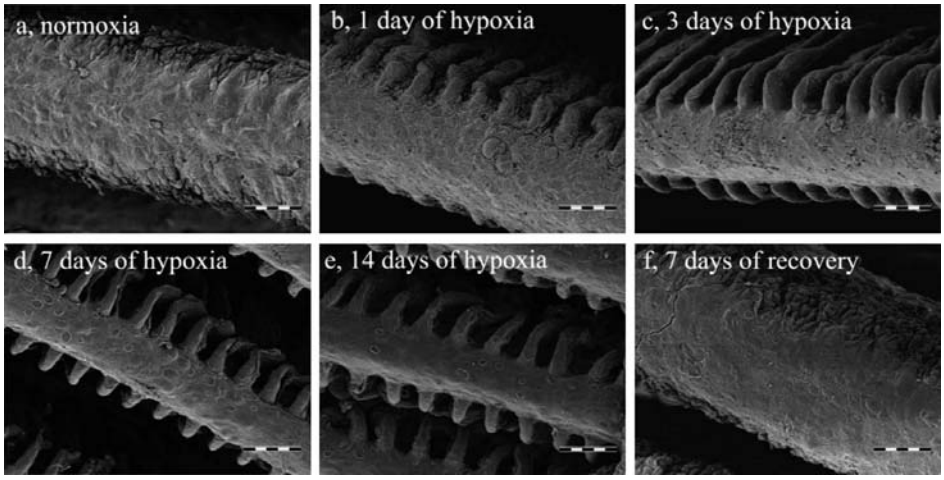

Figure 1. Scanning electron micrographs from the 2nd gill arch of crucian carp kept in normoxic or hypoxic water. In normoxia, the gill filaments have no protruding lamellae (a), but after 1, 3, 7 and 14 day of hypoxia exposure the lamellae gradually appear (b,c,d,e). After a further 7 days of recovery in normoxic water, the gills again lack protruding lamellae (f). Scale bar is 50 µm. Adapted from Sollid *et al.* (2003).

Sollid *et al.* (2003) studied this phenomenon immunohistochemically in order to reveal the underlying cellular mechanisms. They found that under normoxic conditions the spaces between the gill lamellae were filled with a mass of cells they named the interlamellar cell mass (ILCM). This cell mass would be reduced in size when fish were being held in hypoxia (Fig. 2). Further, mitotic (S-phase) cells and apoptotic cells in the ILCM were identified using immunohistochemistry for BrdU (Bromodeoxyuridine – an externally introduced marker of DNA synthesis, and thus an S-phase marker) and TUNEL staining (Fig. 2). The number of cells in S-phase dropped significantly, and the number of TUNEL positive apoptotic cells increased transiently after exposure to hypoxia (Fig. 3). These effects combined appeared to bring about the change in gill morphology.

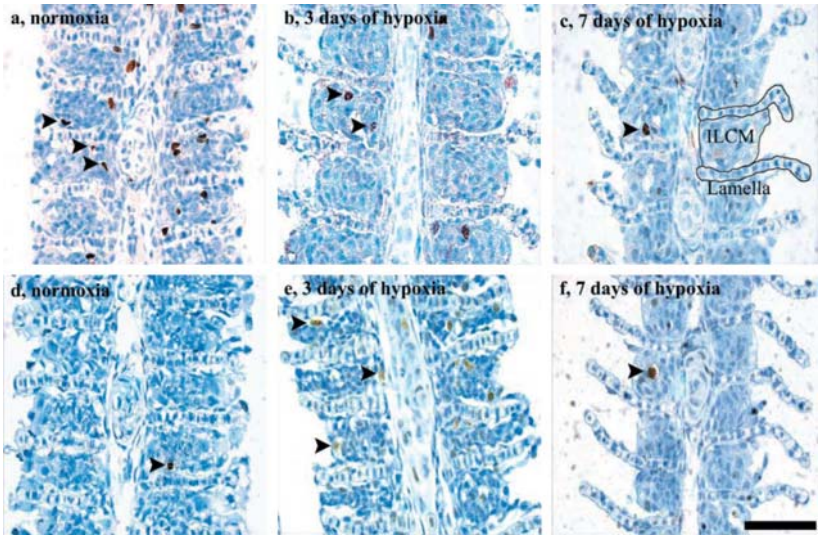

Figure 2. Light micrographs of gills stained for S-phase cells (BrdU) (a-c) and apoptotic cells (TUNEL) (d-f) in normoxia (a,d), 3 days of hypoxia (b,e) and 7 days of hypoxia (c,f). Arrows point out some of the positively stained cells. ILCM; interlamellar cell mass. Scale bar, 50 µm. Adapted from Sollid *et al.* (2003).

An interesting point is that the cell proliferation did not drop to zero; 0.58% of the cells were still actively dividing after 7 days of hypoxia. This is indeed surprising as the animals had been kept at oxygen levels far below the point where mammalian cells are known to halt cell cycle progression (Pettersen and Lindmo, 1983).

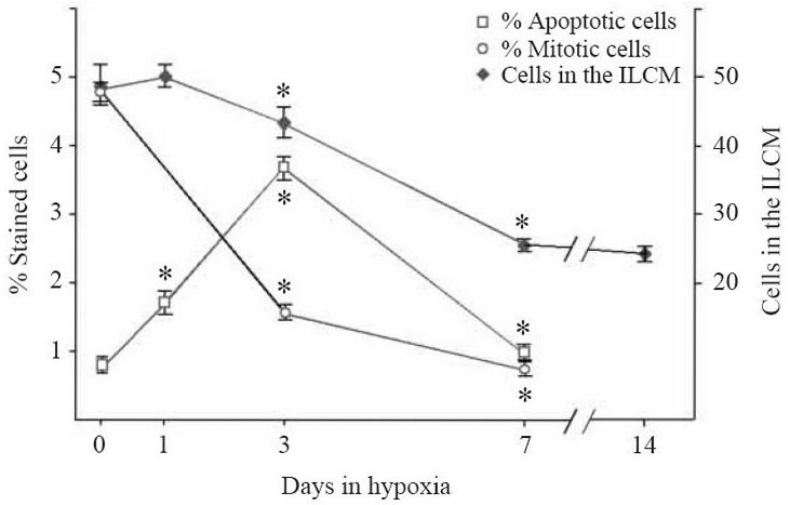

Figure 3. The percentage of apoptotic cells and S-phase cells (left y-axis) and the total cell number in a central cross-section of the interlamellar cell mass (ILCM; right y-axis) after hypoxia exposure. Values are means ± S.E.M. Values that are significantly different from previous or later time point are marked with an asterisk ($P<0.01$). From Sollid *et al.* (2003).

Sollid *et al.* (2005b) also studied the gill morphology of crucian carp exposed to anoxia. Interestingly there was no difference in morphology between the gills of normoxic fish and fish that had spent 7 days in an anoxic environment (Fig. 4). This does make sense, as there is no oxygen to extract from the environment in this case, and maintaining the ILCM saves energy on osmoregulation and ion transport.

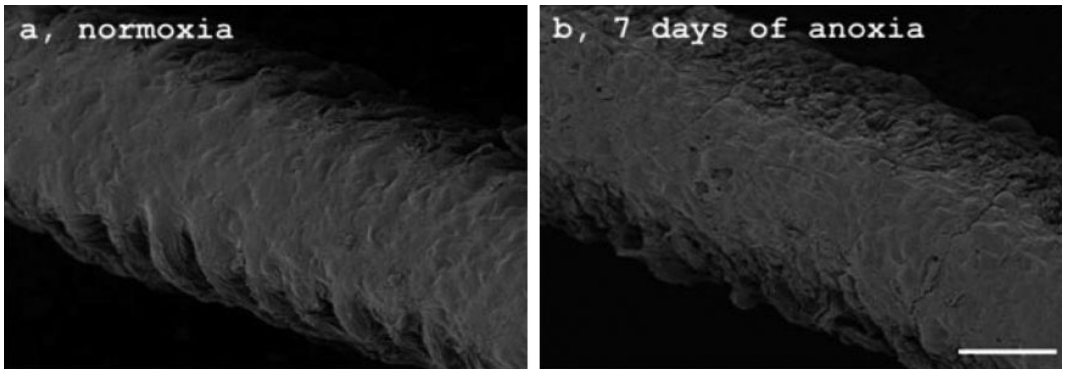

Figure 4. Scanning micrograph of crucian carp gill filament in normoxia (a) and after 7 days of anoxia exposure (b). There were no apparent differences in gill morphology between the two exposure groups. Scale bar = 50 µm. From Sollid *et al.* 2005b.

Once again the gills were studied on a cellular level with immunohistochemistry for BrdU. Proliferative activity was significantly down-regulated, but after 7 days completely without oxygen, 5.0% of the ILCM cells were still going through S-phase (Fig. 5) (this number is higher than that of the hypoxic experiment, but the anoxia experiment was conducted at a slightly higher temperature, and a higher general metabolism and cell proliferation rate is to be expected). The same study (Sollid *et al.,* 2005b) also showed that cell proliferation continued in liver and intestine during anoxia.

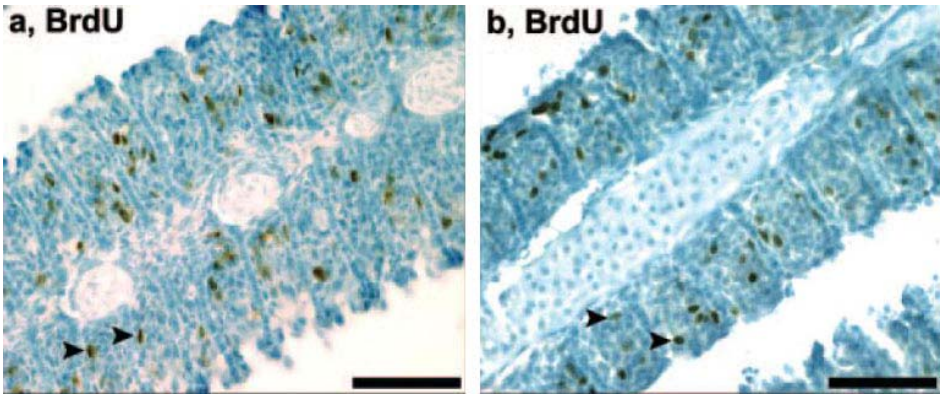

Figure 5. Light micrographs of gills stained for BrdU from fish taken from normoxic (a) and 7 days of anoxia (b). Scale bars = 50 µm. Adapted from Sollid *et al.* (2005b).

In mammals, hypoxia and anoxia have profound effects on cell cycle progression. Exposure of mammalian cell cultures to an atmosphere containing < 4 ppm O_2, under which respiration is inhibited (Froese, 1962) results in immediate arrest of cells in S-phase. Several proteins are involved in the oxygen dependent regulation of the cell cycle, but one of those with a direct effect on cell cycle progression is ribonucleotide reducase (RNR). RNR is the enzyme responsible for converting the four standard ribonucleotides to deoxyribonucleotides needed for DNA synthesis (Thelander and Reichard, 1979; Eklund *et al.,* 2001). This reaction is the rate-limiting step of DNA synthesis (Eriksson *et al.,* 1984; Engström *et al.,* 1985), and thus inhibition of RNR halts progression through the S-phase. The vertebrate RNR consists of 4 subunits (α_2 β_2) (Figure 6). The two β-subunits (R2), harbours a di-iron centre each, involved in formation of a tyrosyl radical responsible for the reduction of ribonucleotides. The formation of this radical is an oxygen dependent reaction, and in the absence of oxygen the radical has a half-life of 30-60 minutes at room temperature (Chimploy *et al.,* 2000; Nyholm *et al.,* 1993). Thus, in the absence of oxygen, RNR activity is rapidly lost and DNA synthesis stops and cell cycle progression is halted (Graff *et al.,* 2004; Probst *et al.,* 1984; Probst *et al.,* 1988). The levels of R1 are stable throughout the cell cycle due to a half life of more than 20 hours, but the R2 subunit shows S-phase specific expression and has a half life of only 3 hours, probably regulated by controlled degradation (Chabes and Thelander, 2000).

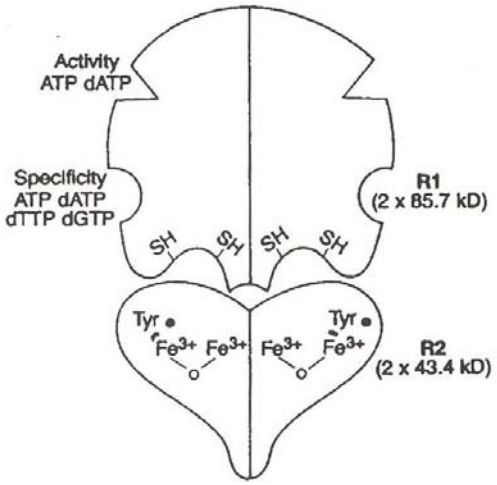

Figure 6. An overview of RNR showing the dimmer of the R1 (α_2) and R2 (β_2) subunits. In the R2 subunit the tyrosyl radicals are indicated. From Reichard 1993.

As DNA synthesis continues in anoxic crucian carp, the crucian carp RNR might have bypassed the oxygen dependence of the tyrosyl radical formation. Sollid *et al.* (2005b) partially cloned the crucian carp RNR R2 subunit and aligned it with the same sequences from zebra fish (*Danio rerio*), *Xenopus laevis* and mouse (*Mus musculus*) (Fig. 7). There was a high degree of sequence homology, and all the amino acids involved in coordinating and creating the tyrosyl radical, as identified in a 3D model of the mouse RNR R2 were conserved in all four species compared. Thus, the three-dimensional structure of the crucian carp RNR R2 is almost identical to its mammalian counterpart, which is known to be oxygen dependent.

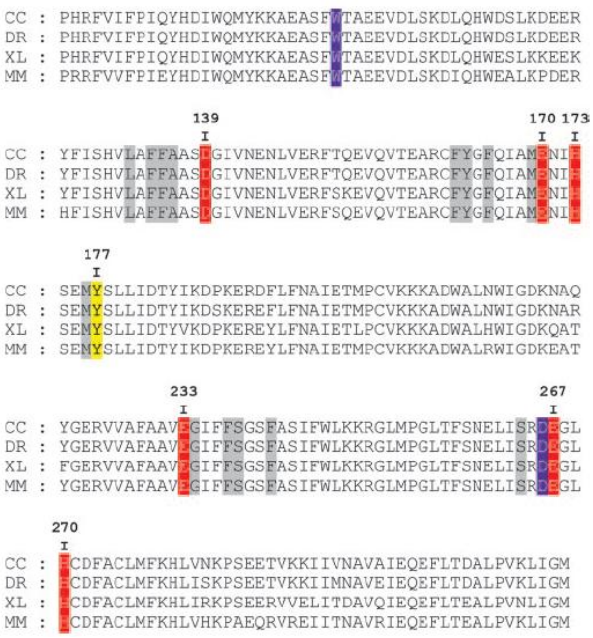

Figure 7. Amino acid sequences of the RNR R2 subunits of CC: crucian carp (*Carassius carassius*), DR: zebrafish (*Danio rerio*), XL: African clawed frog (*Xenopus laevis*) and MM: mouse (*Mus musculus*). Amino acids holding the iron centre are highlighted in red. The radical is generated on Tyr177 (yellow). Amino acids involved in transport of the radical to the active site on the R1 subunit are highlighted in blue, while amino acids involved in generating the entrance to and surrounding the radical site are highlighted in grey. From Sollid *et al.* (2005b).

Sollid *et al.* (2005b) also quantified the amount of RNR R2 mRNA in crucian carp gills in normoxia and anoxia, performing real time PCR, and found no significant difference between the groups, even though the number of S-phase cells was reduced. There does not appear to be any anoxia-induced regulation of R2 on the transcriptional level.

It thus appears that the crucian carp, in contrast to other investigated vertebrate species, is able to maintain DNA synthesis in the absence of oxygen. Surprisingly, amino acid residues, especially those involved in coordinating the tyrosyl radical, are identical in crucian carp and mouse RNR R2. Also there is no change in RNR R2 mRNA in crucian carp exposed to anoxia for 7 days. There is a possibility that the crucian carp RNR R2 is more effective in keeping the tyrosyl radical stable. However, a difference in stability from 3 hours in mammals at 25 °C to several days in crucian carp at 10 °C is not likely. Still, since no structural or spectroscopic data are presently available for crucian carp RNR, no direct measurements of the radical stability in this species exist.

Another possibility is that the crucian carp uses another RNR or R2 subunit for ribonucleotide reduction either at all times or when exposed to anoxia. The cloning was

done based on sequences of RNRs of other species, and might not have captured alternative RNR variants. The crucian carp genome is tetraploid, so it harbours multiple versions of several proteins, where the different versions often have divergent properties.

In conclusion, the crucian carp is capable of maintaining DNA synthesis and cell proliferation in the absence of oxygen. How this is done, however, still remains a mystery.

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Hypoxia-tolerance in a tropical elasmobranch: does adenosine trigger a multi-system protective response?

by

G.M.C. Renshaw[1].

Life in a marine setting poses a particular set of physiological challenges to be met and responded to, whether it is surviving on the reef or surviving man-made changes to the environment. Few vertebrates can survive prolonged hypoxia or anoxia and most of those studied evolved their tolerance at temperatures close to freezing when metabolic rates are lower. A physiologically more severe challenge occurs when normoxia or anoxia is encountered at normothermic temperatures. Activation of retaliatory or pre-emptive protective mechanisms in response to a physiological stressor can confer cross protection to other types of physiological stressors, particularly if molecular chaperones such as heat shock proteins are upregulated. So it is possible that some of the protective mechanisms elicited in fish to prolong survival in hypoxia and anoxia may be also switched on to prolong their survival in temporarily toxic environments. Consequently, a comparative examination of the repertoire of physiological mechanisms that have evolved to prolong survival may be useful not only in providing a deeper understanding of evolutionary processes but also help to develop conservation strategies to counter a variety of environmental stressors such as eutrophication and pollution which provide increasingly serious risks to fish populations.

When O_2 supply is diminished, high energy purines such as ATP, ADP and AMP can not be re-synthesised at a rate to match their usage with the result that increased adenosine is formed from AMP or via the IMP and inosine pathways in what Lutz *et al.* (2003) term the "energetically compromised brain." The rising adenosine level can be both friend and foe. While a rise in adenosine can signal imminent destruction of tissue via necrosis or apoptosis, with the most vulnerable tissues being the brain and heart, an elevated level of adenosine can act as a switch to conserve energy. In hypoxia- and anoxia-tolerant animals adenosine may reduce metabolic rate and neuronal activity, as well as stimulate glycolysis to increase available energy which ultimately delays the onset of tissue damage (Lutz *et al*., 2003). In the brain, the action of adenosine on its receptor conserves neuronal energy because it clamps the resting membrane potential and inhibits transmitter release making it less likely that the neuron will respond to or generate an action potential resulting in decreased energy utilisation.

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Stimulation of adenosine receptors increases hypoxia and anoxia tolerance by triggering metabolic depression in vertebrates that evolved their tolerance at 0^{o}C (Lutz *et al.*, 2003). Adenosine plays a key role in hypoxia and anoxia tolerant teleosts such as Crucian carp (*Carassus carrassus*) that evolved their tolerance to diminished oxygen in conjunction with 'over wintering' at 0^{o}C, making it extremely difficult to disentangle cytoprotective strategies from a metabolic shut-down triggered in response to freezing. It is easier for an organism to tolerate anoxia when freezing temperatures have already depressed energy consumption. Hypoxia and anoxia provide a greater challenge at tropical temperatures; a few species of fish in Brazil (Almeida-Val, 1995; Val and Almeida-Val, 1995; Chippari-Gomez *et al*., 2005) and Australia (Renshaw and Dyson, 1999; Renshaw *et al*., 2002; Nilsson and Ostlund-Nilsson, 2004) have evolved mechanisms to enable them to successfully cope with severe hypoxia and anoxia.

Some reef platforms on the Great Barrier Reef in Australia can be subject to extreme fluctuations in dissolved oxygen levels. Heron Island reef platform (23^{o}27'S, 151^{o}55"E) is surrounded by a fringing reef and dissolved oxygen levels range from over 150% saturation at midday to 30% saturation on some nocturnal low tides (Kinsey and Kinsey, 1967). During nocturnal low tides when the water on the reef platform is cut off from the surrounding ocean water by a fringing reef and prevailing wind conditions do not cause mixing and re-oxygenation of the surface waters, dissolved oxygen levels can fall to 19% saturation (Renshaw, unpublished observations). This extreme environment provides cycles of nocturnal low tides that could potentially pre-condition its inhabitants to hypoxia. The epaulette shark (*Hemiscyllium ocellatum*) is a nocturnal feeder that successfully exploits this sheltered habitat. In previous studies we have shown that the epaulette survives severe hypoxia, 0.39mg O_2 l^{-1} for 2 hours, without delayed neuronal apoptosis (Renshaw and Dyson, 1999) and at least one hour of anoxia (Renshaw *et al.*, 2002) without a deleterious decrease in brain energy charge. More recent experiments have established that animals recover from 5-6 hours of anoxia (Chapman and Renshaw, unpublished results). The metabolic and ventilatory depression that occurs in response to hypoxia (Routley *et al*., 2002) and anoxia (Renshaw *et al*., 2002) may serve to match energy consumption to reduced ATP generation in the epaulette shark because blockade of adenosine receptors with aminophylline, a non-specific adenosine antagonist, lowered brain energy charge (Renshaw *et al*., 2002). We demonstrated that hypoxia- and anoxia-tolerance could be increased in the epaulette by prior exposure to preconditioning episodes of sub-lethal hypoxic (Routley *et al*., 2002) or anoxic exposure (Renshaw *et al*., 2002), a phenomenon first described in goldfish (Prosser *et al*., 1957).

This paper examines the evidence that adenosine switches on potentially protective mechanisms in the tropical hypoxia and anoxia tolerant epaulette shark and makes an appreciable difference in conserving brain energy charge during anoxia at tropical temperatures. In a series of separate studies, we focused on the effect of adenosine on the control of: i) ventilation rate; ii) heart rate; iii) blood supply to the gills, heart and brain; iv) neuronal activity and brain energy charge; and v) the level of molecular chaperones. It is clear that adenosine is capable of triggering a multi-system response to diminished oxygen levels. However the full extent of the protective extent of adenosine, during exposure to hypoxic and anoxic conditions, is not completely understood. In some but not all systems, adenosine appears to be a major retaliatory

molecule to pre-empt energy failure and increase natural repair systems both of which would serve to reduce cell damage.

The effect of hypoxia and adenosine on ventilation rate

To examine the role of adenosine in controlling heart rate and blood flow to the heart, gills and brain during a hypoxic response, anaesthetised epaulette sharks were exposed to either hypoxic (0.34 mg O_2 l^{-1}) or normoxic conditions with and without pharmacological intervention. Separate groups of unanaesthetised animals were exposed to progressive hypoxia in a closed system respirometer or to sudden anoxia.

Increasing the rate of buccal pumping serves to increase the speed and volume at which water passes over the epithelium of the secondary lamellae, ultimately maintaining the oxygen gradient between the water surrounding the gills and the blood (Randall and Daxbroek, 1982). However during hypoxia and anoxia, this increased ventilatory activity reduces the energy budget at a time when oxidative phosphorylation is limited due to hypoxemia. In such environments, ventilatory depression is adaptive and prolongs survival time. Examination of the changes in ventilatory rate during progressive hypoxia revealed that the $[O_2]_{crit}$ of the epaulette shark occurred at 2.30 mg O_2 l^{-1} and ventilatory depression occurred between 1.5 and 1.0 mg O_2 l^{-1}. Furthermore, ventilatory depression coincided with a significant elevation in lactate (Routley *et al*., 2002). An increase in the level of the anaerobic byproduct, lactate, suggests that dependence on anaerobic pathways was increased as oxidative pathways slowed due to the diminished availability of oxygen. Under such conditions, it is likely that adenosine levels rose and thus could be implicated in triggering a decrease in the ventilation rate.

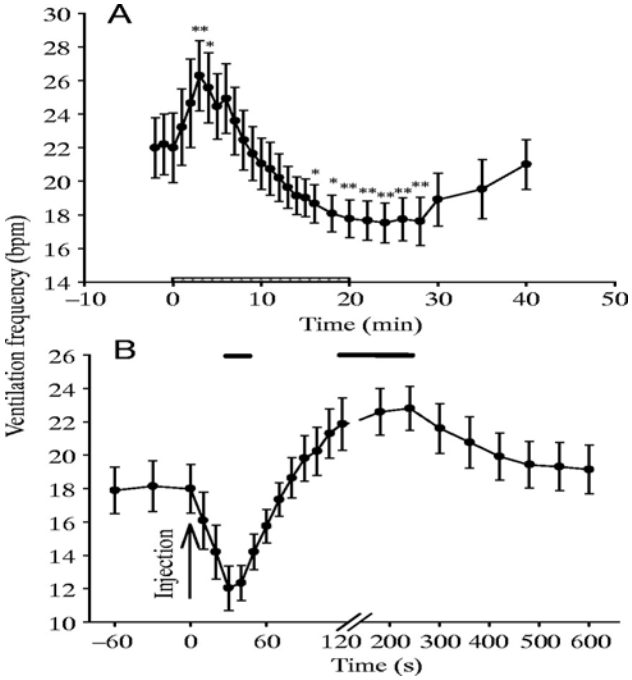

Figure 1. Effects of (A) hypoxia and (B) adenosine (1 μmol kg^{-1}) on ventilation frequency. The horizontal line indicates a significant time interval that differed from the last normoxic value; non-parametric ANOVA (Freidman

test) with Dunn post-test. Values are mean ± S.E.M. (Adapted from Stenslokken et al., 2004)

A bi-phasic ventilatory response was observed during the exposure of anaesthetised sharks to hypoxia (0.35 mg O_2 l^{-1}) (Fig. 1A). Initially, ventilatory movements steadily increased and then significantly decreased, indicating that ventilatory depression had occurred in response to hypoxia (Stenslokken *et al*., 2004). While this confirms previous findings in un-anaesthetized epaulette sharks (Routley *et al*., 2002), it was noted that ventilatory movements were at a lower frequency in anaesthetised animals.

However, when we examined the effect of adenosine administration (1 µmol kg^{-1}) on anaesthetised animals, the ventilatory response elicited by adenosine administration was the reverse of that observed in response to hypoxic exposure. Instead of an initial increase in ventilation, there was an initial pronounced decrease followed by a delayed increase (Fig. 1B). This is the first indication of the involvement of adenosine in a respiratory reflex in any fish (Stenslokken *et al*., 2004).

Furthermore, all ventilatory movement ceased when aminophylline (30 mg kg^{-1}) was injected into anaesthetised sharks. This finding is in sharp contrast to the lack of effect of aminophylline on the ventilation rate of unanaesthetised sharks exposed to anoxia (Fig. 2). Both aminophylline (30 mg kg^{-1}) and saline treated controls exhibited a similar immediate and significant decrease in ventilatory rate that was unaffected by aminophylline (Renshaw *et al*., 2002).

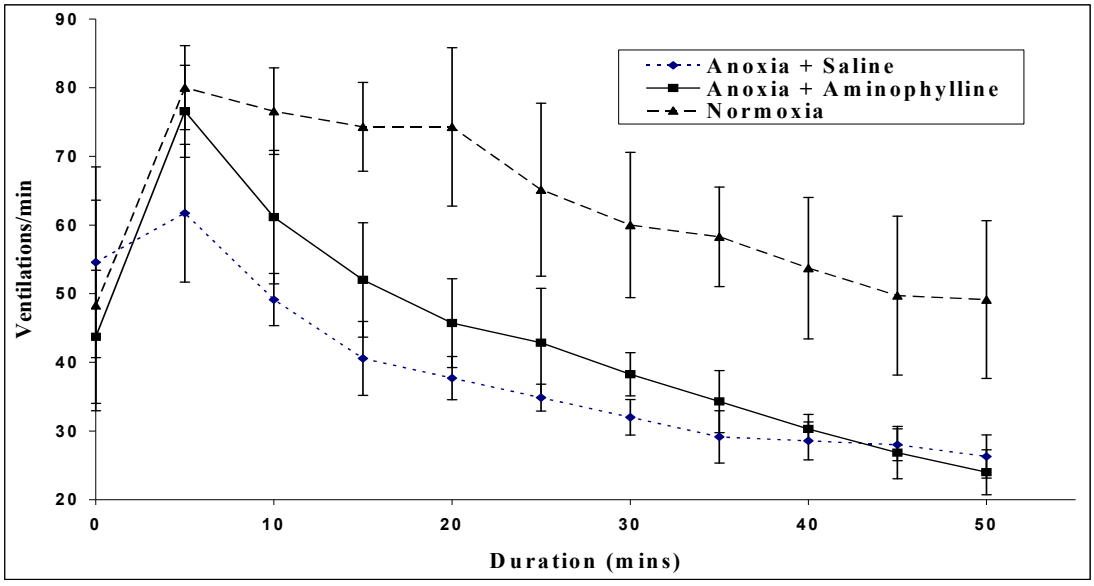

Figure 2. The ventilation rate of anaesthetised epaulette sharks held at normoxia or at anoxia after the prior administration of aminophylline (30 mg kg^{-1} in saline) or saline alone. After initial an initial increase in ventilation rate most likely due to capture stress, the steady decline in ventilation rate was similar for

aminophylline and saline treated animals. (Kerrisk, Nilsson and Renshaw, unpublished results).

The discrepancy between the effect of aminophylline on anaesthetised and unanaesthetised sharks may have occurred due to the interaction of the Benzocaine anaesthetic, used in the study by Stenslokken *et al*. (2004), with GABA receptors present in cardiorespiratory centres (Mulvey and Renshaw, unpublished). It is suggested here that if GABA receptors are blocked by anaesthetic, then adenosine may be needed to maintain respiratory drive. Furthermore, we have shown that there is an overall decrease in the level of neuronal activity in the brainstem in response to hypoxic preconditioning (Mulvey and Renshaw, 2000), and a significant concomitant increase in GABA in hypometabolic brain nuclei (Mulvey and Renshaw, unpublished observations). More specifically, hypometabolism and a concomitant increase in GABA were evident in an important cardiorespiratory centre, the dorsal vagal nucleus. Since adenosine levels are likely to increase during hypoxia, the potential synergism of adenosine and GABA in the ventilatory control of these sharks during hypoxia needs further attention using specific adenosine receptor subtypes to determine under what conditions adenosine plays a role in controlling ventilation.

The effect of hypoxia and adenosine on heart rate

Hypoxia induced bradycardia in the epaulette shark (Soderstrom *et al*., 1999). The heart rate decreased from 58.44 $\pm$ 0.97 beats per min (bpm) to 39.03 $\pm$ 2.61 bpm over a 15 minute period with a significant concomitant drop in blood pressure in the dorsal and ventral aorta and an 84% decrease in erythrocyte velocity indicating a reduced cardiac output (Stenslokken *et al*., 2004). In contrast, bradycardia was evident within 1 min of hypoxic exposure in the non-hypoxia tolerant spiny dogfish (*Scyliorhinus canicula*) (Taylor *et al*., 1977). The slow onset of hypoxia-induced bradycardia in the epaulette shark suggests that there is a non-nervous component to the response (Stenslokken *et al*., 2004). While aminophylline alone did not block bradycardia in response to hypoxia, neither did muscarinic antagonists revealing that this shark differs from other elasmobranchs and teleosts studied so far. However, the administration of adenosine (1 μmol kg^{-1}) during normoxia did mimic bradycardia (Fig. 3) and its action could be blocked by aminophylline (10 mg kg^{-1}) indicating that at present, we cannot rule out a cardioprotective role for adenosine during hypoxia. Further work is needed to clarify whether specific adenosine receptors have a cardioprotective role, during a hypoxic exposure or subsequent reperfusion, or whether there is a specific time point at which a short-term effect of adenosine could be observed.

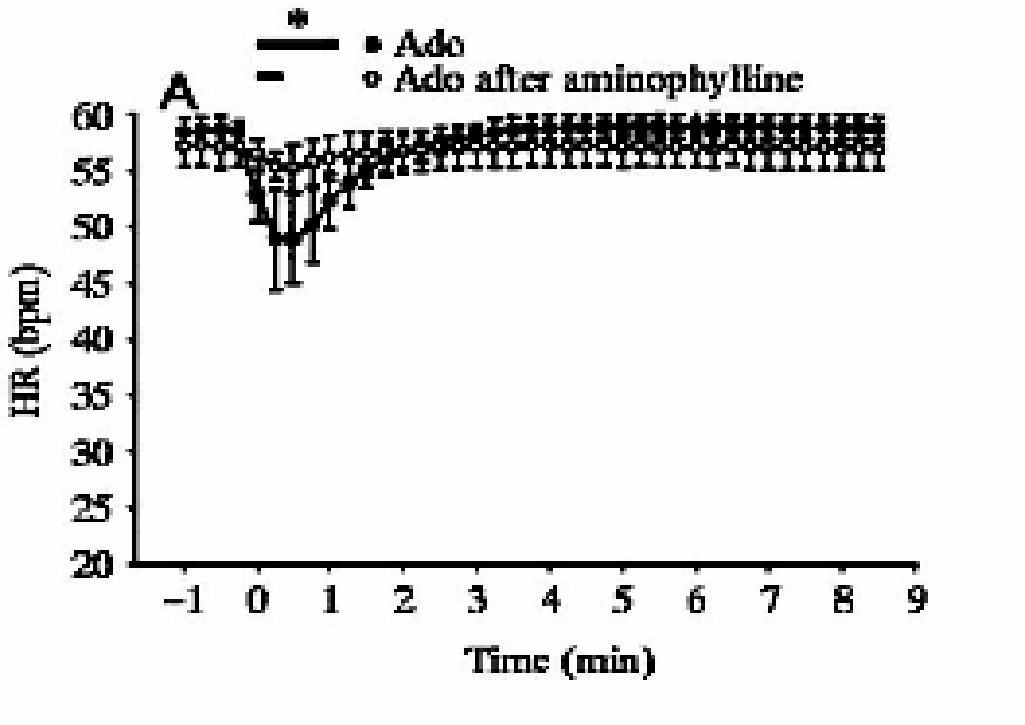

Figure 3. Adenosine administration (1 mmol kg $^{-1}$), during normoxia in anaesthetised epaulette sharks, mimicked the effects of hypoxia. Heart rate fell significantly ($P<0.05$) from 58.60 $\pm$ 1.01 beats min $^{-1}$ to 48.87$\pm$4.49 beats min^{-1} and could be blocked by aminophylline (10 mg kg^{--1}). Time dependant changes were tested using a repeated measures ANOVA with a Dunnet post-test. Lines indicate the time periods that differ significantly from the last normoxic value ($P<0.05$). (From Stenslokken *et al*., 2004).

The effect of hypoxia and adenosine on the regulation of blood supply to the gills, heart and brain.

In teleosts, adenosine is one of the key regulators of blood flow to the gills and acts to constrict branchial circulation (Colin and Leray, 1981; Sundin and Nilsson, 1996). Both hypoxic exposure and adenosine administration during normoxia caused bradycardia and a fall in the blood pressure in the dorsal and ventral aorta (Stenslokken *et al*., 2004). Epi-illumination microscopy revealed that after 4 - 5 minutes, two parallel longitudinal blood vessels opened. Figure 4 shows a video micrograph of the free tip of a gill filament showing one of the longitudinal vessels, which opened when blood pressure dropped during hypoxia. The direction of blood flow (black arrows) in the longitudinal vessel (outlined in black) was toward the base of the filament. These longitudinal vessels extended from the filament base to its tip, where the free tip attached to the septum. The white arrows indicate the position of anastomoses where the blood started flowing in response to hypoxia or adenosine injections (1 μmol kg $^{-1}$). Such anastamoses are also present in the spiny dogfish (Olsen and Kent, 1980) but not in the small spotted cat shark

(Laurent, 1984; Randall, 1985). After approximately 12-15 min of hypoxic exposure, which is the approximate time course for the development of hypoxia-induced bradycardia, blood flow stopped in the longitudinal vessels even though the openings were still visible at high magnification. While the control mechanisms to open these anastomoses are not known, it was suggested that they could be under adenosinergic control (Stenslokken *et al*., 2004).

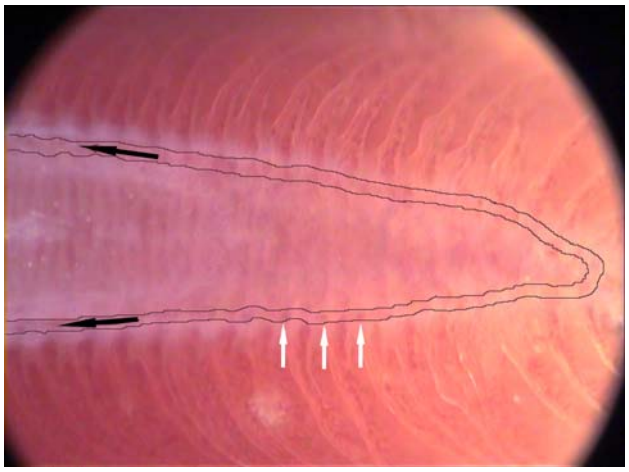

Figure 4. The longitudinal vessel is outlined in black with the direction of blood flow indicated by black arrows and was toward the base of the filament. These longitudinal vessels extended from the filament base to its tip, where the free tip attaches to the septum. White arrows indicate the position of anastomoses where the blood started flowing in response to hypoxia or adenosine injections (1μmol kg^{-1}). (From Stenslokken *et al*., 2004).

Since blood in the longitudinal vessels drains directly into the heart via the arterio-venous circulation, the initial effect of hypoxia or adenosine in recruiting these normally collapsed longitudinal vessels could be expected to provide a short term increase in the blood supply to the heart and protect it before hypoxia- and anoxia-induced bradycardia spare cardiac energy consumption (Stenslokken *et al*., 2004). This indicates a potential role for adenosine in achieving hypoxia and anoxia tolerance in this successfully tolerant species.

While blood pressure dropped by 50 % in the epaulette shark during severe hypoxia (0.35 mgO_2 l^{-1} at 24°C), cerebral blood flow, measured by epi-illumination microscopy, was unaffected indicating that compensatory vasodilation or autoregulation was switched on in response to hypoxia (Soderstrom et al., 1999). During normoxia, we observed an increase in cerebral blood flow velocity when the normoxic brain was superfused with adenosine and this increase could be blocked with aminophylline. This finding reveals that sharks *may be the oldest vertebrate group in which adenosinergic control of cerebral blood flow occurs.* However, during hypoxia neither adenosine nor aminophylline had an effect upon the maintenance of cerebral blood flow (Soderstrom et al., 1999). There was no evidence of an adenosine-mediated increase in cerebral blood flow in the epaulette shark that corresponded to that observed in freshwater turtles and cyprinid fish (Lutz et al., 2003), so our present results are in contrast to the effect of

adenosine in other hypoxia- and anoxia-tolerant species that evolved their tolerance at temperatures close to freezing. Further investigation of the key neurotransmitters and neuromodulators involved in maintaining cerebral blood flow is needed with particular attention paid to more specific antagonists of adenosine receptors.

The effect of hypoxia and adenosine on the maintenance of brain energy charge.

To examine the role of adenosine in maintaining brain energy charge in anoxia-preconditioned sharks during an anoxic challenge, unanaesthetised epaulette sharks were placed into sudden anoxia (< 0.02 mg O_2 l^{-1}) until they lost their righting reflex, this constituted the preconditioning phase (episode 1, E1). The time to loss of righting reflex was measured and used as the end point for episode 1. Sharks were returned to a normoxic holding pool and allowed to recover in it for 24 hours. Sharks were pair matched for endurance time and 15 min prior to a second anoxic challenge (episode 2, E2), one shark from each pair was injected IP with aminophylline (30 mg kg^{-1} in saline) and the other with saline alone, and then the time to loss of righting reflex was measured.

The mean time to loss of righting reflex in the first episode (E1) of anoxia was 47 minutes. The mean time to loss of righting reflex varied significantly with treatment and Figure 5 shows the percentage change in time to loss of righting reflex in E2 in relation to the mean time to loss of righting reflex in E1. Saline treated sharks lost responsiveness and their righting reflex 66% faster in E2 than E1 ($P<0.001$). While aminophylline treated sharks remained alert and retained their righting reflex 143% longer than they had in E1 ($P<0.001$).

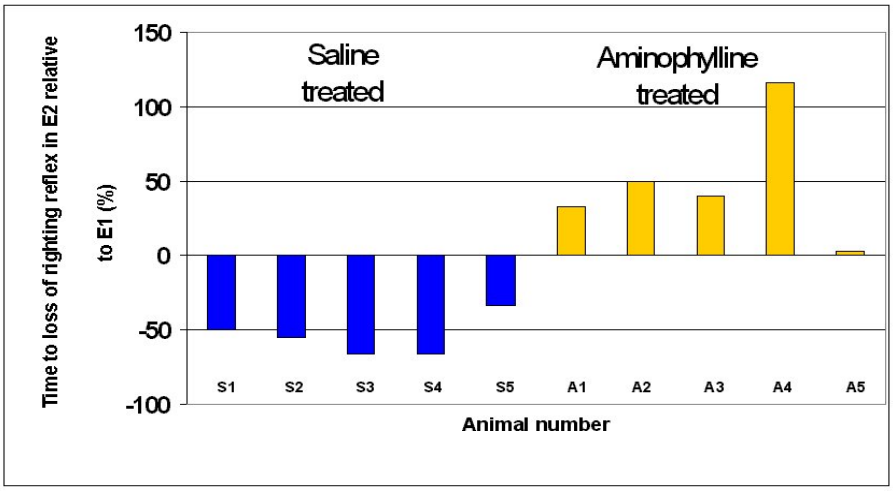

Figure 5. The time to loss of righting reflex for 5 pairs of sharks in episode 2 (E2). Each histogram represents the percentage of time to loss of righting reflex in E2 relative to episode 1 (E1) shown as the baseline. Sharks were pair matched for their time to loss of righting reflex in E1, allowed to recover in a normoxic holding tank then 24 hours later one animal from each pair was injected with aminophylline (30 mg kg^{-1} in saline) and the other with saline alone. Animals were moved to sudden anoxia 15 minutes after injection and the time to loss of right reflex was measured. (Adapted from Renshaw *et al.*, 2002).

Since the righting reflex is under cerebellar control it is likely that the loss of the righting reflex indicated the time at which neuronal activity in the cerebellum was significantly reduced. This appears to have occurred more rapidly when adenosine was allowed to act at its receptor in saline treated sharks than when aminophylline blockade was present. Decreased neuronal activity is an energy sparing adaptive mechanism that appears to be under adenosinergic control in the epaulette shark and mirrors the adaptation of other anoxia tolerant vertebrates (reviewed by Lutz *et al.*, 2003).

In a parallel set of experiments, the time in episode 2 was for a set period of 50 minutes and a group of untreated sharks were held at normoxia for corresponding periods of time in E1 and E2. At the conclusion of this experiment, anoxic animals, their pair matched controls and untreated normoxic sharks were anaesthetised and the brainstems were rapidly frozen and then used to measure energy charge and adenosine levels (*via* HPLC) or to measure molecular chaperone Heat shock protein 70 (Hsp70) levels (*via* semi-quantitative western blotting).

Adenosine levels were 3.5 fold higher in the animals exposed to 50 minutes of anoxia in E2 than in controls exposed to normoxia for 50 minutes demonstrating that the level of the anoxic challenge was sufficient to deplete high-energy purines. Measurements of brain energy charge (Fig. 6) showed that while the energy charge in both saline and aminophylline treated brains was significantly lower than in untreated animals exposed to anoxia for the same period of time, the greatest decrease occurred when aminophylline blockade was in place. The mean energy charge of anoxia treated sharks was 0.736 and of aminophylline treated sharks was 0.687 $\pm$ 0.008 which was significantly lower than untreated sharks held at normoxia ($P<0.001$) and saline treated controls exposed to anoxia ($P<0.05$). These results revealed that the delayed time to loss of righting reflex detrimentally affected the energy budget and that adenosine has an effect in suppressing neuronal metabolism to conserve energy charge. Therefore, the loss of the righting reflex in this species, may be an energy conserving strategy.

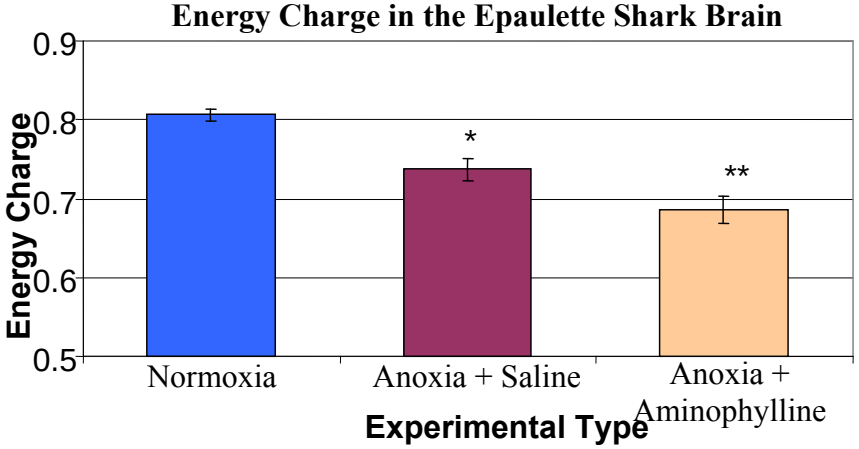

Figure 6. The mean energy charge of brains from epaulette sharks exposed to two episodes of normoxia or anoxia 24 hours apart. The mean energy charge of aminophylline treated sharks was 0.687$\pm$0.008 which was significantly lower

than untreated sharks held at normoxia (P<0.001) and saline treated controls exposed to anoxia (P<0.05). (From Renshaw *et al*., 2002)

The effect of hypoxia and adenosine on the level of a neuroprotective molecular chaperone: heat shock protein 70

Changes in gene expression are associated with switching to a protected phenotype in response to environmental and/or physiological stress. Ubiquitous molecular chaperones from the heat shock protein superfamily confer neuronal protection that can be blocked by anti Hsp70 antibodies (Nakata *et al*., 1993). Activation of the Hsp70 promoter responds to negative cellular energy balance (Kiang and Tsokos, 1998) and oxidative stress (Das *et al*., 1995).

We examined the effect of anoxia and aminophylline treatment of the level of Hsp70, using the methods described above. Figure 7 shows that while the constitutive level of Hsp70 in untreated controls was low, the level of Hsp70 was significantly higher in the brain of anoxia treated epaulette compared to untreated controls (P<0.005) (Renshaw *et al*., 2004). When aminophylline was administered 15 minutes prior to the second episode of anoxia, the level of Hsp70 was significantly higher than in anoxia treated animals (P<0.01) and represented a 7.4 fold increase above the mean constitutive level of Hsp70 in untreated controls (Renshaw *et al*., 2004). This indicates that Hsp70 may also act as an energy sensor and a useful marker of reduced energy charge.

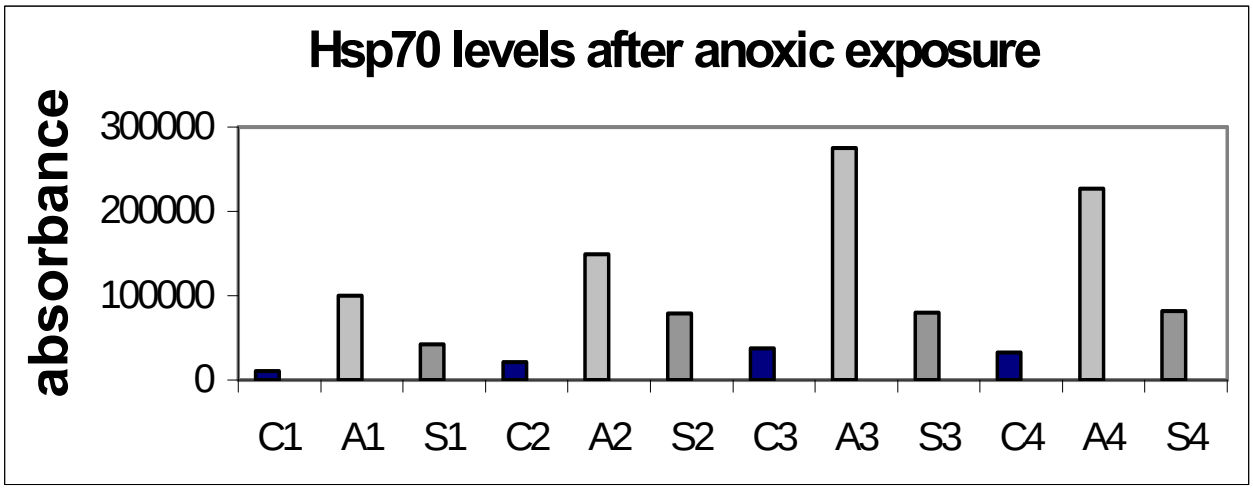

Figure 7. The level of Hsp_{70} in the brain of epaulette sharks after exposure to normoxia (series C) or two episodes of normoxia or anoxia 24 hours apart. Sharks were injected with aminophylline in saline (series A) or saline alone (series S) 15 minutes prior to the second anoxic exposure, Adapted from Renshaw *et al*., 2004).

Conclusions

While this series of studies has demonstrated that elevated adenosine levels can initiate protective responses such as ventilatory depression, bradycardia, increased cerebral blood flow and increased blood flow to the heart, the regulatory role of elevated adenosine levels during hypoxia needs further clarification. The use of selective adenosine antagonists prior to hypoxic exposure is expected to provide a better understanding of the retaliatory and pre-emptive effects of adenosine.

There is compelling evidence that elevated adenosine levels, via acting on its receptor in the brain, resulted in the temporary loss of cerebellar responsiveness because the righting reflex was lost 66% earlier after anoxic-preconditioning. The loss of the righting reflex in response to anoxia appears to have a neuroprotective functional correlate because the administration of aminophylline prolonged the time to loss of the righting reflex with a significant decrease in brain energy charge. Furthermore, the significantly higher levels of neuroprotective Hsp70 in aminophylline treated animals, which had significantly lower brain energy charge than controls, indicate that increased cellular stress occurred when adenosine receptors were blocked. Taken together these results reveal that elevated adenosine associated with anoxic exposure did provide a pre-emptive state of metabolic depression, which served to conserve ATP in this successfully hypoxia and anoxia tolerant species.

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Behavioural, respiratory, ionoregulatory, and N-metabolic adaptations to low environmental O_2, and the influence of body size in the hypoxia-tolerant Amazonian oscar (*Astronotus ocellatus*)

by

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Introduction

The Brazilian Amazon, a "giant piece of amphibian land" (Val and Almeida-Val, 1995), has been an evolutionary testing ground for strategies of hypoxia-tolerance in fish. Due to the dramatic annual cycles in water level, fish entering the flooded jungle ("igapo") and grasslands ("varzea") to feed and reproduce encounter environments that fluctuate greatly in oxygen levels due to photosynthesis of algae and respiration of submerged organic matter (Fink and Fink, 1979; Junk *et al*., 1983; Val and Almeida-Val, 1995). As the water recedes, many become trapped and have to migrate over land back to the main river, activities facilitated by the evolution of a variety of air-breathing organs and locomotory strategies (Graham, 1997). Others survive by metabolic and behavioural adaptations that allow them to cope with severely hypoxic conditions without having to actually enter the aerial environment. These include down-regulation of metabolic rate, anaerobic metabolism, seeking out less hypoxic environments, periodic bouts of aquatic surface respiration (ASR, "skimming") to exploit the more oxygen-rich surface film (Kramer and Mehegan, 1981; Kramer and McClure, 1982; Kramer, 1987), and changes in social and foraging behaviour (Val and Almeida-Val, 1995). These adaptations may be viewed as compromises to maximize fitness at times of adversity, as they may increase susceptibility to predation and/or decrease feeding or reproductive success, while at the same time increasing the chances of surviving oxygen lack so as to be able to grow and reproduce in the future. One such species is the oscar (Acará-açu; *Astronotus ocellatus*; Cichlidae) which is renowned for its hypoxia tolerance; adults are reported to survive up to 6h of complete anoxia, and can tolerate levels of 5-20 % air saturation for 20 –50 h (Muusze *et al*., 1998; Almeida-Val *et al*., 2000; J.G. Richards, pers. comm.). We have

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used this species to investigate a number of different aspects of physiology and behaviour in response to environmental hypoxia.

Throughout these studies, a particular theme has been the influence of body size on hypoxia tolerance. Within a number of teleost species, smaller individuals are more tolerant of hypoxia than larger individuals (Smale and Rabeni, 1995; Burleson *et al*., 2001; Robb and Abrahams, 2003). This observation has given rise to the idea that hypoxia can serve as an ecological refuge where smaller individuals can avoid larger predatory fish which are less tolerant of low oxygen levels (Kolar and Rahel, 1993; Chapman *et al*., 1996; Robb and Abrahams, 2003). However, this may not always be the case, and for the oscar, exactly the opposite may be true. Almeida-Val *et al*. (2000) reported that small oscars (~16 g) survived fairly severe hypoxia (~ 30 torr) for only about 9 h whereas larger individuals (~230 g) survived under identical conditions for approximately 35 h. The greater anaerobic potential of larger fish was indicated by higher concentrations of lactate dehydrogenase and malate dehydrogenase in a variety of tissues (Almeida-Val *et al*., 2000). Thus a scaling effect on hypoxia tolerance has been proposed where larger oscars are better physiologically equipped for coping with hypoxic conditions. We therefore hypothesized that larger individuals would be able to preserve various aspects of physiological homeostasis for a longer period and/or down to a lower PO_2 threshold than smaller individuals. Similarly, we hypothesized that larger oscars would postpone "risky" behaviours such as ASR or leaving shelter to search for better oxygenated waters to a similar degree.

A second theme has been the influence of hypoxia on two key aspects of gill function – ionoregulation and nitrogenous waste excretion. To date, impacts of hypoxia on these processes have received little attention, but there are several reasons for believing that these functions may be particularly sensitive to low environmental oxygen.

Firstly, there is a well-documented respiratory-osmoregulatory compromise at the gills, such that effective gill area and diffusion distance are adjusted to provide the permeability required for gas exchange, while minimizing the permeability for diffusive ion losses and osmotic water gain (Wood and Randall, 1973a,b; Gonzalez and McDonald, 1992). It is probable that lamellar recruitment and gill vasodilation would occur during hypoxia to help sustain oxygen uptake (Holeton and Randall, 1967a,b). We therefore hypothesized that increases in ion efflux rates to the water, and a dilution of plasma ion levels would also occur.

Secondly, ionoregulation is a costly process in fish, with estimates generally falling in the range of 2-20% of resting metabolism at the whole animal level (reviewed by Febry and Lutz, 1987). The very dilute nature of many Amazonian waters ("slightly contaminated distilled water" – Sioli, 1984) may exacerbate these costs, and a general tendency for reduced ion levels in the plasma of Amazonian teleosts has been noted (Mangum *et al*., 1978). At the cellular level, it is generally accepted that the cost of ion-pumping is second only to that of protein synthesis, and both processes are markedly turned down during hypoxia in model species such as the turtle and crucian carp which are capable of severe hypometabolism (reviewed by Hochachka and Lutz, 2001;

Boutilier, 2001). This is accomplished by both channel arrest and down-regulation of Na^+,K^+-ATPase activity. Furthermore, gill ionocytes which are directly exposed to the external water will be on the front line of hypoxia exposure and the very first cells to experience the oxygen deficit. Studies with a water-ventilated and saline-perfused trout gill preparation (Wood *et al*., 1978) demonstrated that gill tissue normally acquires about 50% of its oxygen uptake directly from the external water, and the other 50% from the perfusate, but it must be remembered that this perfusate has itself just been oxygenated in the arterial-arterial pathway of the gill lamellae before perfusing the filaments where the majority of the ionocytes are located (Olson, 2002; Wilson and Laurent, 2002). Therefore, it seems likely that ionocytes will receive little benefit from internal oxygen stores in the bloodstream during hypoxia. In total, the rate of oxygen utilization by perfused and ventilated gill tissue amounts to about 4 -12% of resting oxygen uptake by the whole animal (Wood *et al*., 1978; Lyndon, 1994; Morgan and Iwama, 1999) – i.e. in the same range as estimates of the costs of ionoregulation. Therefore, we hypothesized that active ion influx rates from the water would fall during hypoxia, perhaps in a two-step process, the first in response to oxygen starvation of the working ionocytes, and the second as a result of down-regulation of uptake channels and Na^+,K^+-ATPase activity to save metabolic costs.

Lastly, the gills excrete more than 80% of the metabolic ammonia production in fish and the mechanism is thought to be linked in some way to the active uptake of Na^+ (discussed by Wood, 1993; Wilson, 1996; and Wilkie, 1997, 2002). Original ideas about direct Na^+/NH_4^+ exchange coupling in the gill cells (e.g. Krogh, 1939; Maetz and Garcia-Romeu, 1964; Wright and Wood, 1985), while not entirely disproven, have given way in recent years to the concept that the coupling is indirect (Avella and Bornancin, 1989; Lin and Randall, 1995). Thus an H^+ pump on the apical membrane provides the electrical gradient needed to drive Na^+ uptake from the water through coupled Na^+-channels, and the associated acidification of the gill boundary layer enhances the "diffusion-trapping" of NH_3 as NH_4^+, thus sustaining the PNH_3 gradient for diffusive NH_3 efflux (Wilson *et al*., 1994; Clarke and Potts, 1998). If hypoxia interferes with the Na^+ uptake-H^+ pumping mechanism (or Na^+/NH_4^+ exchange), ammonia excretion may be inhibited. At the same time however, the rate of ammonia production may be reduced by down-regulation of aerobic metabolism (i.e. less deamination of amino acids for fuel; Kutty, 1972; van Waarde, 1983). Therefore we hypothesized that ammonia excretion would fall during hypoxia and become uncoupled from Na^+ uptake, and that changes in plasma ammonia levels would indicate which of the excretion or the production processes were impaired to the greater extent.

The present paper provides an overview of the experiments performed on oscar to test these hypotheses, some of which were confirmed, while others were disproven or modified as outlined below. The studies are reported in detail in Sloman *et al*. (2006) and Wood *et al*. (2007), and all methodological information is given in these two papers. When reference is made to "small" and "large" oscar, the animals were about 20 g and 200 g respectively. Experimental temperature was 28 $\pm$ 1.5ºC , and all tests were performed in very dilute, very soft water taken from a well on the INPA campus in Manaus. The water is typical of the Amazon region, with the following composition: Na^+

= 19, Cl^- = 21, K^+= 16, Ca^{2+} = 11, Mg^{2+} = 2 μmol.L^{-1}, pH = 6.5, dissolved organic carbon = 0.6 mg C L^{-1}.

Respiratory Responses to Hypoxia - the Influence of Body Size

Under normoxic conditions, routine mass-specific MO_2 was more than twice as high in small oscars than large oscars (Fig. 1). However these rates were only about half of those recorded for teleosts in general at comparable size and temperature (Clarke and Johnston, 1999), indicating that low metabolic rate in itself may serve as a general adaptation to frequently hypoxic conditions in this species. When environmental PO_2 was progressively reduced in a closed system, large fish exhibited no change in MO_2 until PO_2 fell to a threshold of approximately 50 torr. Similarly, Muusze *et al*. (1998) working with adult oscars of unspecified size found a threshold of about 30 torr. In contrast, small oscars showed no clear threshold with MO_2 falling progressively with PO_2 right from normoxia, though the first statistically significant decrease in MO_2 occurred at about 70 torr (Fig. 1). By 10 torr, MO_2 had fallen to values of about 20% and 10% of normoxic rates in large and small fish respectively. Thus in accord with our initial hypothesis, larger fish were able to maintain O_2- independent respiration (Hughes, 1981) down to a lower threshold PO_2 than smaller fish, and support it to a higher degree below that point. Additional fish were available to extend the mass range from 9g to 308g, and allowed the derivation of a three-dimensional model, predicting MO_2 (μmol O_2 h^{-1}) as a function of body mass (kg) and environmental PO_2 (torr) at 28^OC:

Log MO_2 = 3.20686 + 0.786*log(mass)-0.88913*0.96371^{PO2} (0.05 – 0.3 kg) (r^2 = 0.948)
Log MO_2 = 3.28076 + 0.786*log(mass)- 0.92331*0.97024^{PO2} (0.01-0.05 kg) (r^2= 0.928)

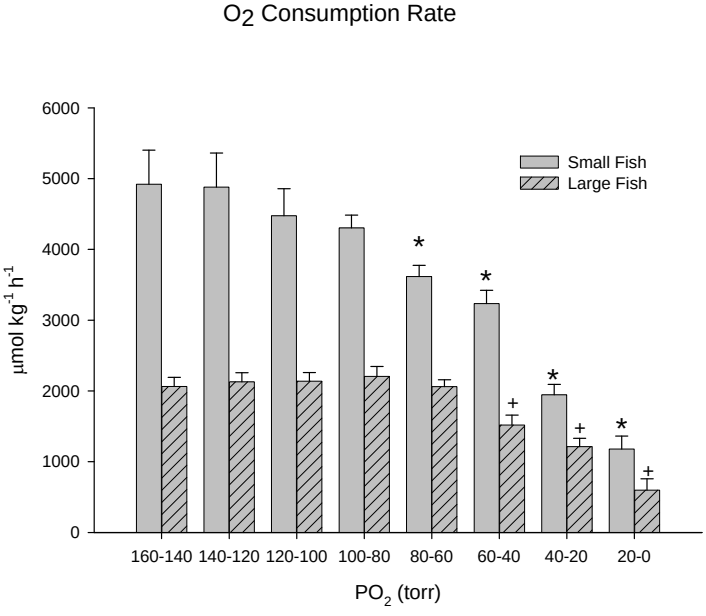

Figure 1. The influence of water PO_2 on MO_2 in large versus small oscars. (Data from Sloman *et al*., 2006).

Thus not only do large oscars have a much greater anaerobic capacity and survival time during severe hypoxia (Almeida-Val *et al*., 2000), they also have a greater ability to maintain MO_2 under more moderate hypoxia, thus avoiding or postponing the need to resort to more costly or dangerous metabolic or behavioural survival strategies. Unlike many other species (e.g. yellow perch, *Perca flavescens*, Robb and Abrahams, 2003; largemouth bass, *Micropterus salmoides*; Burleson *et al*., 2001), *Astronotus ocellatus* shows a positive relationship between physiological tolerance of hypoxia and mass.

Behavioural Responses to Hypoxia – the Influence of Body Size

In light of the above findings, it was initially surprising that when progressive hypoxia trials were run in an apparatus that allowed the fish to voluntarily move to the surface to exploit ASR, individual fish mass was positively correlated, and not negatively correlated with the PO_2 at which this behaviour first occurred ($p=0.04$), in contrast to one of our original hypotheses. Indeed, on average, this behaviour was postponed to a much lower environmental PO_2 (22.3 ± 3.7 torr) in small oscars than large ones (49.6 ± 9.8 torr). Therefore the PO_2 threshold for ASR corresponded almost perfectly with the PO_2 threshold for MO_2 decline in large fish (i.e. 50 torr *versus* 50 torr) , and not at all for small fish, where there was a large discrepancy (70 torr *versus* 22 torr) (Fig. 1).

However a likely explanation for this difference, was provided by a second behavioural experiment run in a long shallow arena tank. An oxygen gradient was set up by bubbling nitrogen into one end and air into the other, as illustrated in Fig. 2A. At the most hypoxic end of the tank (approximately 30 torr), shelter was created by the addition of some floating plants, *Pistia stratiotes* (shaded area). Fish which were naïve to the arena were added individually at the middle of the gradient (at ~65 torr), and observed for 10 minutes. Large oscar spent approximately equal amounts of time under the hypoxic shelter, and exploring the less hypoxic (but exposed) parts of the arena (Fig. 2B). In contrast, small oscars spent essentially the entire period under the hypoxic shelter and did not explore the less hypoxic exposed areas. When the fish were pre-exposed to this same level of hypoxia (30 torr) in a separate tank for 60 min prior to the test, the pattern of exploratory behaviour by the large fish did not change significantly, whereas the small fish abandoned the hypoxic shelter, spending about 80% of their time in the more normoxic but exposed areas (Fig. 2B).

A possible interpretation of these results is that at least down to a PO_2 of 22 torr, ASR has more negative consequences over the short term for fitness of the small fish than remaining in a hypoxic environment because it exposes them to aerial predators (Kramer *et al*., 1983; Randle and Chapman, 2005) and other predatory air-breathing fish (Wolf, 1985). Therefore they choose to remain under the shelter for virtually the entire experimental period and accept the associated physiological cost of exposure to hypoxia. However, when pre-exposed for one hour at 30 torr, their "reserves" of physiological tolerance, which are lower in small oscar because of their lower anaerobic (Almeida-Val *et al*., 2000) and oxygen regulation capacities (Fig. 1), become exhausted so that they are

forced to choose more oxygenated waters at the sacrifice of shelter. Indeed, Shingles *et al*. (2005) recently demonstrated that ASR is a behavioural O_2-chemoreflex that can be modified by the risk of predation in the flathead grey mullet, *Mugil cephalus*. These observations fit with the ideas of Claireaux *et al*. (1995) who studied the behaviour and physiology of cod in fluctuating salinity and oxygen conditions, and concluded that in responding to environmental factors, fish may simply be constrained into choosing the lesser of two evils.

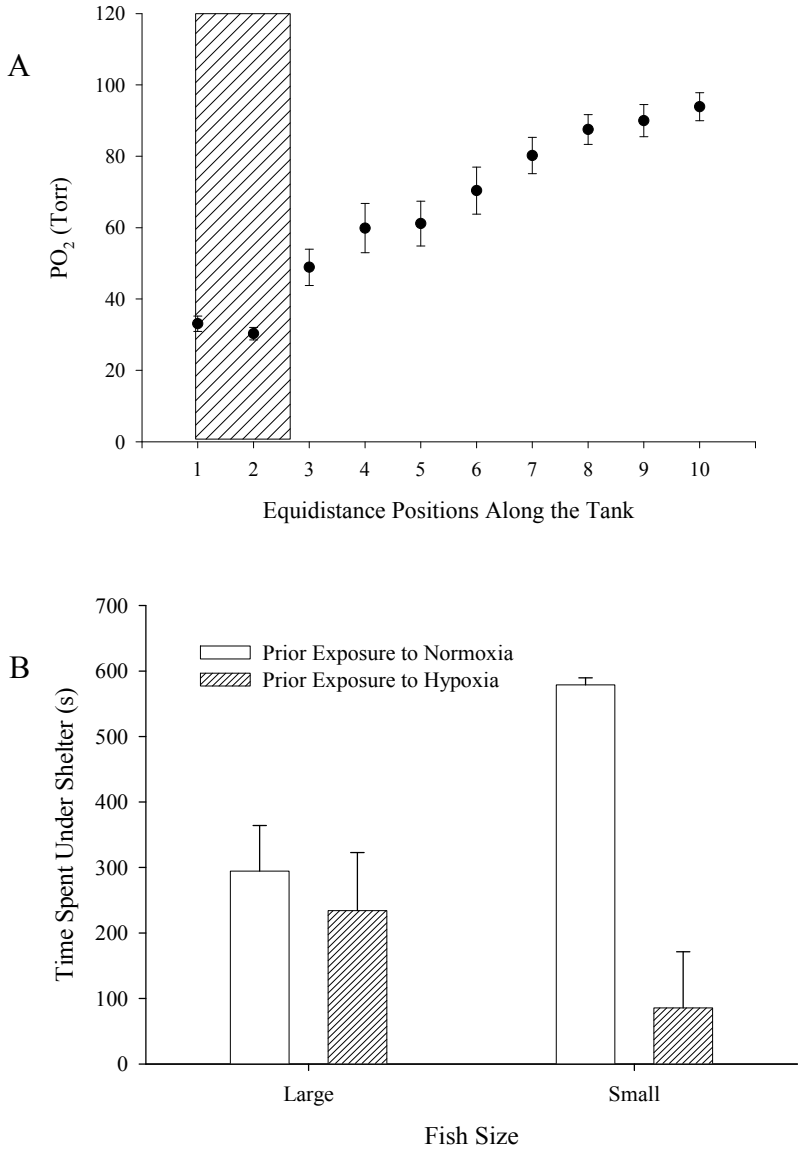

Figure 2. (A) Gradient of PO_2 in the test arena: the cross-hatched area represents shelter by floating plants. (B) Time spent under shelter at the most hypoxic end of the gradient by large versus small fish with 1 hour prior exposure to either normoxia (130 torr, open bars) or hypoxia (40 torr, hatched bars). (Data from Sloman *et al*., 2006).

Another behavioural test examined the effect of progressive hypoxia on spontaneous activity level. Social groups of four fish in a large aquarium were established for a one hour period under normoxia. Behaviour was then examined, firstly under normoxia (136 torr), then at 80 torr, and finally at 40 torr, with the reductions between each level occurring steadily over 60 min periods. Vertical and horizontal movements as

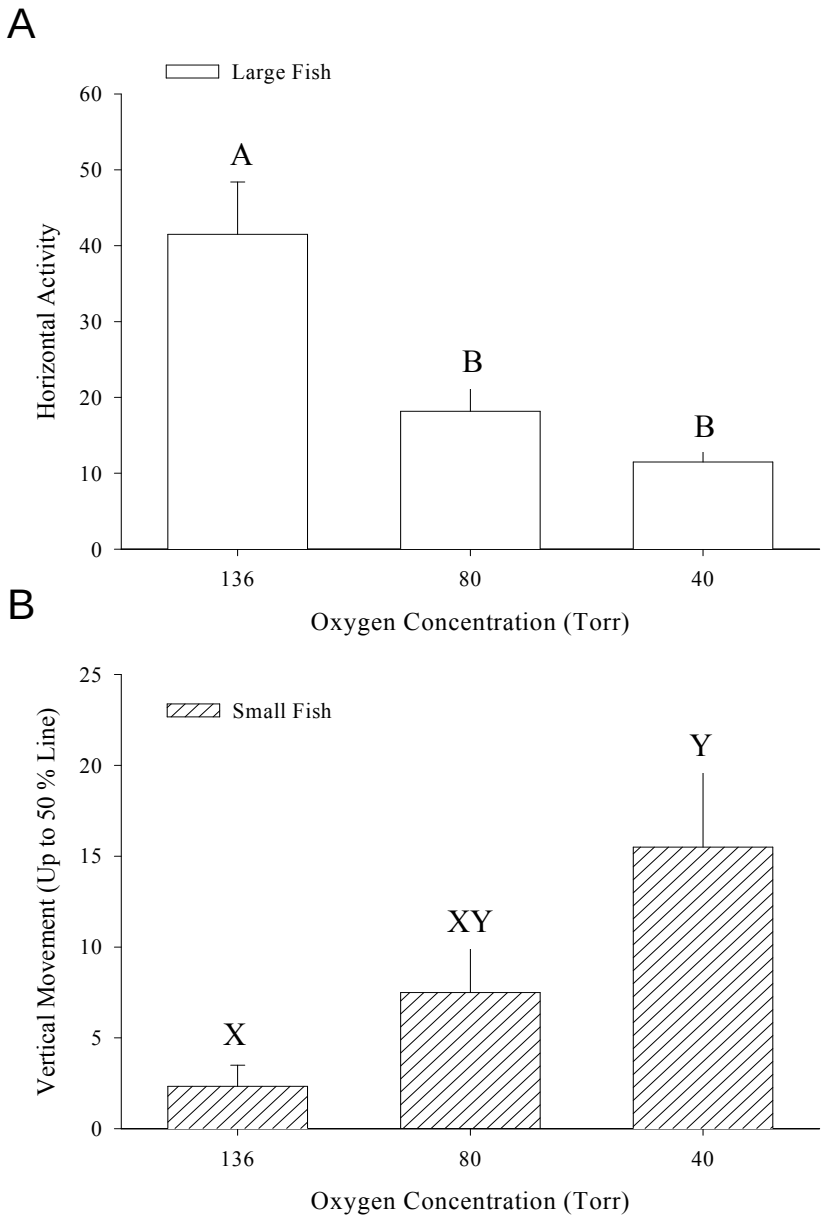

Figure 3. (A) Change in horizontal activity in large oscars during progressive hypoxia. (B) Change in vertical activity in small oscars during progressive hypoxia. (Data from Sloman *et al*., 2006).

well as acts of aggression were scored for 10 min periods at each level. Large oscars were more active than small oscars under normoxic conditions, and formed clear social hierarchies with many acts of aggression, whereas none were observed in the small fish. Aggression was not influenced by hypoxia, but large oscars exhibited a decrease in movement in the horizontal plane with decreasing oxygen tensions (Fig. 3A), whereas small fish showed an increase in activity in the vertical plane during hypoxia (Fig. 3B); activities in the two reciprocal planes were not affected. However the increase in vertical activity in smaller fish occurred only up to the 50 % depth line, not up to the water surface (as might be expected at tensions less than 22 Torr). Thus it seems likely that larger fish reduce their level of activity to aid metabolic suppression (Boutilier and St-Pierre, 2000) but do not sacrifice the behaviour needed to maintain their social status, whereas smaller fish, which are not reproductively mature, do not "waste" metabolic reserves on aggression, but do increase their activity during moderate hypoxia, potentially in the hope of finding areas less devoid of oxygen (Domenici *et al*., 2000). Clearly, the trade-offs between behaviour and physiology in relation to ultimate fitness during hypoxic exposure are both size-related and complex.

Ionoregulatory Responses to Hypoxia - the Influence of Body Size

In these experiments, unidirectional and net fluxes of Na^+ between the water and the fish were measured using standard radio-isotopic (^{22}Na) techniques whereby uptake (influx) is determined by disappearance of the radio-label from the water, net flux by change in total Na^+ in the external water, and efflux by difference (Wood and Randall, 1973a,b; Kirschner, 1970; Wood, 1992). Water-to-fish volume ratios, radio-isotopic specific activity, and measurement periods were optimized as a compromise between measurement sensitivity and the need to avoid errors due to isotopic-recycling ("backflux"). In practice, this meant that the period of total measurement was limited to 7h, so in various experiments, "snap-shots" of different periods during hypoxic exposure and recovery were taken. Our initial intention was to expose both small fish and large fish to the same severe hypoxia (~ 10 torr) for 3 h, but in preliminary experiments it was found that some small oscars succumbed at this level, so a less severe hypoxia (~20 torr) was used for the latter.

In contrast to rates of oxygen uptake (Fig. 1) and ammonia excretion (Fig. 4C,F), mass-specific unidirectional Na^+ flux rates were of similar magnitude in small (Fig. 4B) and large fish (Fig. 4E), though net balance tended to be more negative in the former. The fact that mass-specific ion-turnover rates are not greater in the expected fashion (e.g. Bianchini *et al*., 2002) may in itself represent a cost-saving adaptive strategy for these small fish in ion-poor water. The fact that these rates are low in both large and small fish relative to many other temperate and tropical species appears to be typical of Amazonian cichlids, and reflects a low gill permeability which is adaptive to ion-poor and acidic waters (reviewed by Gonzalez *et al*., 2005), as well as to periodic hypoxia. In both large and small oscars, Na^+ influx rates declined substantially during hypoxia (Fig. 4B,E), in accord with our original hypothesis that this expensive process would be limited by oxygen availability.

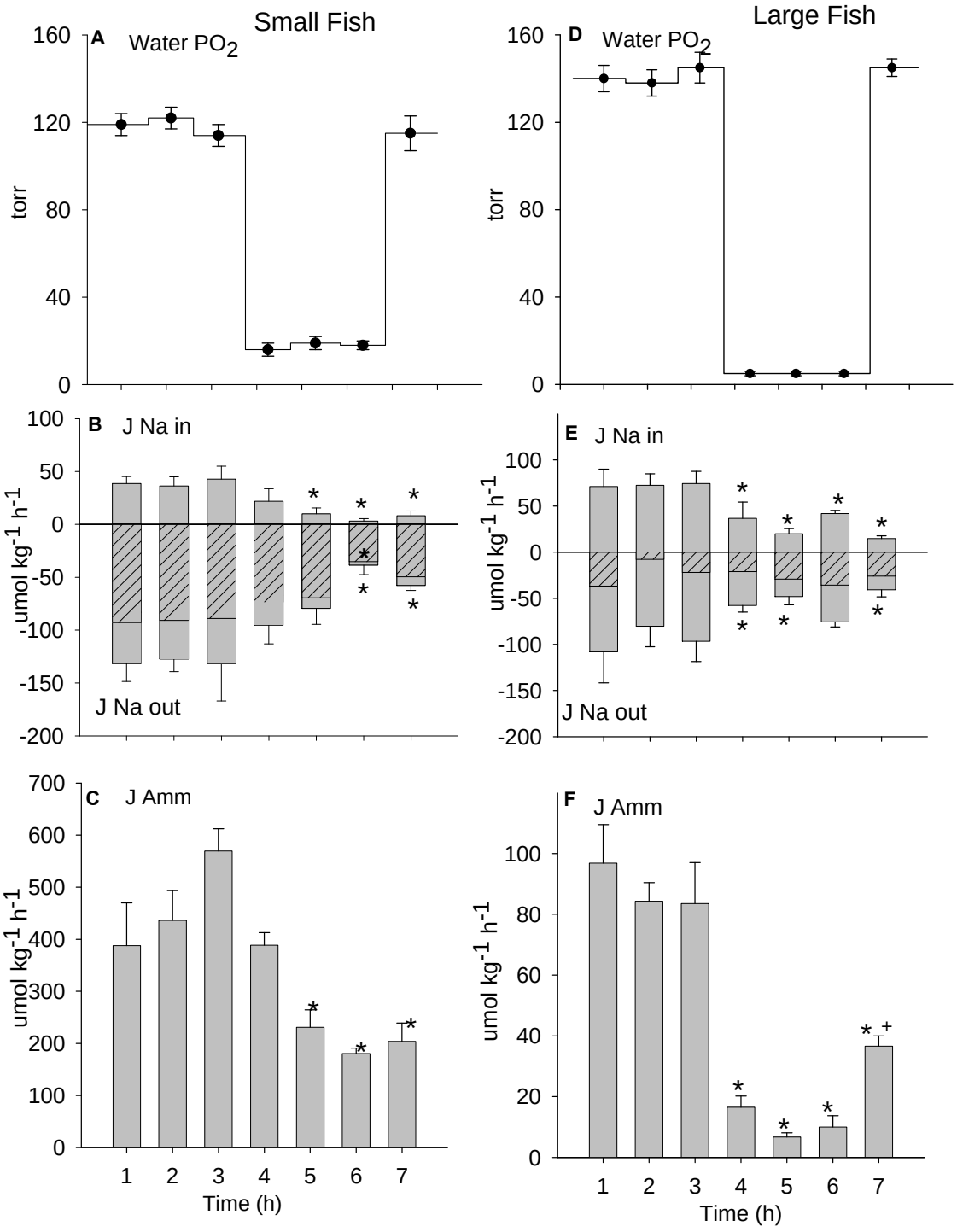

Figure 4. The responses of small oscars (left panels) and large oscars (right panels) to a step induction of severe hypoxia for 3 hours followed by a step restoration of normoxia. (A,D) Water O_2 tension; (B, E) unidirectional influx (upward bars), efflux (downward bars), and net flux (hatched bars) of Na^+ across the gills; and (C, F) net excretion rate of ammonia. (Data from Wood *et al*., 2007).

However, rather than increasing during hypoxia, unidirectional Na^+ efflux rates also declined in both large and small fish, so that net balance remained unaltered (Fig. 4B,E). Furthermore in a separate experimental series with large fish, rather than the predicted decrease in plasma ions, a slight but significant increase in plasma Na^+ and Cl^- concentrations was seen during hypoxia (Fig. 5B), perhaps attributable to hemoconcentration due to a water shift into lactate-producing tissues as occurs after exhaustive exercise (e.g. Wang *et al*., 1994). These observations on Na^+ efflux rates and on plasma electrolytes directly contrast with our hypothesis based on the respiratory-osmoregulatory compromise, and suggest that other factors come into play.

The reductions in Na^+ influx and efflux rates during hypoxia became significant more rapidly in large fish (first hour versus second or third hour; Fig. 4B,E), but in view of the different hypoxia exposure levels and data variability, it is difficult to say whether this is a true size-specific difference. To pursue this further, more gradual, graded hypoxia exposure trials were performed. In large fish, reductions in both Na^+ influx and efflux rates first became significant at a threshold PO_2 of about 40 torr, while in small fish the PO_2 threshold for significant reduction was about 20 torr, though a non-significant reduction was seen at 40 torr (data not shown). Thus it appears that large oscars are at least slightly better able to implement this cost-saving response earlier, at a higher PO_2, without compromising ionic homeostasis (i.e. no change in net Na^+ balance), another indicator of their better hypoxia tolerance relative to small oscars.

Three possible explanations come to mind for the observed decreases in Na^+ efflux rates during hypoxia (Fig. 4B,E). The first is that exchange diffusion is turned down during hypoxia, so that the reduced Na^+ efflux is directly coupled to the reduced Na^+ influx. In the exchange diffusion phenomenon, the same transport mechanism may perform both "futile" Na^+/Na^+ self-exchange and vectorial transport (*e.g.* Na^+/H^+ exchange). Exchange diffusion has been seen during normoxia in many freshwater teleosts and crustaceans (*e.g.* Shaw, 1959; Wood and Randall, 1973b), including about half of the Amazonian teleosts surveyed by Gonzalez *et al.* (2002), but to our knowledge, has never been studied during hypoxia. However, as pointed out by Potts (1994), the phenomenon can be equally well explained by an exchange protein or a selective channel mechanism linked to an electrogenic pump, such as the H^+-pump, Na^+-channel model of active Na^+ uptake discussed earlier (Avella and Bornancin, 1989; Lin and Randall, 1995). This brings us to the second possibility, that channel arrest, a well-documented phenomenon at the cellular level in hypoxia-tolerant species (Hochachka and Lutz, 2001; Boutilier, 2001), could also occur at the gills, thereby reducing both Na^+ influx and Na^+ efflux. Surprisingly, this has not been previously investigated in gill tissues during hypoxia exposure to our knowledge, but will be discussed in greater detail subsequently. The third possibility is that a hypoxia-tolerant species such as the oscar actually reduce gill area and permeability during hypoxia by reducing lamellar perfusion so as to reduce ionoregulatory costs in a situation where the potential for oxygen uptake from the water has become very slight.

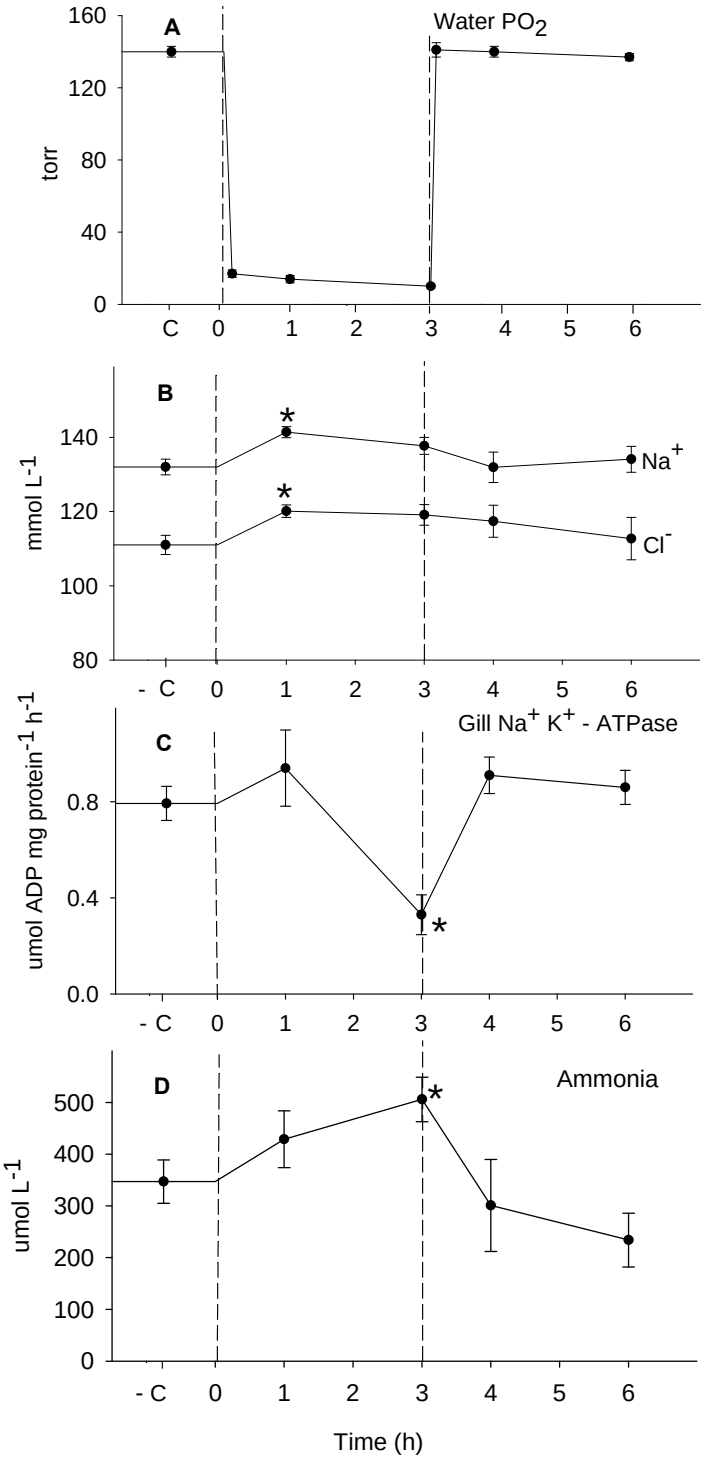

Figure 5. Changes in (B) plasma Na^+ and Cl^- concentrations; (C) gill Na^+, K^+-ATPase activity; and (D) plasma total ammonia concentration in large oscars subjected to 3 hours of severe hypoxia followed by 3 hours of normoxic recovery as illustrated in (A). (Data from Wood *et al*., 2007).

It seems unlikely that this final idea is the complete explanation, because both Na^+ influx and efflux rates remained depressed during the first hour of return to normoxia (Fig. 4B,E), despite the fact that blood measurements demonstrated almost complete clearance of the blood lactate load during this first hour (data not shown). To allay concerns that this was a not a measurement artifact in the final hour of the "flux window", an additional 7h experiment was performed on large fish, focussing on the recovery period as its mid-point, and confirmed that restoration of Na^+ influx and efflux rates was delayed for one hour following the re-establishment of hypoxia (data not shown).

Several other lines of evidence point to at least a partial temporal disconnection between the oxygen regime and the simultaneously measured ion fluxes, suggesting that two (or more) mechanisms may be causing the reduction in Na^+ turnover during hypoxia, in accord with one of our initial hypotheses. The first is that in the large fish experiment of Fig. 5, branchial Na^+,K^+-ATPase activity was fully maintained at the end of 1 h of hypoxia (Fig. 5C), despite the fact that Na^+ influx and efflux rates had already declined (Fig. 4 E). Na^+,K^+-ATPase activity did fall greatly (by about 60%) by 3h of hypoxia, but had fully recovered by 1h of normoxia re-establishment (Fig. 5C), yet unidirectional flux rates remained depressed during this first hour of recovery (Fig. 4E). Secondly, in a separate experiment on large fish, unidirectional Na^+ flux rates were measured during hours 1 and 2 of hypoxia, and again at hour 8 of continued hypoxia. Both influx and efflux values were further reduced at this latter time, again pointing to the involvement of more than one mechanism (data not shown). Thirdly, the dependence of Na^+influx rate on external Na^+concentration ("kinetics"; Wood and Goss, 1990; Potts, 1994) was measured during normoxia and again during prolonged hypoxia (2h to 10h at ~ 10 torr) in the same large fish (Fig. 6A). The maximum Na^+ influx rate (Jmax) was significantly depressed by about 60 % during hypoxia as might be expected from the observed 60% reduction in branchial Na^+,K^+-ATPase activity (Fig. 5C). There was no significant change in affinity (Km; Fig. 6A). Notably, these Km values are high relative to many Amazonian teleosts surveyed by Gonzalez *et al.* (2002), and this low affinity (i.e. high Km's) is in accord with the low permeability, low Na^+ turnover, and high Km values recorded in other cichlids collected in this region (reviewed by Gonzalez *et al*., 2005).

In summary, the data are consistent with down-regulation of Na^+ influx (and efflux) rates by at least two mechanisms during hypoxia. One is clearly by a delayed reduction in Na^+,K^+-ATPase activity. Notably, this appears to occur by post-translational modification of enzyme activity, because specific Na^+,K^+-ATPase mRNA and protein abundance did not fall in a comparable experiment on *Astronotus ocellatus* in which branchial Na^+,K^+-ATPase activity was depressed by about 50% after 4h of hypoxia (J.G. Richards, pers. comm.). There is also clearly another mechanism (or mechanisms) which is/are more rapid and more persistent, but additional work will be required to determine the relative contributions of oxygen starvation, channel closing, changes in lamellar perfusion, and alterations in the activity of other proteins (e.g. H^+-ATPase, Na^+/H^+ and Na^+/Na^+ exchangers) in the observed responses. Regardless, the bottom line is that *Astronotus ocellatus* can withstand severe hypoxia without a marked disturbance of internal ion status, by simultaneously reducing Na^+ pumping and leak rates at the gills.

As with other adaptations, this ability appears to be at least slightly better developed in large fish.

Nitrogen Metabolism Responses to Hypoxia - the Influence of Body Size

Similar to most other teleosts, *Astronotus ocellatus* is strongly ammoniotelic; urea-N excretion rates are less than 10% of ammonia-N excretion rates. As with MO_2 (Fig. 1), mass-specific ammonia excretion rates (J_{Amm}) were much higher in small fish than in large fish (Fig. 4C, F). In general, the relative difference was even greater than in MO_2, suggesting that small oscars rely on protein oxidation to a greater extent (van den Thillart and Kesbeke, 1978; Lauff and Wood, 1996). Ammonia excretion rates (J_{Amm}) were also much higher on an absolute basis than Na^+ influx rates, especially in small fish (Fig. 4B,C,E,F), so any coupling of ammonia excretion to Na^+ uptake, if it occurs, must be indirect. Nevertheless, during normoxia, there was clear evidence of some sort of coupling, because J_{Amm} exhibited a Michaelis-Menten type dependency on external Na^+ concentration in both large oscars (Fig. 6B) and small oscars (data not shown). Interestingly however, the apparent Km was significantly lower (i.e. affinity for Na^+ was higher) than for the simultaneously determined Michaelis-Menten dependence of Na^+ influx on external Na^+ concentration, whereas the Jmax values were similar (Fig. 6A,B).

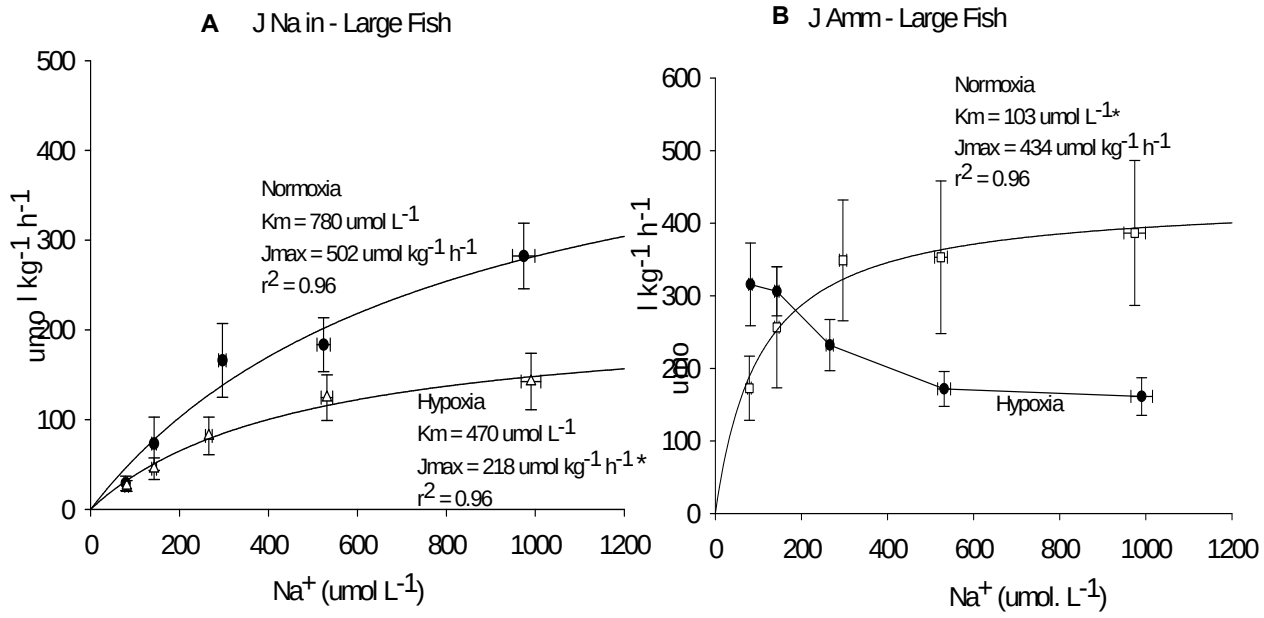

Figure 6. (A) Michaelis-Menton kinetics of Na^+ influx as a function of external Na^+ concentration in large oscars during normoxia or severe hypoxia. Note the decrease in Jmax during hypoxia. (B) Michaelis-Menton kinetics of ammonia excretion as a function of external Na^+ concentration in large oscars during normoxia. Note the lack of a Michaelis-Menton relationship during severe hypoxia. Note also the difference in Km between the Na^+ influx relationship (A) and the ammonia excretion relationship (B) in normoxia. (Data from Wood *et al*., 2007).

During hypoxia, J_{Amm} was reduced in a similar fashion to Na^+ influx and efflux rates in both small fish (Fig. 4B,C) and large fish (Fig. 4E,F). Again the reduction occurred more quickly in large oscars. Notably, as with Na^+ influx, recovery did not occur during the first hour of restoration of normoxia (Fig. 4C,F). Furthermore, in the prolonged hypoxia experiment with large fish, the reduction in J_{Amm} at hour 8 was significantly greater than during hours 1 and 2 (data not shown). Overall, these responses were very similar to those seen in unidirectional Na^+ fluxes, arguing for some sort of common mechanism. However, the apparent kinetic coupling of J_{Amm} to external Na^+ concentration was completely lost during prolonged hypoxia (Fig. 6B). While all these observations were in accord with our original hypotheses, it remains to be determined whether the declines in J_{Amm} were driven primarily by a down-regulation of the ammonia production rate, or by specific hypoxia-induced blockade of a branchial ammonia excretion mechanism, such as "diffusion-trapping" linked to an H^+-pump/Na^+-channel system or Na^+/H^+ exchange (see Introduction).

For several reasons, it appears likely that both phenomena were involved, and that the latter predominated. Urea-N excretion, although it represented only a small fraction of N-waste excretion, was reduced whenever J_{Amm} was reduced during hypoxic exposures (Fig. 7). Urea arises from different metabolic pathways than ammonia in teleost fish (uricolysis or arginolysis rather than trans-deamination or adenylate breakdown; see Wood, 1993, Wilkie, 2002 for reviews). This suggests that a general reduction in metabolic N-waste production occurs during hypoxia, in accord with a general suppression of metabolic rate. However the slope of the regression relating the relative reduction in urea-N excretion to that in ammonia-N excretion was only 0.57, and the intercept was significantly greater than zero, indicating that ammonia-N excretion was more strongly depressed at any given level (Fig. 7). Secondly, if the reduction in J_{Amm} were simply a consequence of reduced production, it seems unlikely that it should persist during the first hour of normoxia restoration when aerobic metabolism was likely restored (Fig. 4C,F). And most cogently, plasma total ammonia concentration increased significantly by 3h of hypoxic exposure in the blood sampling experiment (Fig. 5D), suggesting that the excretion mechanism was inhibited to a greater extent than the production mechanism. This conclusion is in accord with early observations that ammonia-N production is reduced to a lesser extent than aerobic metabolic rate when teleosts are exposed to hypoxia (Kutty, 1972; van den Thillart and Kesbeke, 1978; van Waarde, 1983). Notably however, the change in plasma ammonia was not large, so again the oscar appears to very good at maintaining internal homeostasis during severe hypoxia.

Concluding Remarks

In general, research on hypoxia in fish has emphasized the respiratory and metabolic responses, with less concern for how behaviour and other physiological systems may be impacted. By focusing on an extremely hypoxia-tolerant Amazonian species (*Astronotus ocellatus*), we have found important responses in behaviour, in

ionoregulation, and in N-waste excretion which help to preserve homeostasis and thereby ensure survival during severe environmental hypoxia. Many of these adaptations are

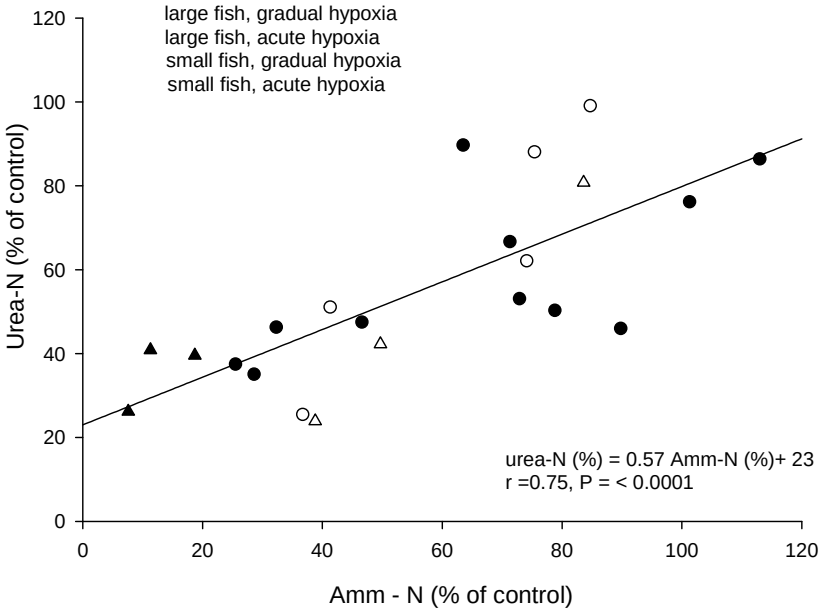

Figure 7. The relationship between the mean relative urea-N excretion rate (as a percentage of the normoxic control value) and the mean relative ammonia-N excretion rate in individual periods of various hypoxic exposure experiments with large and small oscars. (Data from Wood *et al*., 2007).

size-dependent. In future, it will be of interest to examine these same areas during more moderate hypoxia exposure in hypoxia-intolerant model species, such as salmonids.

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Effect of winter hypoxia on fish in small lakes with different water quality

by

A. Tuvikene[1], L. Tuvikene and M. Viik

Introduction

The most common cause of natural fish kills is lack of oxygen in surface water. A lack of oxygen can asphyxiate most fish species. Oxygen deficiency may occur in shallow lakes with thick ice and snow cover. Light is shut off by ice and snow, and the plants, including planktonic algae, consume more oxygen than they produce. Due to temperature stratification in the water under ice cover, and the most intensive degradation of organic matter at the bottom water layer, the concentration of dissolved oxygen starts to decrease first at near-bottom water layers. Usually in such lakes, temperate zone oxygen deficiency extends to the whole water column by the second half of winter – February or March. Massive fish deaths occur most often in shallow eutrophic lakes where a high content of organic matter results in extensive oxygen depletion. For example, in shallow eutrophic Lake Võrtsjärv, the rate of mean oxygen decrease under the ice cover can reach 100 mg/m^2/day in March (Tuvikene *et al.,* 2002). Substances released into the water after massive fish deaths affect local water quality, and can temporarily intensify eutrophication (Kasumyan and Tuvikene, 2004).

Low oxygen concentrations are known to modify metabolism, growth rate and feeding efficiency of fish (Pichavant *et al.,* 2000). At low concentrations, oxygen can act as a limiting factor for growth and, in some conditions, oxygen may be a more important limiting factor than food (Kramer, 1987).

During the severe winter of 2002/2003 fish populations in many Estonian lakes suffered from hypoxia, and massive fish kills happened. During 2003-2005 we performed a fish survey with multi-size gillnets on several small lakes with different water qualities (conductivity, nutrients) to assess fish numbers and relative biomass (CPUE) as well as species composition and condition factor (CF). The aim of the survey was to estimate the natural recovery of fish populations in different lakes within 3 years after the massive fish kill.

Material and methods

During 2003-2005 we performed a fish survey with multi-size gillnets to assess fish numbers and relative biomass (CPUE, catch per unit effort), species composition and

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condition factors (CF, Fulton's coefficient of condition, CF = total weight · standard length^3/100). The lakes studied were: Prossa (surface area 33 ha, average depth 2.2 m), Järvi Pikkjärv (5 ha, 1.8 m), Pillejärv (7 ha, 3.6 m), Kahala (346 ha, 1.0 m), Endla (180 ha, 2.4 m), Lahepera (100 ha, 2.4 m) and Eistvere (24 ha, 1.5 m). The lakes differ from each other mainly by the mineralization level (Fig. 1). Fish were caught with multisize monitoring gillnets (14 different sections with mesh size between 6.25 and 75 mm). Calibration studies with repeated fish seining and fish marking, and following recapturing showed that 1000 g CPUE corresponds roughly 100 kg fish/ha.

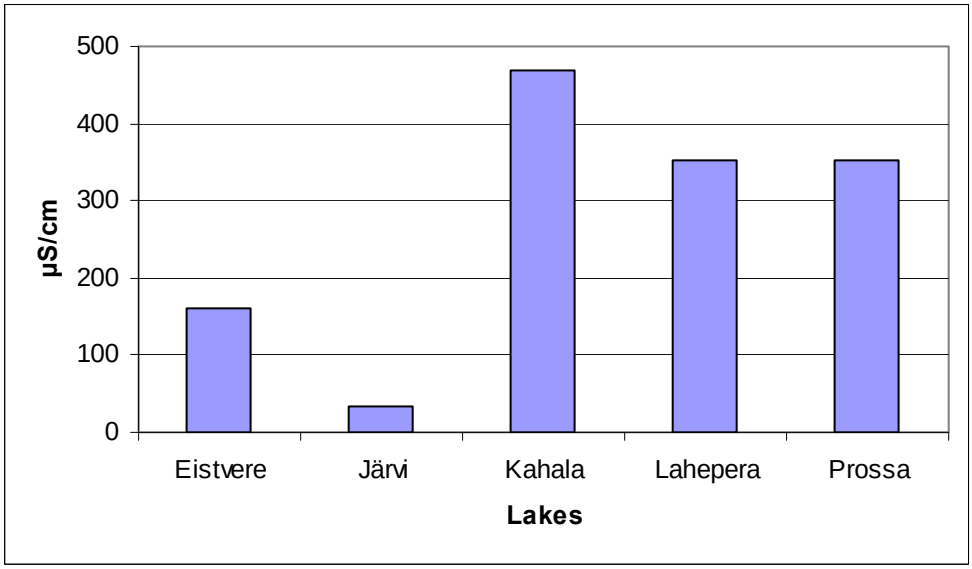

Figure 1. Water conductivity in lakes during winter 2005.

To assess the dependence of fish hypoxia tolerance on low water mineralization, we investigated, in the laboratory, the surface respiratory behavior of the roach (*Rutilus rutilus*) adapted during 24 hours in Lake Järvi Pikkjärv water (low mineral content) and Lake Kahala water (high mineral content). In the aquarium experiments, we registered the oxygen content in water when fish switched to continuous surface respiration. We also investigated the emission of nutrients into the water from dead fish (killed by asphyxia) during the first 24 h. Emissions of nutrients from dead fish were measured at 25 ^{o}C and 4 ^{o}C, representing summer and winter condition, respectively.

Results and discussion

Oxygen conditions in studied lakes

Winter hypoxia occurs most often in small and shallow lakes, as seen in Lake Prossa and Lake Eistvere (Fig. 2).

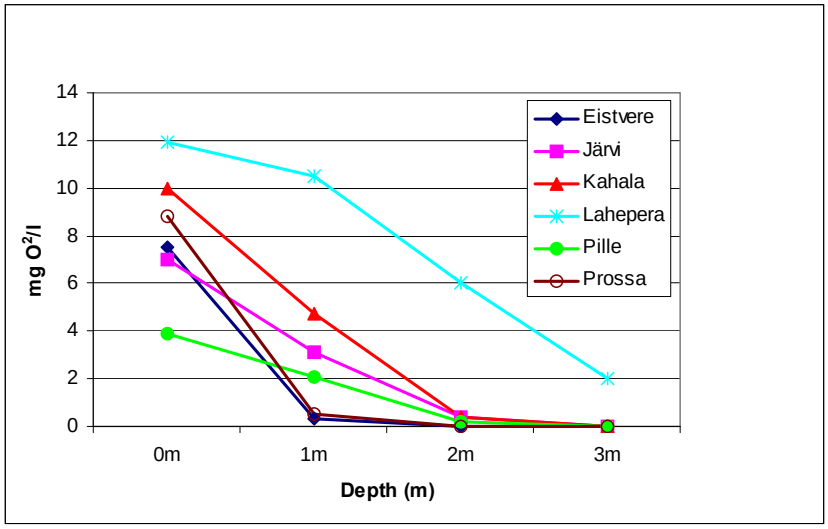

Figure 2. Content of dissolved oxygen under the ice in Estonian small lakes in March 2004.

Biomass of fish in studied lakes

The highest fish biomasses were recorded in the larger lakes Endla and Lahepera (Fig. 3) where the biggest changes of biomass also occurred. The lowest CPUE was recorded in the soft water lake Järvi Pikkjärv. The prehypoxia levels of biomass were achieved in some lakes within three years of recovery. The very soft water lake Järvi Pikkjärv (conductivity 30 µS/cm), suffered the most severe fish population decline with deaths of hypoxia tolerant crucian carp (*Carassius carassius*) and gibel carp (*C. gibelio*) recorded (Fig. 3). Perhaps this is due to a low ion content of the water resulting in a greater osmoregulatory energy cost. The number of fish species in the lakes varied between 3 and 9, being lower in the lakes suffering most often from hypoxia (3 in Lake Prossa) and low mineralization (3 in Järvi Pikkjärv). In larger lakes the number of fish species was 9 in Lahepera, 8 in Endla, and 7 in Kahala.

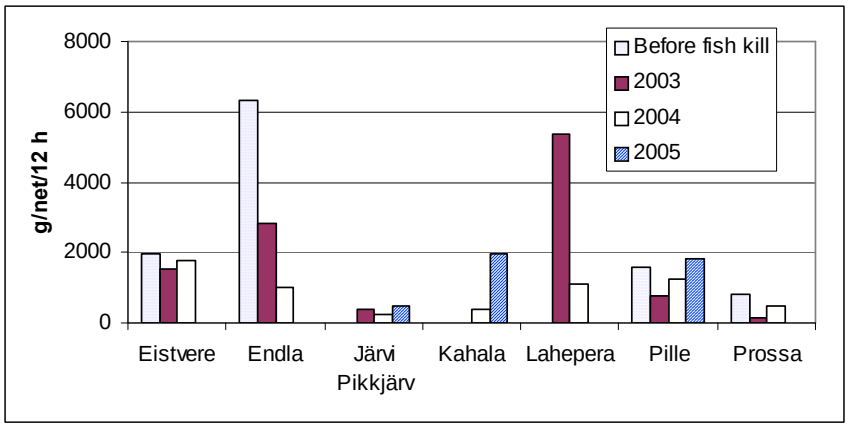

Figure 3. Relative biomass of fish (CPUE) in studied lakes in different years

Condition factor of fish

The condition factor (CF) of fish is a frequently used index in fish studies. It provides important information related to fish physiological state, based on the principle that individuals of a given length, exhibiting higher weight, are in better condition. CF provides a relatively simple and rapid indication of how well a fish copes with its environment. A decline in CF may reflect a change in feeding pattern, which could be a behavioral response to low oxygen, or an increase in metabolic rate in response to stress caused by hypoxia. Hypoxia has been shown to reduce food intake, thus resulting in slower growth (Randall and Yang, 2004) and a smaller CF.

The CF of many fish species is lower in lakes which are often hypoxic when compared to the same species in lakes where hypoxia is rare (Table I). Condition factors were also low in the soft water lake Järvi Pikkjärv. This could be due to elevated osmotic stress, resulting in less energy for growth. It was also found that the number of fish species was reduced in lakes which were often hypoxic (Table I).

Table I. Condition factors of fish.

Lake	**Eistvere**	**Endla**	**Järvi**	**Kahala**	**Lahepera**	**Pille**	**Prossa**
Gibel carp, *Carassius auratus*	3.2		2.5	3.0	3.2	3.7	
Crucian carp, *Carassius carassius*			2.8	3.2			
Roach, *Rutilus rutilus*	1.9	1.9		2.0	1.7	1.8	1.6
Perch, *Perca fluviatilis*		1.6	1.6	2.1	1.9	1.9	1.7
Pike, *Esox lucius*	0.9			1.0	0.9	0.9	

Hypoxia tolerance in water with low mineralization

All freshwater fishes devote a significant portion of their basal metabolic rate to maintain their internal salts and other dissolved substances at concentrations different than those in their environment. Salts are very efficiently resorbed from the urine of freshwater teleosts, e.g., 99.9% of the Na^+ and Cl^- ions (Bone *et al.,* 1995). Freshwater teleosts also have a high-affinity salt-uptake mechanism at the gills. The efficiency of this

mechanism becomes obvious from the low rate of salt loss in freshwater teleosts in comparison with marine teleosts, and by the accumulation of ions from very dilute solution, e.g., $Na^+ > 10^{-4}$ M or 2.3 mg/l (Bone *et al.,* 1995).

The energy costs of osmotic and ionic regulation mean that less energy can be allocated to growth (Matey, 1996). In water with low mineralization, fishes experience osmotic stress. Large water uptake results in high rates of dilute urine excretion, and ion losses must be compensated for by energetically costly ion pumping (Sollid *et al.,* 2003).

When acclimated to soft water, fish experience a proliferation of the ion-transporting chloride cells of the branchial epithelium, which contributes to the maintenance of ionic homeostasis by enhancing branchial ion uptake (Matey, 1996).

In spring 2003 many crucian carps and gibel carps died in lake Järvi Pikkjärv. These fish species are known to be highly tolerant to hypoxia. In addition to hypoxia, the reason for their death in lake Järvi Pikkjärv could be the osmotic stress resulting from a very low ion content in the lake water (Table II). Lake Järvi Pikkjärv is a forest lake and there is no evidence of any direct anthropogenic influence on this lake. An alkalinity of at least 20 mg/l is good for the well-being of fish (Wedemeyer and Yasutake, 1977).

Table II. Water parameters in soft water lake Järvi Pikkjärv and in hard water lake Kahala.

	Unit	**Järvi Pikkjärv**	**Kahala**
Na^+	mg/l	2.1	8.4
K^+	mg/l	0.73	3
Ca^{2+}	mg/l	5	70
Alkalinity	mg/l	15	156
Conductivity	µS/cm	29-53	470

The use of the aquatic surface respiration is one of the few alternatives to aerial respiration, which allow fish to survive in conditions of extreme hypoxia. Surface respiration behavioral studies were conducted with roach *Rutilus rutilus* exposed to the soft water of lake Järvi Pikkjärv and the hard water of lake Kahala water. Fish adapted in the water of lake Järvi Pikkjärv showed higher thresholds for aquatic surface respiration, than fish adapted in water of lake Kahala (Fig. 4). This was most likely due to a higher osmotic stress in water with low mineralization.

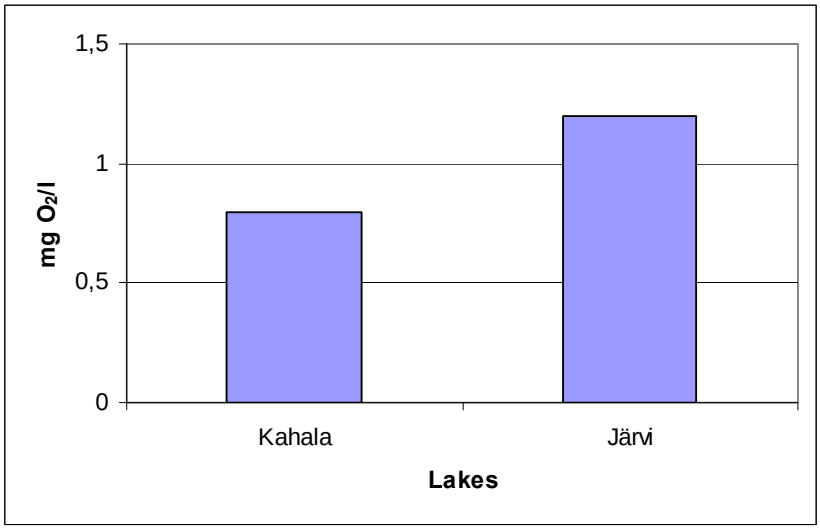

Figure 4. Oxygen content at 25 ˚C when roach switched to surface respiration (statistically significant difference, $P<0.05$, $N=3$).

Nutrients from dead fish

Dead fish are a source of chemical substances that appear in the surrounding water shortly after fish deaths (Kasumyan and Tuvikene, 2004). Dead fish may cause a rapid increase in the content of dissolved organic matter in the surrounding water (Table III). In turn, this can result in a dissolved oxygen deficit that aggravates other unfavorable conditions, and can lead to massive fish mortality. Nutrients from dead fish accelerate eutrophication of a water body.

Table III. Average emission of nutrients (µg/g/h) from dead fish during first 24 hours (see text for more details).

	4˚C	**25˚C**	**Difference (folds)**
Total nitrogen	6.1	116.2	20
Nitrate	0	0	0
Nitrite	0.05	0.75	15
Ammonia	2.0	40.2	20
Total phosphorus	1.0	27.0	27
Reactive phosphorus	0.5	21.9	44

Average fish biomass in Estonian lakes is around 200 kg/ha (10 g/m^3 if the lake is 2 m deep). If all the fish in a lake die, then of all of the nutrients released from dead fish at 25 ˚C (Table III), only total and reactive phosphorus are important from the point of view of eutrophication. They can raise the phosphorus content in water (Table IV) by 30%. The nitrate, nitrite, and ammonia is negligible (2% increase in ammonia, other nitrogen compounds less). In winter conditions, at 4˚C, the quantity of released nutrients from dead fish is negligible (all nutrients less than 1%).

Table IV. Chemical parameters of the upper water layer measured in spring 2006.

	Järvi Pikkjärv	Kahala
N-tot (mg/L)	1.28	1.47
NH_4-N (mg/L)	0.68	0.48
NO_2-N (mg/L)	<0.01	0.01
NO_3-N (mg/L)	0.10	0.01
P-tot (mg/L)	0.03	0.04
PO_4-P (mg/L)	0.005	0.015
BOD_5 (mg O/L)	1.5	1.7

Summary

During the severe winter of 2002/2003 in Estonia, fish populations in many lakes suffered from hypoxia/anoxia, and massive fish kills occurred. In several lakes the fish populations recovered within the following three years. In a very soft water lake even hypoxia tolerant crucian carp and gibel carp died from hypoxia in conjunction with elevated osmotic stress due to very low ionic content of the lake water. In the summer, only the amounts of total and reactive phosphorus released from dead fish are significant from the point of view of eutrophication while the quantity of nitrogen ions is negligible. Under the ice cover, the quantity of released nutrients from dead fish is negligible. Hypoxia or/and low mineralization of lake water result in unfavorable conditions for fish.

Acknowledgements

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Interdemic variation in gill morphology of a eurytopic African cichlid

by

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Introduction

The physical environment has a major influence on the ecology of organisms. For fishes, the availability of dissolved oxygen (DO) is one abiotic factor that can exert a strong selective force by affecting habitat quality, growth, survival, and reproduction. Oxygen scarcity (hypoxia) occurs naturally in systems characterized by low levels of ambient light and mixing. For example, heavily vegetated swamps, flooded forests, and the hypolimnion of deep lakes are habitats particularly prone to oxygen limitation. Low DO can be acute in (but not limited to) tropical waters where high temperatures elevate rates of organic decomposition and reduce oxygen tensions in the water. Unfortunately, environmental degradation is increasing the occurrence of hypoxia as the influx of municipal wastes and fertilizer runoff accelerates eutrophication and pollution of water bodies (Prepas and Charette, 2003). Increasing hypoxia is a threat to fresh waters and coastal waters worldwide; and oxygen depletion in deeper waters, one side effect of this process, can lead to fish kills and a massive reshaping of fish communities (Prepas and Charette, 2003). It has therefore become increasingly important to understand the effects of hypoxia on aquatic organisms.

Despite much interest in the physiological and biochemical adaptations of fishes to hypoxic stress, the significance of dissolved oxygen in driving divergence among populations remains largely unexplored. Strong selection for hypoxia tolerance may lead to variation among populations that experience divergent aquatic oxygen environments. This may lead to further diversification if the benefits accrued by higher respiratory performance in hypoxic habitats lead to sub-optimal performance in normoxic waters. Our studies of East African fishes have demonstrated that alternative dissolved oxygen (DO) environments provide a strong predictor of interpopulational (interdemic) variation, particularly with respect to respiratory traits (e.g., gill size) and associated characters. In a series of studies comparing populations from low- and high-oxygen environments, we found total gill size (surface area and/or total gill filament length) to be larger in swamp-dwelling populations of the cyprinid *Barbus neumayeri* (Chapman *et al.*, 1999; Schaack and Chapman, 2003), the mormyrids *Gnathonemus victoriae* and *Petrocephalus catostoma* (Chapman and Hulen, 2001), the cichlid

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Pseudocrenilabrus multicolor victoriae (Chapman *et al*., 2000; Chapman *et al*., 2002), and the air-breathing African catfish *Clarias liocephalus* (McCue, 2001) relative to open-water populations. This interdemic variation in gill traits could be purely genetically based or due to environmental influences on gene expression (i.e. phenotypic plasticity; DeWitt and Scheiner, 2004). If there is phenotypic plasticity, it may be determined either at a critical period of ontogeny, or be a phenotypic response that remains labile throughout an individual's lifetime.

Recent studies support an element of environmentally-induced plasticity in gill morphology in response to DO regime. In a study of the African cichlid *P. multicolor*, we compared gill size of a population from a stable hypoxic habitat with one of a stable well-oxygenated habitat (Chapman *et al*., 2000). In addition, we compared siblings (split-brood) raised under hypoxic or well-oxygenated conditions. The response to hypoxia was an increase in gill area, both in the field (29%) and in the plasticity experiment (18%). The difference in the magnitude of the response between field and experimental fishes may reflect differences in selection pressures between populations, and/or a combination of inherited changes and plasticity (Chapman *et al*., 2000). For the sea bass (*Dicentrarchus labrax*) Saroglia *et al*. (2002) reported higher gill surface area associated with lower oxygen partial pressure of the water in which the bass were reared for 3 months, providing further evidence of phenotypic plasticity in fish gills in response to DO availability. The maintenance of plasticity in gill morphology of divergent populations may preserve the possibility for future evolutionary responses. This could be particularly relevant in aquatic systems of the Lake Victoria basin of East Africa that is characterized by a volatile history, peppered by volcanic explosions and changing lake levels (Beadle, 1981; Stager *et al*., 1986; Johnson *et al*., 1996).

The species flock of endemic haplochromine cichlids in Lake Victoria represents one of the most rapid, extensive, and recent radiations of vertebrates known (Kaufman 1992; Kaufman *et al*., 1997; Seehausen *et al*., 2002). By contrast, a small number of eurytopic cichlid species inhabit a broad range of habitats (rivers, streams, lakes, and swamps) throughout the Lake Victoria watershed and adjacent areas. Mechanisms facilitating the eurytopic distribution of these species remain largely unknown; however, strong patterns of morphological variation across populations suggest locally adapted phenotypes (Smits *et al*., 1996; Chapman *et al*., 2000). In this study, we quantified interdemic variation in the gill morphology of the widespread African cichlid *Astatoreochromis alluaudi*. We compared gill metrics of a population from a hypoxic lake habitat with one of a well-oxygenated hyper-eutrophic lake (Chapman *et al*., 2000). In addition, we compared siblings (split-brood) reared under hypoxic or well-oxygenated conditions for one population.

Study Site and Species

Astatoreochromis alluaudi is a widespread haplochromine cichlid that can be found in a range of habitats in the Lake Victoria basin of East Africa including fast flowing rivers, lakes, wetlands, and streams (Greenwood, 1959; Chapman *et al*., 1996a,b). It is a species well known for a high degree of plasticity in the pharyngeal jaw apparatus and associated muscles in response to the nature of its prey base. When introduced to a hard prey diet (e.g., mollusks) *A. alluaudi* develops a large pharyngeal mill with hypertrophied muscles; while a softer diet is associated with a smaller jaw and trophic muscles (Greenwood, 1965a; Huysseune *et al*., 1994). Smits *et al*. (1996) reported that *A. alluaudi* feeding on snails showed a 31% increase in

head volume compared to fish that fed on insects. Given the widespread distribution of this species and the extraordinary plasticity of its pharyngeal jaw apparatus, we anticipated a similarly high level of variation in its gill apparatus in response to variation in aquatic oxygen. We selected two field populations of *A. alluaudi* from Uganda. Both are lake populations but one (Lake Nabugabo) experiences very low oxygen conditions in dense wetland bays and the other (Lake Saka) is found in areas of high oxygen.

Lake Nabugabo, a satellite of Lake Victoria (24 km^2, approximately $0^o45'$ S and $31^o45'$ E), is characterized by an extensive stretch of shoreline macrophytes (mainly *Miscanthidium violaceum* and *Vossia cuspidata*), interrupted by stretches of forests (dominated by *Ficus* spp.) and sand beaches. Small bays surround the east side of the lake and provide an ideal environment for the development of the bladder-wort *Utricularia.* The lake lies within an extensive wetland that was formerly a bay on the western shore of Lake Victoria (Fig. 1, Greenwood, 1965b). Long shore bars that isolate Lake Nabugabo from Lake Victoria were created during water-level fluctuations about 4,000 years ago (Greenwood, 1965b). Today, water from Lake Nabugabo drains southeastward via the Lwamunda swamp before it seeps through the sand bar into Lake Victoria. *Astatoreochromis alluaudi* was first reported from Lake Nabugabo by Greenwood (1965b) based on a survey conducted by a Cambridge expedition. However, only one specimen was collected at that time with no records of habitat use. In recent studies, *A. alluaudi* has been reported in the wetland ecotones of the lake and the papyrus-choked Juma River, the main tributary to the lake (Chapman *et al*., 1996a,b; Rosenberger and Chapman, 1999; Schofield and Chapman, 1999). In a quantitative survey of habitat use of fishes in Lake Nabugabo conducted in 2000 (see Chapman *et al*., 2003 for details), we found that *A. alluaudi* were restricted in their distribution to wetland ecotones, primarily in small bays characterized by dense growth of *Ceratophyllum* and bordered by emergent wetland grasses. In the 2000 survey, the DO level where *A. alluaudi* were captured averaged 3.5 mg l^{-1} (upper 50 cm of the water column) with an average water temperature of 24.4 ^oC. This current distribution pattern of this species may reflect the introduction of the predatory Nile perch in the early 1960's. Many haplochromine cichlids were largely restricted to wetland ecotones, which serve as refuges from Nile perch predation (Chapman *et al*., 1996a,b, 2002, 2003).

Lake Saka is found at the northernmost extreme of the crater lakes in western Uganda (0^o 40'N and 30^o 15'E, elevation of 1,520 m) and has a maximum length of 1.4 km and maximum width of 1.0 km. A small crater forms an embayment at the southeast corner of the lake (Melack, 1978). Several wetlands drain into Lake Saka, and the drainage basin has been almost totally cleared for agricultural production by subsistence farmers and a large Ugandan government prison farm (Crisman *et al*., 2001). Unlike Lake Nabugabo, this is a highly eutrophic lake characterized by supersaturated DO conditions during the peak light of the day. Crisman *et al*. (2001) reported DO in surface waters often exceeding 15 mg l^{-1} (180% saturation) during 1995-1998. In a quantitative survey of the fish fauna of Lake Saka conducted in 2000, we found that *A. alluaudi* was most abundant in wetland ecotones with average surface DO concentration of 10.4 mg l^{-1}. Nile perch were also stocked into Lake Saka in the early 1970s, but currently persist at low numbers.

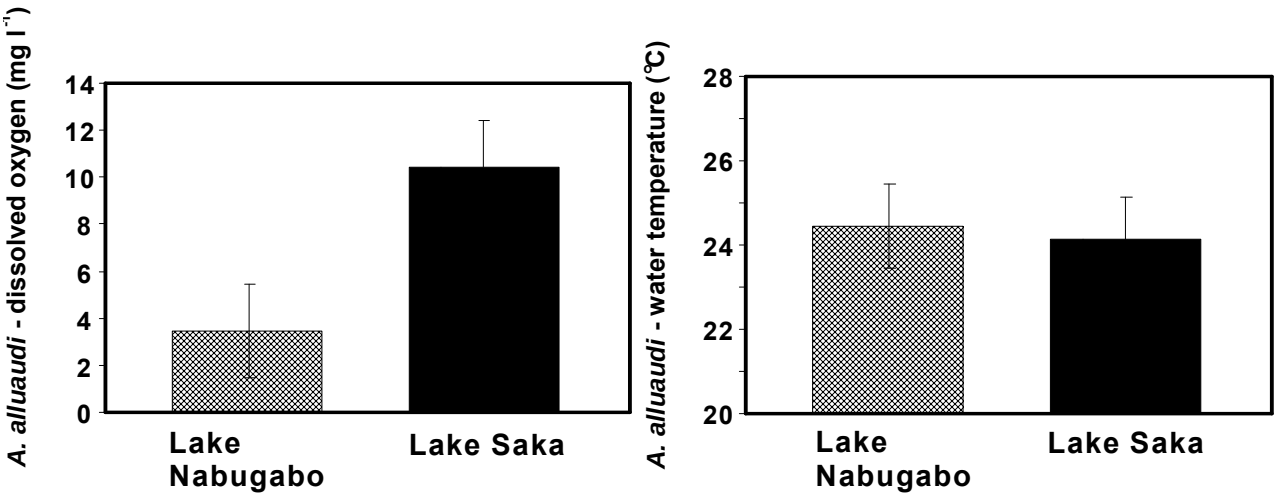


Figure 1. Mean levels of dissolved oxygen concentration (mg l^{-1}) and water temperature (^{o}C) measured in the upper 50 cm of the water column at sites where *Astatoreochromis alluaudi* were captured in a survey (2000) of Lake Nabugabo and Lake Saka, Uganda. (Unpublished data from Chapman and colleagues.)

Methods

Field Collections

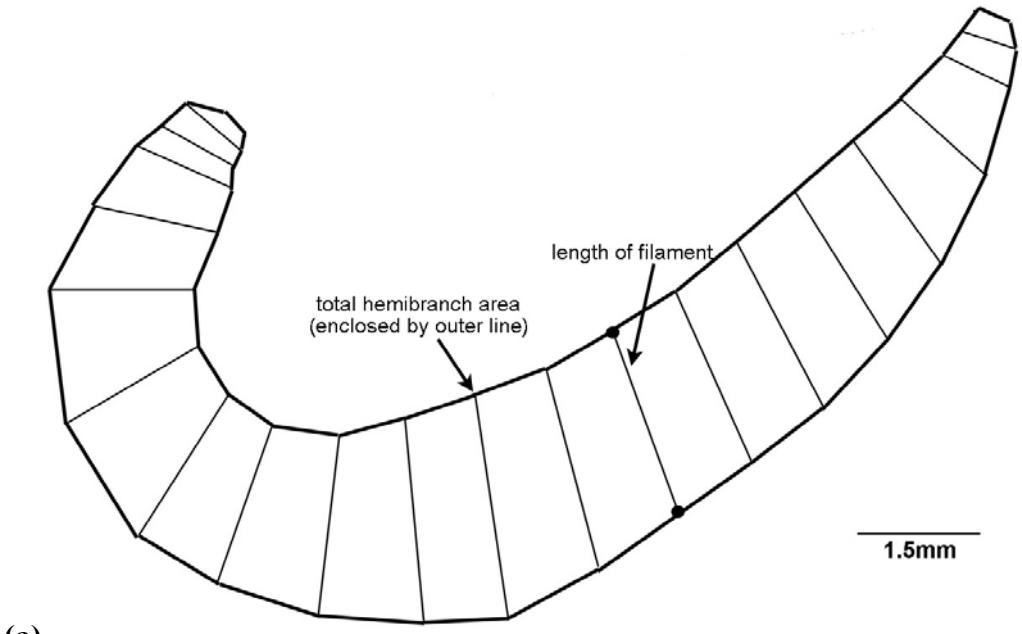
In both lakes (Nabugabo and Saka), *A. alluaudi* were captured using metal minnow traps. Fish were euthanized with an overdose of MS222 in the field and preserved in buffered paraformaldehyde (40 g l^{-1}).

Rearing Experiment

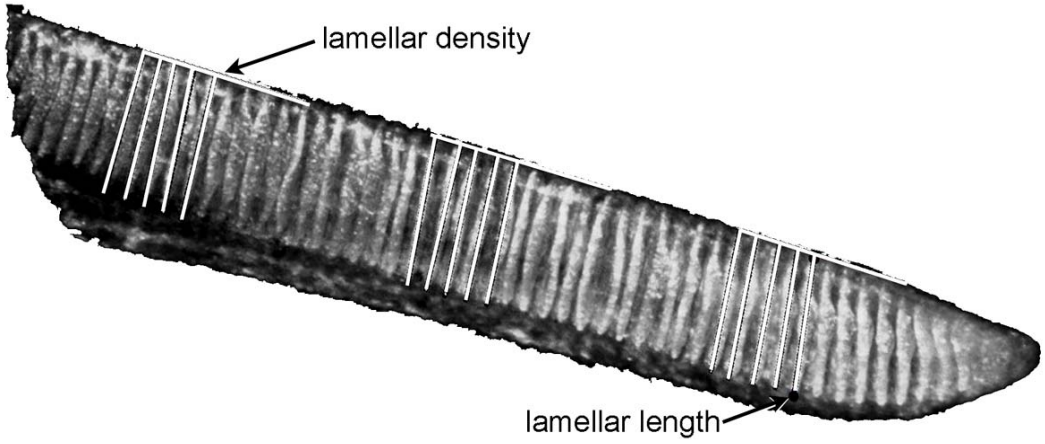
As part of a larger lab-rearing experiment to explore direct and indirect tradeoffs between trophic structures and the respiratory apparatus of *A. alluaudi*, F1 offspring from Lake Nabugabo were raised under low (≈1.3 mg l^{-1}) and high (≈7.4 mg l^{-1}) DO. Water temperature averaged 24.5 ^{o}C. We used a split brood design with the F1 offspring of three sets of parents to provide family level replication; but the number of families was limited due to complexity of key target traits (gill surface area). Brooding pairs were held in separate, normoxic aquaria until young were released from the female's mouth. When a brood was released from a female, each brood was divided into two groups of 10 individuals and groups randomly allocated to one of 6 aquaria (20-l) between two treatments. After 2 months, each tank was cropped to 6 individuals, by randomly removing and euthanizing offspring. *Astaoreochromis alluaudi* in Lake Nabugabo are not molluscivorous, as snails are extremely rare in this system (Beadle, 1981; Efitre *et al*., 2001). In the rearing experiment reported in this paper, fish from Lake Nabugabo were fed tetramin food flakes once per day. Fish were raised for approximately 1 year and then euthanized (MS222) and preserved in buffered paraformaldehyde.

Gill morphometry

Gill metrics were estimated for 10 specimens from each field population. We measured the following gill characters: total gill filament length (TGFL), average lamellar density (ALD), average lamellar area (ALA), total hemibranch area (THA), and total gill surface area (TGSA). For the rearing experiment, we measured three specimens from each family, and we included only measures for TGFL and THA. TGSA and lamellar characters on lab-reared fish will be the focus of future studies. Total gill filament length was measured using standard methods modified after Muir and Hughes (1969) and Hughes (1984a). For each fish, the branchial basket was removed, and the four gill arches from the left side of the basket were separated. For each hemibranch of the gill arches, the length of every 5^{th} gill filament was measured (Fig. 2a). Two successive measurements along a hemibranch were averaged and multiplied by the number of filaments in the section between the two filaments. Filament lengths were summed for the four hemibranchs and multiplied by 2 to produce an estimate of total gill filament length (TGFL). Lamellar density was measured in the dorsal, middle, and ventral parts of every 10^{th} filament of the second gill arch on the left side (Fig. 2b). The total number of lamellae (on one side of the filament) and average lamellar density (ALD) were estimated using a weighted mean method that takes into account the difference in length of different filaments (Muir and Hughes, 1969; Hughes and Morgan, 1973). For every 10^{th} filament starting at filament 5, the length and height of 5 secondary lamellae was measured at the top, middle, and bottom sections of the filament (Fig. 2b). Average values of these characters for each filament were converted to estimates of lamellar area using a regression determined through the dissection of several lamellae from various sections of the second gill arch from two or more specimens from each population. The sum of the total lamellar area for all sections of the second arch was divided by the total number of lamellae and multiplied by 2 to produce a weighted average bilateral surface area on one side of the filament (ALA). Total gill surface area (TGSA) was determined using the formula: TGSA = TGFL x 2 x ALD x ALA. To estimate hemibranch area, we digitized the area of the gill filaments on the 8 hemibranchs on 1 side of the fish. This was multiplied by 2 to produce an estimate of the total hemibranch area (THA, not including the bony arches, Fig. 2a). For all characters, images were captured with a Leica stereoscope and Infinity I camera, and linear and areal dimensions measured with Motic Images software version 2.0.



(a)



(b)

Figure 2. Illustration of measurements used for analyses of gill size and shape of *Astatoreochromis alluaudi.* (a) The length of filaments was used to estimate total gill filament length (TGFL); the total hemibranch area (THA) was estimated from the outer line. (b) Average lamellar density (ALD) was estimated by measuring the length encompassed by 10 filaments at the base, middle, and upper part of selected filaments. Lamellar length (shown here) and height (not shown here) were measured for 5 lamellae at the base, middle, and upper part of selected filaments and used in the calculation of average lamellar area (ALA).

Palzenberger and Pohla (1992) reviewed the literature on gill morphometry of fishes. From their data set for 28 non-air-breathing freshwater species (with multiple populations for eight species), they extracted the mean slope of significant regressions for gill morphometric parameters and body weight. They set the lowest and highest mean values within each parameter range to 0% and 100% respectively to create a range of values for each gill character. This permitted them to express the values of a species as a percentage within the range of values for freshwater fishes. We used their parameter estimates for total gill surface area and total gill filament length to estimate these characters for each field population expressed as a percentage of freshwater fishes.

Results

Field populations

Gill characters were measured on 10 fish from each field population selected to maximize range in body mass (Lake Nabugabo: mean body mass = 5.20 g, range = 1.427 g to 11.277 g; Lake Saka: mean = 10.14 g, range= 2.486 to 15.06 g). For the two populations, total gill filament length, average lamellar area, hemibranch area, and total gill surface area were positively correlated with body size (Table I). Average lamellar density was negatively related to body size in both groups (Table I). ANCOVA indicated no difference in the slopes of the bilogarithmic relationships between *A. alluaudi* of the Lake Nabugabo and Lake Saka populations for total gill filament length, total hemibranch area, lamellar density, or lamellar area, though there was trend toward heterogeneity in slopes for the latter (Table I). Intercepts differed for all four of these characters between field populations (Table I). When adjusted for body mass total gill filament length, hemibranch area, and lamellar area were greater in fish from the hypoxic waters of the Lake Nabugabo wetland, than in fish from the well-oxygenated waters of Lake Saka (Table I, Fig. 3a,b). Interestingly, gill lamellar density was lower in *A. alluaudi* from Lake Nabugabo (Table I, Fig. 3c). The slopes of the bilogarithmic relationship between total gill surface area and body mass differed between the two populations, with gill surface area increasing more slowly with body size in the Lake Saka population. Given heterogeneity in the slopes, we did not test for a difference in the intercepts; however, for a fish of the average size of the two populations, and using the independent regressions for each population (5.8 g), total gill surface area was estimated at 35.87 cm^2 for *A. alluaudi* from Lake Nabugabo and 15.65 cm^2 for *A. alluaudi* from Lake Saka (Fig. 3d).

Data on *A. alluaudi* were converted into a percentage of the estimated range of all species following Palzenberger and Pohla (1992) for total gill filament length and total gill surface area. Total gill filament length, expressed as a percentage of freshwater fishes averaged 67% for Lake Nabugabo, and 56% for Lake Saka. For total gill surface area, *A. alluaudi* from Lake Saka averaged only 20% of the range for freshwater fishes, while those from Nabugabo averaged 54% of the range.

Table I. Summary of linear regression analyses and analyses of covariance (ANCOVA of relationships between gill morphometric characters and body mass (g) for *Astaoreochromis alluaudi* from Lake Nabugabo (low-oxygen site) and Lake Saka (high-oxygen site). Both gill characters and body mass were $\log_{10}$ transformed. The mean values represent antilogged adjusted means calculated from the ANCOVA analyses (sample means adjusted for a common mean body mass of 6 g and a common regression line). If slopes were heterogeneous, then we did not test for a difference in intercepts (Int.). *For total gill surface area, the means represented the predicted value from the population-specific regression lines, since slopes were heterogeneous.

Character	Site	n	Slope	Int.	r	p	ANCOVA F	Slope p	ANCOVA F	Int. p	Adj. means
Total gill filament length (mm)	Nabugabo	10	0.641	2.012	0.978	<0.001	2.008	0.176	146.665	<0.001	2552.70
	Saka	10	0.471	2.891	0.979	<0.001					1782.38
Hemibranch Area (mm^2)	Nabugabo	10	0.671	2.184	0.995	<0.001	0.342	0.567	150.676	<0.001	504.66
	Saka	10	0.526	3.002	0.994	<0.001					321.36
Lamellar Density (no. per mm)	Nabugabo	10	-0.17	1.589	0.807	0.008	0.195	0.665	29.269	<0.001	28.71
	Saka	9	-0.14	1.677	0.786	0.007					36.90
Lamellar area (mm^2)	Nabugabo	10	0.641	-2.467	0.893	0.001	4.429	0.053	23.83	<0.001	0.0099
	Saka	9	0.339	-2.488	0.869	0.001					0.0055
Total gill Surface area (cm^2)	Nabugabo	10	0.723	1.003	0.97	<0.001	8.669	0.01	*****	*****	30.76*
	Saka	9	0.529	0.791	0.971	<0.001					15.49*

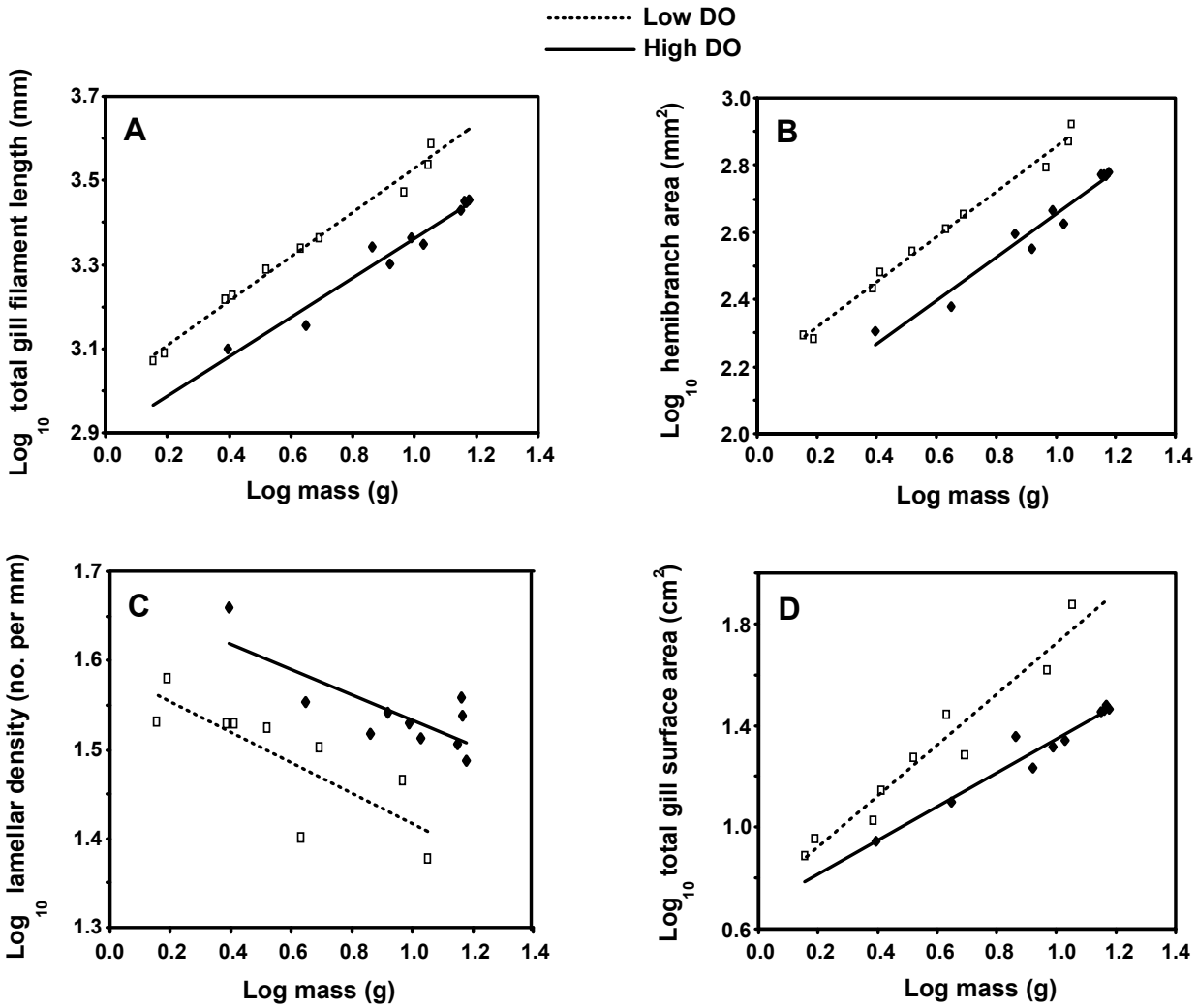


Figure 3. Bilogarithmic plots of gill metrics and body mass for *Astatoreochromis alluaudi* from two field populations: Lake Nabugabo (low oxygen), Lake Saka (high oxygen). (a) total gill filament length (mm), (b) total hemibranch area (mm^2), (c) average lamellar density (number of lamellae per mm), and (d) total gill surface area (cm^2).

Lab-rearing study

For our lab-rearing study, full siblings whose parents originated from Lake Nabugabo were raised under low- and high-DO. Total gill filament length and total hemibranch area were measured for 3 fish per family per treatment selected to maximize range in body size (normoxia: mean body weight=5.4 g, range=1.7 to 10.4 g; hypoxia: mean=4.8 g, range=3.4 to 9.2 g). For both the normoxia- and hypoxia-raised fish, total gill filament number and hemibranch area were positively related to body mass (Table II). Analyses of covariance indicated no difference in the slopes of the bilogarithmic relationships between the hypoxia and normoxia groups for total gill filament length and hemibranch area (Table II); however, the intercepts differed between groups. For a fish of a given body mass, total gill filament length

and hemibranch area were greater in fish grown under extreme hypoxia than in fish grown under normoxia (Table II, Fig. 4a,b).

Table II. Summary of linear regression analyses and analyses of covariance (ANCOVA) of relationships between gill morphometric characters and body mass (g) for F1 offspring of *Astaoreochromis alluaudi* from Lake Nabugabo. Both gill characters and body mass were $\log_{10}$ transformed. The mean values represent antilogged adjusted means calculated from the ANCOVA analyses (sample means adjusted for a common mean body mass of 6 g and a common regression line).

Character	DO	n	Slope	Intercept	r	p	ANCOVA F	Slope p	ANCOVA F	Intercept p	Adj. means
Total gill	Hypoxia	9	0.422	3.094	0.905	<0.001	0.537	0.476	122.776	<0.001	2371.4
Filament	Normoxia	9	0.48	2.92	0.985	<0.001					1733.8
length (mm)											
Hemibranch	Hypoxia	9	0.528	2.302	0.902	<0.001	2.904	0.11	156.36	<0.001	449.78
area (mm^2)	Normoxia	9	0.677	2.021	0.994	<0.001					295.80

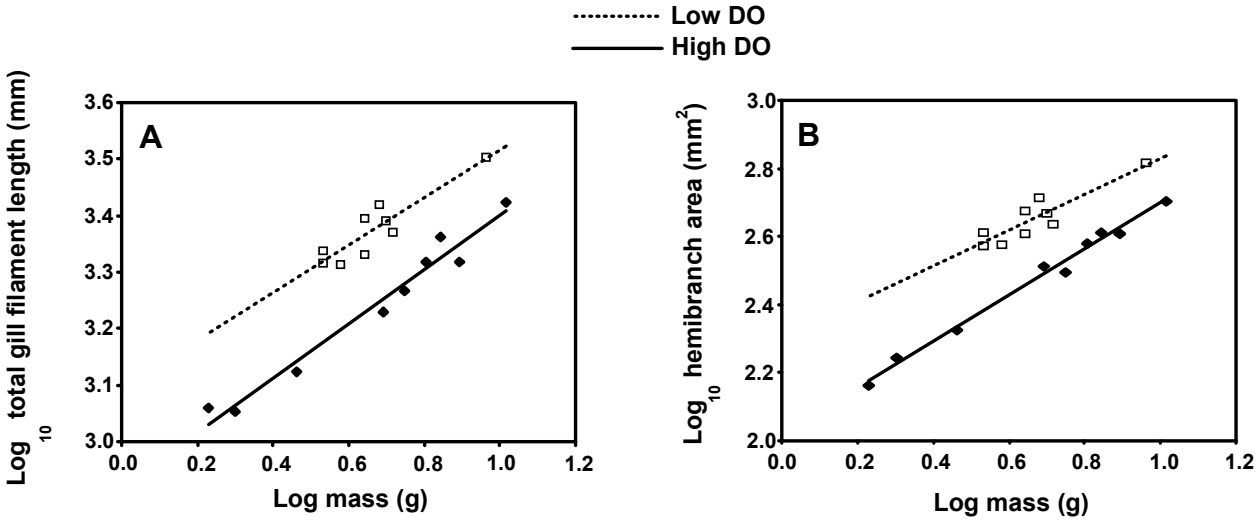


Figure 4. Bilogarithmic plots of gill metrics and body mass for F1 offspring *Astatoreochromis alluaudi* from Lake Nabugabo raised under low- and high-oxygen conditions. (a) total gill filament length (mm) and (b) total hemibranch area (mm^2).

Discussion

Aquatic Oxygen as a Predictor of Morphological Divergence

Several studies based on interspecific comparisons of non-air-breathing fishes have suggested that large gill respiratory surface may reflect hypoxic conditions in their environment (Gibbs and Hurwitz, 1967; Galis and Barel; 1980, Fernandes *et al*., 1994; Mazon *et al*., 1998). There is now a growing body of evidence to support similar patterns of variation within species. Significant variation in total gill surface area and/or other metrics of gill size has been reported among populations of widespread species that inhabit alternative oxygen environments, including representatives of several families: Cichlidae, Cyprinidae, Mormyridae, and Poeciliidae (Chapman *et al*., 1999, 2000, 2002; Chapman and Hulen, 2001; Schaack and Chapman, 2003; Timmerman and Chapman, 2004). In *A. alluaudi*, patterns of variation in gill metrics between populations from alternative aquatic oxygen environments support this trend. *Astatoreochromis alluaudi* is one of the few extremely widespread haplochromine cichlids in East Africa, in stark contrast to the enormous number of stenotypic endemic cichlids in great lakes of the region. The remarkable morphological variation in its trophic and respiratory characters may have facilitated its eurytopic distribution. The haplochromine cichlid *Pseudocrenilabrus multicolor* inhabits a similarly broad range of habitats (rivers, streams, lakes, and swamps), and is also characterized by strong interdemic variation in gill morphology, trophic morphology, and other morphological traits (Chapman *et al*., 2000, 2002).

The difference in total gill filament length between *A. alluaudi* from lakes Nabugabo and Saka (30%) was much lower than the difference in total gill surface area (50%), and notably the total gill surface area of fish from Lake Saka fell within the lower range (20^{th} percentile) of freshwater fishes (derived from equation in Palzenberger and Pohla, 1992). Lakes Nabugabo and Saka differ not only in dissolved oxygen availability but also in other site characters that may contribute to differences in gill morphology between lakes. For example, Lake Saka is subject to very high concentrations of a number of potentially toxic blue-green algae (aka cyanobacteria), including *Microcyctis aeruginosa*, *Oscillatoria* sp. and *Cylindrospermopsis* sp., with *M. aeruginosa* the dominant species by biomass (E. Phlips and L. Chapman unpubl. data). Since the gill comprises over half the body surface area of a fish and is characterized by a thin barrier between the blood and the water, most chemical transfer between the fish and the aquatic environment occurs across the gills (Hughes, 1984b; Wood and Soivio, 1991; Randall and Brauner, 1993). Thus, fishes living in waters with high levels of algal toxins may be selected for decreased gill surface area. To provide additional support for the hypothesis of oxygen caused population differentiation, we performed the rearing experiment to directly test for oxygen effects on gill morphology while holding other environmental parameters constant.

Developmental Plasticity in Fish Gills

Phenotypic plasticity often evolves because it allows organisms to mitigate environmental variation (DeWitt and Scheiner, 2004). We found a strong element of developmental plasticity in total gill filament length and total hemibranch area in *A. alluaudi* in response to the dissolved oxygen environment in which it was raised. *Astatoreochromis*

alluaudi grown under normoxia exhibited a total gill filament length and a hemibranch area smaller (27% and 34%, respectively) than fish raised under hypoxic conditions. Two lines of evidence to suggest this plasticity in gill morphology is adaptive. First the induced morphological responses are in the direction one would predict to increase oxygen uptake capacity. Second, the response was similar in the lab-reared fish to that observed between the two field populations from alternative oxygen environments. The next steps in this work will focus on quantification of gill surface area for this rearing experiment and the interaction of environmentally-induced variation and population effects by comparing fish from both lakes Nabugabo and Saka reared under low- and high-oxygen conditions.

The maintenance of plasticity in these divergent populations may preserve the possibility for future evolutionary responses and foster population colonization and persistence in novel environments (Schlichting and Pigliucci, 1998; Yeh and Price, 2004). Swamps grade into lakes and rivers in the Lake Victoria basin, and small changes in water levels can produce large changes in available habitat and in connectivity. Thus, fish lineages may experience alternative oxygen environments either within or among generations. We found high levels of developmental plasticity in the gill morphology of two other species of East African fishes that persist in variable DO environments. These other species include the cichlid *P. multicolor* (Chapman *et al*., 2000) and the cyprinid *Barbus neumayeri* (L. Chapman unpubl. data). And, other studies have demonstrated high levels of plasticity in gill traits in fishes (Schwartz, 1995; Sargolia *et al*., 2002) and larval salamanders (Bond, 1960; Burggren and Mwalukoma, 1983). Thus, environmentally-induced gill proliferation may be a widespread response to sub-lethal hypoxic stress.

The degree of developmental plasticity in response to alternative DO environments in *A. alluaudi* may differ depending on other features of the environment or the natal history of the population. When fed on hard prey (e.g., molluscs) *A. alluaudi* will develop a massive pharyngeal mill with hypertrophied muscles, whereas a softer diet leads to reduction in pharyngeal jaw size and associated musculature (Greenwood, 1965a; Huysseune *et al*., 1994). Smits *et al*. (1996) found that the total head volume in snail-eating *A. alluaudi* was 31% larger than in fish from an insect-eating population, and they reported internal reallocations of the respiratory apparatus (change in the shape of the gills). Thus, gill proliferation may be compromised to some degree when *A. alluaudi* is faced with dual challenge of hypoxia and a mollusk-dominated diet. We are currently exploring this interaction in *A. alluaudi* (Chapman, Galis, and DeWitt, unpubl. data).

Despite an apparent advantage to gill proliferation in response to hypoxic stress, an important issue is understanding what maintains these divergent respiratory phenotypes in the field. Why not have large gills in all environments? Fitness trade-offs, whereby the phenotype with the highest performance in one habitat performs sub-optimally in the alternative environment, may contribute to the maintenance of variation among field populations (Van Buskirk *et al*., 1997; DeWitt and Scheiner, 2004). Trade-offs between feeding and respiratory structures seem very likely in fishes because of their generally compact, laterally compressed head morphology. Our studies on two East African fishes, the cichlid *P. multicolor* and the cyprinid *B. neumayeri,* suggest potential trade-offs between respiratory phenotypes. For example, we demonstrated that adaptive change in gill size (large gills) in fish from hypoxic

waters correlates with reduced size of key trophic muscles and feeding performance relative to small-gilled conspecifics (Chapman *et al*., 2000; Schaack and Chapman, 2003). These trade-offs may lead to fitness costs in the field that impose habitat-specific selection pressures on dispersers.

Recent models of the role of phenotypic plasticity in driving genetic evolution argue that moderate levels of adaptive plasticity are optimal for evolution in novel environments by enhancing population persistence and placing populations under directional selection leading to potentially higher adaptive peaks (Price *et al*., 2003). The widespread distribution of some East Africa cichlids such as *A. allauadi* and *P. multicolor* may reflect broad environmental tolerances due to their phenotypically plastic responses to environmental variation. However, these plastic responses may be assimilated genetically on the long term if populations are under directional selection towards new adaptive peaks (Price *et al*., 2003; West-Eberhard, 2005).

Summary

The significance of variation in dissolved oxygen in driving phenotypic divergence is a largely unexplored aspect of aquatic biodiversity. However, there is now strong evidence that alternative oxygen environments are a strong predictor of intraspecific variation in fishes, particularly in respiratory traits and associated characters. Developmental plasticity seems to play a large role in explaining variation in gill morphology among populations, and may be an important mechanism contributing to the widespread distribution of species that cross strong dissolved oxygen gradients. Future studies on widespread African cichlids that explore the interaction of genetic and environmentally-induced morphological variation in multiple populations should elucidate the potential that an initially plastic response to a novel oxygen environment may be followed by genetic changes in the same direction.

One of the many challenges facing freshwater fishes is the increasing occurrence of hypoxia, which has lead to fish kills, changes in fish distribution, and a massive reshaping of some fish communities. Thus, it has become increasingly important to understand the consequences of low-oxygen stress on fish populations. Interpopulational variation and phenotypic plasticity in respiratory traits may contribute to species persistence in the face of environmental change.

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Hypoxia tolerance in coral reef teleosts

by

G.E. Nilsson[1] and S. Östlund-Nilsson

Introduction

Water-breathing animals run a much greater risk of experiencing hypoxia than air-breathers, as the concentration of oxygen in air-saturated water is only about 3 – 5 % of that in air, and because oxygen diffuses about 10 000 times faster in air than in water. In many situations, aquatic organisms may use up the oxygen in the water before it is replenished by diffusion or photosynthesis. Hypoxia is particularly likely to occur at night, when both plants and animals have to rely on respiration for their energy supply. While tropical freshwater habitats are well known to expose their inhabitants to hypoxia, it has only recently become apparent that hypoxia is a major abiotic factor shaping the teleost fauna on coral reefs.

Hypoxia tolerance on the Reef

A few years ago, we used closed respirometry to survey hypoxia tolerance, indicated by the critical oxygen concentrations ($[O_2]$crit), in 31 species of teleost fish representing seven families (Apogonidae, Blennidae, Gobiidae, Labridae, Monacanthidae, Nemipteridae, and Pomacentridae) on the coral reef at Lizard Island , Great Barrier Reef, (Table I) (Nilsson and Östlund-Nilsson, 2004). $[O_2]$crit is the lowest oxygen level where a fish is able to maintain its resting rate of oxygen consumption (Prosser and Brown, 1961). All species examined were found to be strikingly hypoxia tolerant, showing $[O_2]$crit values of 13 - 34 % of air saturation (mean being ca 24 %.). Behavioural signs of hypoxic stress were generally not seen until the O_2 level in the closed respirometers fell below 10 % of air saturation, indicating that the fishes were able to compensate for the reduced oxidative ATP production by boosting anaerobic ATP production (i.e. glycolysis), or by reducing ATP demand (metabolic depression), or both.

Until this recent study, hypoxia tolerance was generally not thought to be of importance for coral reef fishes, except in the special case of the epaulette shark (recently reviewed by Nilsson and Renshaw, 2004). Still, the teleosts we examined were a representative selection of the eye catching piscine beauties that people generally associate with coral reefs, all caught in close proximity to living coral in 2 – 5 m deep water.

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That coral reef fishes maintain O_2 uptake in hypoxia at a temperature of about 30 °C can be viewed as a considerable physiological achievement, due to the combined effects of a low solubility of O_2 in warm sea water, and the high rate of oxygen consumption that small fish have at such a high temperature. Most fishes examined weighed less than 10 grams and had resting rates of oxygen consumption (MO_2) of 200 – 700 mg O_2 kg^{-1} h^{-1}, which is several times higher than that of fishes in cold temperate water. In fact, the $[O_2]$crit values shown by the coral reef fishes are similar to those of fishes inhabiting hypoxic tropical freshwaters. African cichlid species like tilapia, *Oreochromis niloticus*, for example, display $[O_2]$crit values of about 20 % of air saturation at 25 °C (Verheyen *et al.*, 1994; Chapman *et al.*, 1995).

When and where do they encounter hypoxia?

During the light hours, hypoxia is probably very rare or absent on coral reefs. However, the situation may change drastically during the night. We now know that coral reef fishes are exposed to hypoxia either when they hide from predators at night by moving into the coral colonies and residing between coral branches, or when they get trapped in tide pools at nocturnal low tides (Nilsson and Östlund-Nilsson, 2004). In *Acropora nasuta* colonies from Lizard Island, kept in outdoor tanks, we found that the average oxygen level between the branches fell to 20 % of air saturation just before sunrise (Figure 1), and oxygen levels as low as 2 % were occasionally measured (Nilsson *et al.*, 2004). Similarly, Goldshmid *et al.* (2004) found that water oxygen levels fell to 10 – 20 % of air saturation in colonies of *Stylophora pistillata* (Esper, 1797) from the Red Sea kept dark in the laboratory. In the same study, Goldshmid *et al*., (2004) obtained results indicating that damselfishes (*Chromis viridis, Dascyllus aruanus* and *D. marginatus*) inhabiting the *Stylophora* colonies perform nocturnal "sleep-swimming" to increase the water flow through the coral in order to reduce the nocturnal hypoxia.

We recently examined oxygen levels on the reef near Lizard Island on low nocturnal tides in calm weather between 2.00 - 5.00 am (G. E. Nilsson, S. Östlund-Nilsson, and J.-P. Hobbs, unpublished observations). When pushing an oxygen electrode some 10 cm into coral colonies where numerous fishes were seen to hide, oxygen levels between 10 and 20 % of air saturation were measured. We also found that in an area of about 1 m^2 of living coral, there could be hundreds of fishes hiding under these severely hypoxic conditions. This habit of hiding in coral at night has previously been described (Fishelson *et al*., 1974; Goldshmid *et al.*, 2004), and is well known to many night divers. During reef walks at nocturnal low tides, we also observed living fishes trapped in tide pools with water oxygen levels as low as 8 - 12 % of air saturation. The fishes observed to reside under the severely hypoxic conditions in coral and in tide pools included surgeonfishes, emperors, coral breams, rockcods, damselfishes, butterflyfishes, wrasses, gobiids, sandperches and cardinalfishes.

Table I. Hypoxia tolerance at 30 °C of fishes at Lizard Island, Great Barrier Reef.

Family / *Species*	N	Weight (g)	Normoxic MO_2 (mg kg-1 h-1)	$[O_2]_{crit}$ (% of air saturation)	$[O_2]_{out}$ (% of air saturation)
Cardinalfishes (Apogonidae)					
Apogon compressus	4	7.0±1.2	179±67	19±5	6.7±1.9
Apogon cyanosoma	1	2.2	259	30	
Apogon doederleini	1	4.4	288	31	
Apogon exostigma	1	3.7	218	26	11.4
Apogon fragilis	14	1.9±0.1	255±17	17±1	7.2±1.0
Apogon leptacanthus	14	1.5±0.1	239±19	19±1	7.0±1.2
Archamia fucata	1	5.8	225	34	
Cheilodipterus quinquelineatus	2	1.8-7.4	244-263	23-31	7.2-11.1
Sphaeramia nematoptera	1	7.3	131	17	10.0
Damselfishes (Pomacentridae)					
Acanthochromis polyacanthus	1	15.4	197	26	6.5
Chromis atripectoralis	5	8.4±2.5	358±84	22±2	8.8±0.8
Chromis viridis	6	2.5±1.1	555±108	23±1.2	7.4±0.9
Chrysiptera flavipinnis	1	2.4	384	30	12.0
Dascyllus aruanus	3	4.1±1.3	306±37	19±0	5.9±0.6
Neoglyphidodon melas	6	32.1±8.8	216±32	25±2	5.6±0.7
Neoglyphidodon nigroris	6	14.9±2.4	162±21	22±3	8.9±1.5
Neopomacentrus azysron	1	3.2	493	32	
Pomacentrus ambionensis	4	12.6±1.7	201±11	22±4	7.1±2.0
Pomacentrus bankanensis	1	7.8	237	19	
Pomacentrus coelestis	6	7.8±3.0	387±85	22±4	9.3±1.0
Pomacentrus lepidogenys	5	3.1±0.6	516±73	31±2	12.5±1.1
Pomacentrus moluccensis	4	5.2±4.0	397±85	25±3	10.4±1.9
Pomacentrus philippinus	2	2.2-6.9	320-348	26-33	9.3-10.5
Gobies (Gobiidae)					
Amblygobius phalaena	1	2.4	333	21	2.8
Asteropteryx semipunctatus	1	1.4	403	26	1.4
Gobiodon histrio	10	1.2±0.2	248±31	18±1	2.8±0.5
Blennies (Blennidae)					
Atrosalarias fuscus	3	7.3±1.9	208±34	18±2	1.6±07
Atrosalarias fuscus juvinile	1	0.29	552	13	1.5
Filefishes (Monacanthidae)					
Paramonacanthus japonicus	1	1.7	486	23	9.5
Breams (Nemipteridae)					
Scolopsis bilineata juvenile	1	1.9	375	28	12.8
Wrasses (Labridae)					
Halichoeres melanurus	1	1.8	394	25	6.8
Labroides dimidiatus juvenile	3	0.56±0.09	736±35	24±5	7.8±0.9

(Normoxic MO_2 = rate of O_2 consumption at a water $[O_2]$ >70% air saturation, $[O_2]_{crit}$ = critical $[O_2]$, below this level MO_2 starts falling and is no longer independent of ambient $[O_2]$, $[O_2]_{out}$ = $[O_2]$ at which the fish showed signs of agitation or balance problems. Values for 3 or more fish are means ± SD. Taxonomy follows Randall *et al*. (1997). From Nilsson and Östlund-Nilsson (2004).

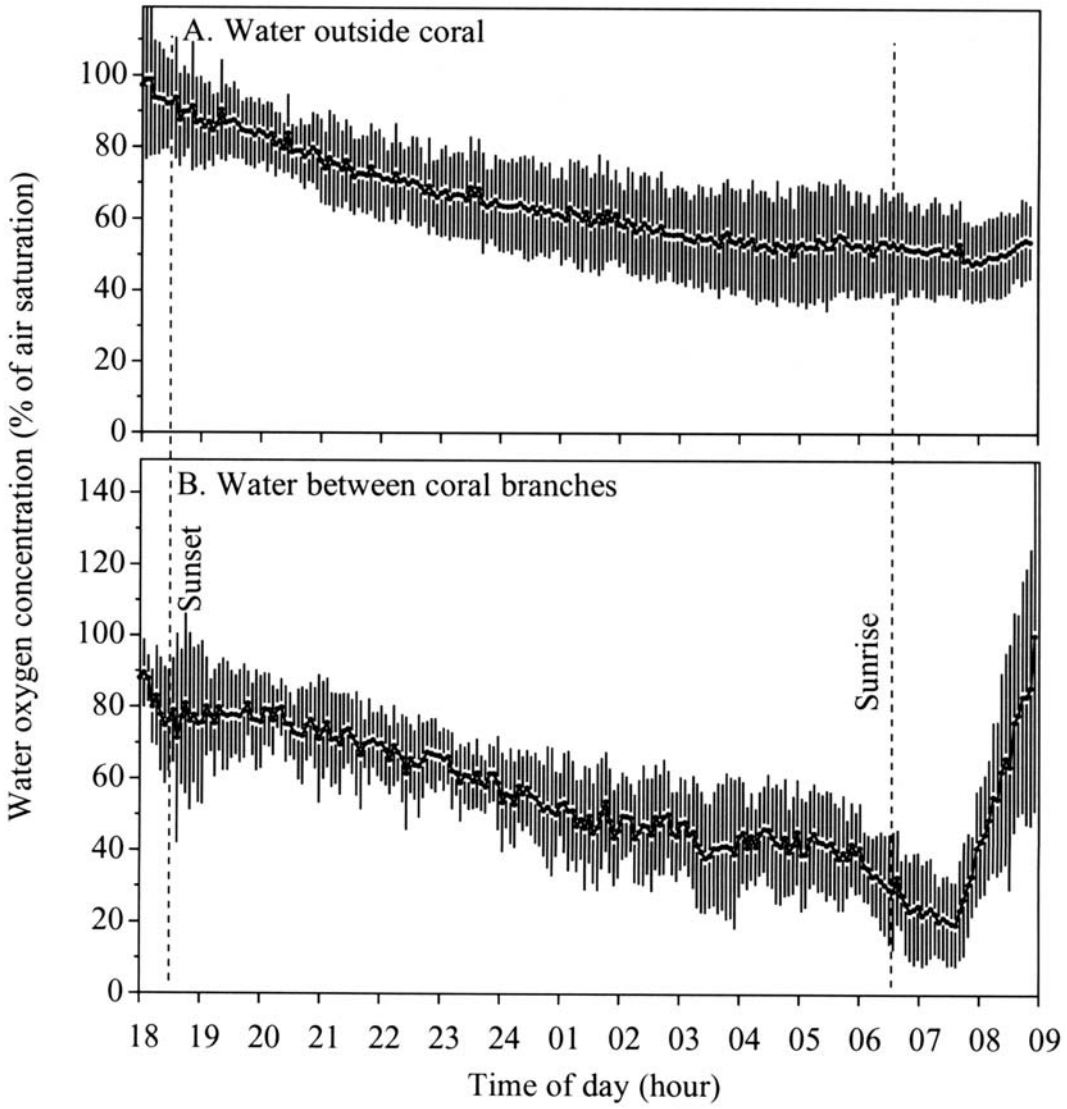

Figure 1. Tracing of the oxygen level (%air saturation) inside the coral *Acropora nasuta*, the habitat where some coral dwelling gobies spend their whole adult life. From Nilsson *et al*. (2004).

Thus, from being a well oxygenized paradise during the day, at night the reef becomes a world where the smaller fish hide between branches in coral colonies, having to endure severe hypoxia.

The extreme coral dwellers

The species of the genera *Gobiodon* and *Paragobiodon* are all obligate coral-dwellers. Thus, they spend most of their lives in narrow spaces formed between the branches of coral colonies (Randall *et al.*, 1997). The *Gobiodon* species are also arguably exceptionally cowardly fishes. Although they are practically inaccessible to predatory fish in their coral home, they still secrete a poisonous mucus that should make them

inedible to most predators. Fish fed with pieces of *Gobiodon* die within a few minutes (Schubert *et al*., 2003). Moreover, these gobies have the ability to repeatedly change sex, so that they do not need to leave the coral to find a mating partner (Munday *et al.*, 1998).

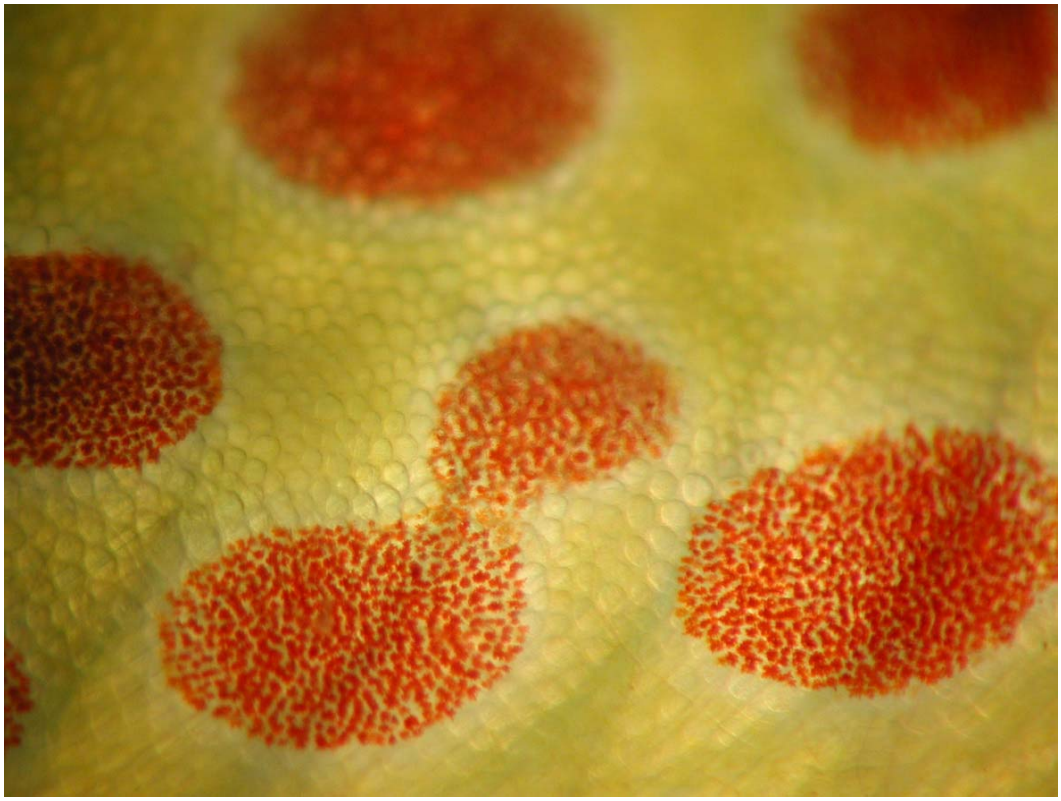

Figure 2. The scaleless skin of the coral dwelling goby *Gobiodon histrio* through which it can take up oxygen during air exposure. Small circular structures that cover the whole surface are poison secreting cells. The width of the picture is 5 mm. Photo by Göran E. Nilsson.

Coral gobies often inhabit coral habitats in shallow water which may become air exposed for several hours at the lowest tides. A study on two *Gobiodon* species revealed not only a higher degree of hypoxia tolerance that the average of coral reef fish, but also excellent air breathing capabilities (Nilsson *et al.*, 2004). This study provided the first evidence that some fishes intimately connected to living coral have evolved excellent air-breathing abilities. It is well known that many fishes living in tropical freshwaters or estuarine habitats have evolved air-breathing capacities to cope with hypoxia or air exposure (Graham, 1997). Apparently, in some situations, it is not enough to be hypoxia tolerant for fishes to survive in a coral habitat, they may also need to have the ability to breathe air. The selection pressure that has given rise to air-breathing in the genus *Gobiodon* is clearly the air exposure that they experience when their coral colonies are out of the water a low tides. By breathing air, they do not have to leave the coral even if it is completely out of the water. Interestingly, continued studies (Nilsson, J.-P. Hobbs, S. Östlund-Nilsson, and P. L. Munday, unpublished) have shown that the air breathing capacities varies with the depth range of the gobiid species. Those that live in shallow-

water corals can maintain the rate of oxygen uptake in air at the same level as in water for up to four hours, while those living deeper can only air breath for about an hour. In fact, the deeper living *Paragobiodon xanthosomus* was completely unable to breath air, which coincided with having a body covered with scales. All the *Gobiodon* species have scaleless bodies, and the major route of oxygen uptake in these fishes appear to be over their skin (Fig. 2).

Cardinalfishes: Fitness versus hypoxic survival

Male cardinalfishes (Apogonidae), one of the more species rich fish families on coral reefs, are faced with a particular respiratory problem. They brood the egg clutch they have received from the females in their mouths for approximately two weeks after fertilization. This egg mass can make up a quarter of their body mass (Östlund-Nilsson and Nilsson, 2004), filling up most of the oral cavity, which makes up about 20-30 % of their body volume (Barnett and Bellwood, 2005). Obviously, one may assume that the egg mass will reduce the ability of male cardinalfish to ventilate their gills. Like other coral reef fish, cardinalfishes generally seek shelter in coral colonies at night and will therefore have to cope with hypoxia. This poses the question: how do male cardinalfish reconcile mouthbrooding with hypoxia? To try to answer this, we compared the respiratory consequences of mouth brooding in two cardinalfish species occurring at the Lizard Island reef: *Apogon fragilis* and *A. leptacanthus* (Östlund-Nilsson and Nilsson, 2004).

Mouthbrooding did not seem to be particularly costly by itself, since the resting metabolic rate was not significantly affected by the presence of the egg clutch in the mouth (after accounting for the oxygen consumption of the clutch it self). By contrast, $[O_2]$crit of the mouthbrooding males was about 32% of air saturation, as compared to 18 % in non-brooding males or females, revealing a significantly reduced hypoxia tolerance during mouthbrooding.

Interestingly, the two species studied were found to differ in the mean egg-clutch mass. The males of *A. fragilis* and *A. leptacanthus* had broods corresponding to 20.0 % and 14.4 % of their body mass, respectively, a difference that clearly affected their ability to cope with hypoxia. Thus, when faced with falling oxygen levels in the closed respirometer, both species eventually spat out the clutch, apparently to save their own life. However, in *A. fragilis* (the species with the larger egg mass), the brood spitting occurred at a higher oxygen level, 21.7 % of air saturation, compared to *A. leptacanthus*. The latter species spat the eggs at 13.0 % of air saturation. Moreover, in contrast to *A. leptacanthus*, mouthbrooding *A. fragilis* were running at their maximal ventilatory frequency already during normoxic conditions, and they were therefore unable to increase ventilation when exposed to hypoxia. Apparently, there is a trade-off situation between brood size and hypoxia tolerance. The successful brooding of a larger clutch should mean a correspondingly larger increase in fitness, and *A. fargilis* seems to be gambling on a brooding period without any severely hypoxic episodes to maximize the fitness gain from each brood. By contrast, *A. leptacanthus* does not seem to take this risk. These different strategies do not seem to be the result of different environmental constrains in the

preferred habitat, because both species often school together in the same habitat, close to branching coral. Still, it is likely that the mouthbrooding strategy will have to influence the choice of nocturnal shelter (more or less deep within coral colonies) or willingness to move out of hypoxic corals. Thus, the mouthbrooding *A. fragilis* males may have to spend their night on the outskirts of coral colonies and the fitness they gain from a large broad may be balanced by a higher risk of being predated.

Landing on the reef: From record swimming to hypoxia tolerance

Coral reef fishes generally start their life as planktonic larvae (Thresher, 1984), spending a few weeks drifting in the open water before they settle on the reef. At the end of their pelagic phase coral reef fish larvae develop extremely impressive capacities for high-speed sustained swimming (i.e. for hours or even days), which they need to reach suitable coral habitats (Stobutzki and Bellwood, 1994; Leis and Carson-Ewart, 1997). Indeed, they appear to be the fastest swimmers of all fishes, since many of them are capable of reaching maximal sustained swimming speeds of 30 - 50 body lengths per second (BL/s) (Stobutzki and Bellwood, 1994; Leis and Carson-Ewart, 1997; Fisher *et al*., 2005). For comparison, it can be mentioned that larvae of temperate fishes do not usually reach sustainable swimming speeds higher than 4 - 5 BL/s (Blaxter, 1986; Meng, 1993), while the adult fishes best known for exceptional swimming performance, which include swordfish (*Xiphias*), tunas (*Thunnus* and *Euthynnus*) the inconnu (*Stenodus leucichthys*) do not exceed 20 BL/s (Aleyev, 1977; Beamish, 1978).

Figure 3. The damselfish, *Pomacentrus ambionensis,* in the late plaktonic stage, just before settlement on a coral reef. This larvae is among fastest swimming

fish, capable of sustaining a speed of about 30 body lengths per second, and during swimming it also displays one of the highest rates of oxygen uptake measured in any fish. The fish is 12 mm long. Photo by Göran E. Nilsson.

Swimming can only be sustained if it is fully aerobic and does not lead to a build up of lactate (Goolish, 1991). We recently constructed a miniature swim respirometer that allowed us to measure maximal rates of oxygen uptake (MO_2max) during high speed swimming in pre- and post-settlement larvae of two species of damselfish (*Chromis atripectoralis* and *Pomacentrus ambionensis*) (G. E. Nilsson, S. Östlund-Nilsson and A. S. Grutter, unpublished). Our measurements showed that *C. atripectoralis* and *P. ambionensis* reach MO_2max of about 5000 and 4000 mg O_2 kg^{-1} h^{-1}, respectively, when swimming at maximal sustained speeds. This is, to our knowledge, the highest MO_2max values ever measured in cold blooded vertebrates. Leis and Carson-Ewart (1997) examined swimming performance in pre-settlement larvae of 17 damselfish species, and found that they reached an average sustained speed of 34 BL/s, with *C. atripectoralis* larvae being the fastest swimmers observed, reaching maximal sustained swimming speeds of 53 BL/s. Pre-settlement larvae of P. *ambionensis* (Figure 3) can be considered to be more average performer among damselfish larvae, reaching sustained speeds of 30 BL/s (Stobutzki and Bellwood, 1994).

Our results allow us to conclude that the extraordinary high sustained swimming speeds of pre-settlement damselfish larvae are paralleled by extraordinary high capacities for rapid oxygen uptake. Obviously, these traits are important for larvae to reach a suitable reef at the end of their planktonic period.

As most fish biologists know, high aerobic capacities of very active fish species preclude hypoxia tolerance, and vice versa (Burggren *et al*., 1991, for review). The underlying reason is probably the opposing demands that a high MO_2max and hypoxia tolerance put on the oxygen carrying properties of haemoglobin. Oxygen uptake in hypoxia requires a haemoglobin with a high oxygen affinity. However, this leads to relatively slow rates of oxygen downloading in the tissues, because oxygen has to be downloaded at a low partial pressure, resulting in a small pressure gradient from blood into the mitochondria and therefore a slow oxygen delivery. Consequently, the haemoglobins of highly active fish show lower oxygen affinities than those of sedentary species (reviewed by Burggren *et al*., 1991).

As we have discussed, adult coral reef fishes are hypoxia tolerant, probably because they need to cope with hypoxia at night, when they avoid predators by moving into coral. Our respirometry studies of post-settlement and juvenile individuals of *C. atripectoralis* and *P. ambionensis* (G. E. Nilsson, S. Östlund-Nilsson and A. Grutter, unpublished) show a striking, almost transient, reduction in MO_2max and $[O_2]crit$ within the first 5-10 days of settlement. Apparently, the larval *C. atripectoralis* and *P. ambionensis*, have to adjust their respiratory capacities to accommodate hypoxia tolerance at the expense of high rates of oxygen uptake. The result is that they are no longer exceptionally fast swimmers, but they should, after this transition, be able to seek shelter within coral colonies at night. It is likely that the transition involves changes in

haemoglobin oxygen affinity, and there are examples of ontogenetic changes in haemoglobin isoform expression in fishes (reviewed by Jensen *et al*., 1998).

Conclusions

For coral reef fishes, as for all animals that strive to survive hypoxia, the key issue is the same: to ensure that ATP levels are maintained so that the integrity of cellular function is not compromised. This means that they have to be able to take up oxygen from the water at as low oxygen levels as possible, and that they can defend their ATP levels with glycolysis combined with reduced ATP utilization, if aerobic metabolism can no longer be sustained. Teleosts on coral reefs are faced with hypoxia when they venture into coral colonies to seek shelter from predators at night, or are trapped in tide pools during nocturnal low tides. While virtually all fishes seen on a coral reef are strikingly hypoxia tolerant, the species most intimately connected to coral are also those that are most hypoxia tolerant, and some of these even have excellent air-breathing capabilities. Most coral reef fishes have planktonic larvae with outstanding swimming abilities. These will have to rapidly transform their respiratory properties when they settle on the reef, from being the fastest swimmers with the highest rates of oxygen uptake of any fishes, to becoming hypoxia tolerant. Since coral reefs contain one of the most biodiverse and fastest evolving vertebrate communities, we can be certain that a wealth of respiratory adaptations remain to be discovered and explored among coral reef fishes.

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Oxygen consumption of fish exposed to hypoxia: Are they all oxyregulators or are any oxyconformers?

by

J.F. Steffensen[1]

Animals exposed to a decreasing partial pressure of oxygen - hypoxia - are traditionally categorized as either oxygen regulators or conformers. Regulators can maintain their standard metabolic rate when exposed to hypoxia until the so-called critical partial pressure of oxygen is reached. At further decreasing partial pressures of oxygen, beyond the critical point, the metabolic rate will decrease and the animal will start utilizing anaerobic metabolism with a concurrent accumulation of lactate (**R** in Fig. 1). Conformers, on the other hand, are not able to maintain standard metabolic rate during hypoxia, and it will decrease linearly with decreasing partial pressure of oxygen (**C** in Fig. 1). Metabolic rate versus hypoxia may also have a trace like **T** in Figure 1. – neither a conformer nor a regulator, but a **T**ypical trace that can be seen in many publications. Reasons for this shape will be discussed below.

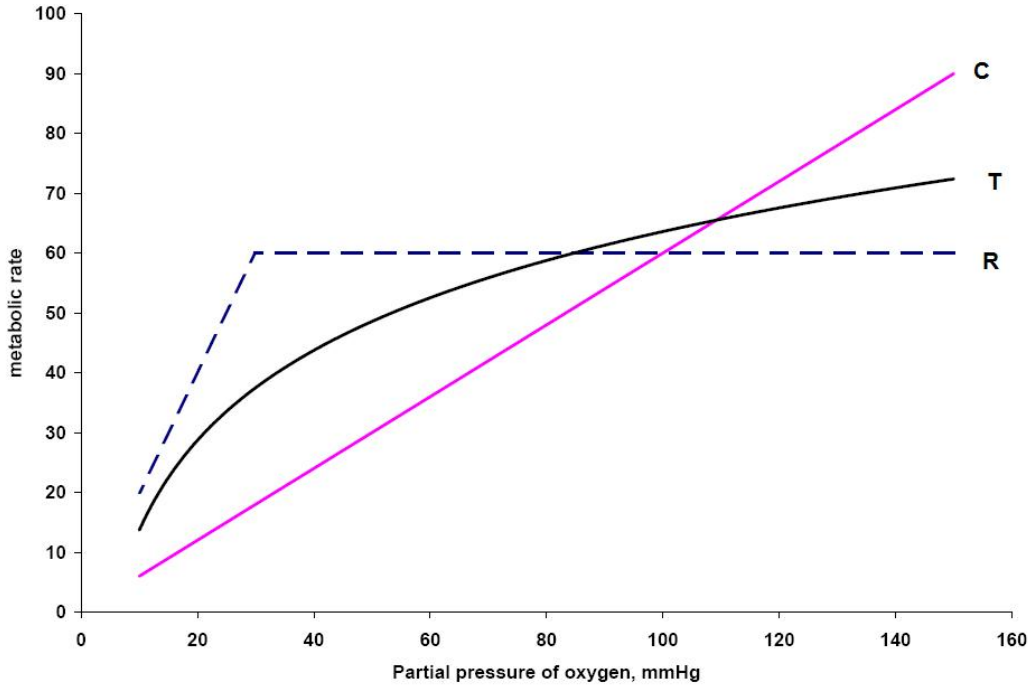

Figure 1. Example of an oxygen conformer (C), an oxygen regulator, and an example of an animal with an intermediate typical (T) metabolic rate vs. hypoxia.

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The sluggish toadfish (*Opsanus tau*) is the classic example of an oxygen conformer (Hall, 1929) and has often been quoted as such for more than 5 decades. Keys (1930), however, questioned Hall's results due to the experimental design, which was based on a modified version of a flow-through respirometer described by Ege and Krogh (1914). While oxygen partial pressure decreased from normoxia to severe hypoxia as the flow of water through the respirometer was decreased, partial pressure of CO_2 increased, but this was ignored by Hall. Keys (1930) also considered that stratification affiliated with this particular flow-through respirometry led to serious errors.

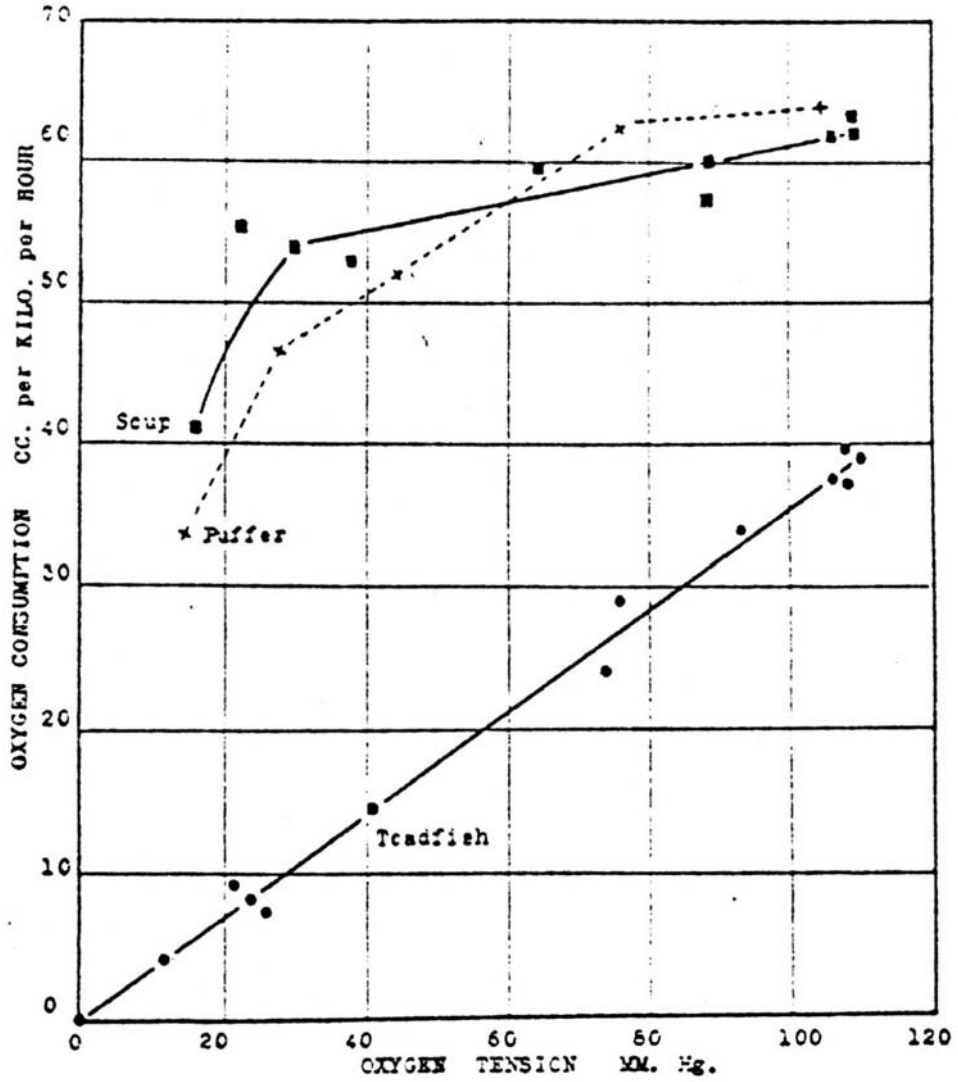

Figure 2. Oxygen consumption of the scup, puffer and toadfish, at different oxygen tensions. The scup and puffer oxygen consumption vs. hypoxia is T-shaped, while the toadfish has a typical conformer C-shape. From Hall (1929).

The sturgeon *Acipenser transmontanus* is another classic example of an oxygen conformer, as reported by Burggren and Randall (1978). They found that gill stroke volume and ventilation rate, and hence gill ventilation decreased with decreasing partial pressure of oxygen (Fig. 3). Since oxygen extraction also decreased, oxygen consumption decreased as well, and it was concluded that the sturgeon clearly was an oxygen conformer. Compared to other teleosts it is unusual that gill ventilation volume decreases with decreasing oxygen partial pressures. It is also unusual that the gill oxygen extraction is highest at the highest gill ventilation volume – usually oxygen extraction is highest in normoxia at which the ventilation volume is normally the lowest. Oxygen consumption was reported to decrease to less than 5 % of the normoxic rates during hypoxia exposure, and because there was no sign of anaerobiosis, it was concluded that this ancient fish reduces total energy expenditure during hypoxic exposure, rather than switching from aerobic to anaerobic metabolism – it simply shuts down metabolism.

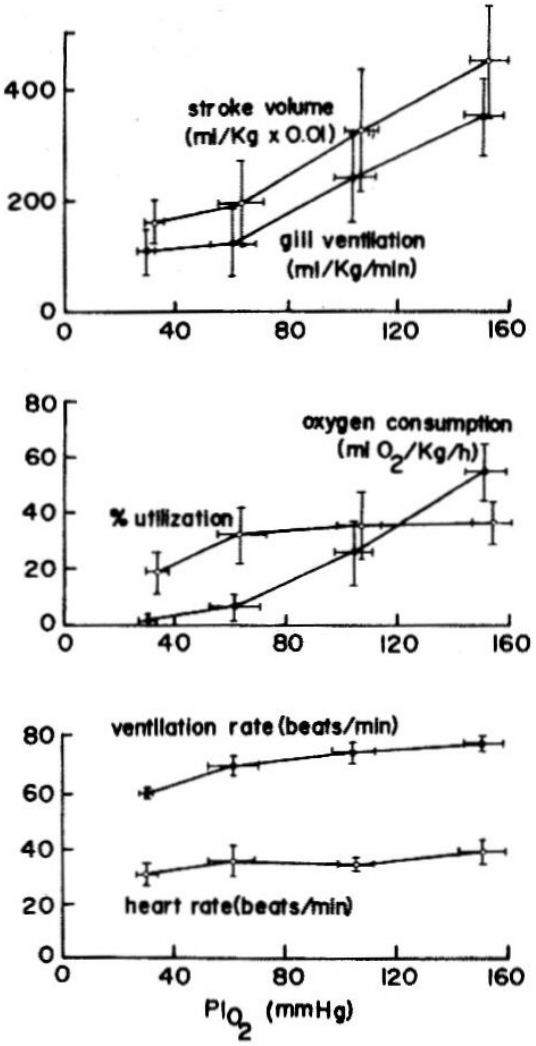

Figure 3. Ventilatory parameters and heart rate in *Acipenser transmontanus* during exposure to 4 levels of hypoxia. (Mean $\pm$ SD, n=6). From Burggren and Randall (1978).

Even though neither Keys' (1930) remarks on the effects of increased CO_2 nor the stratification problems in Hall's (1929) experiments were never specifically addressed, Ultsch *et al*. (1981) revisited the toadfish experiments nonetheless. They also used flow-through respirometry, but rather than causing hypoxia by decreasing the flow through the respirometer, they closely and continuously controlled and regulated oxygen saturation in the water supplying the respirometers. Hence their experimental design among others minimized possible problems with pCO_2. Ultsh *et al*. (1981) hypothesized that there was no reason why any vertebrate with a significant amount of functioning respiratory pigment and a reasonable matching of ventilation and perfusion at the gas exchange surface should not be able to regulate its standard metabolic rate during a considerable decrease in partial pressure of oxygen. The results (Fig. 4) showed that they were correct and that the toadfish actually was a regulator similar to most other fishes (Ultsch *et al*., 1981).

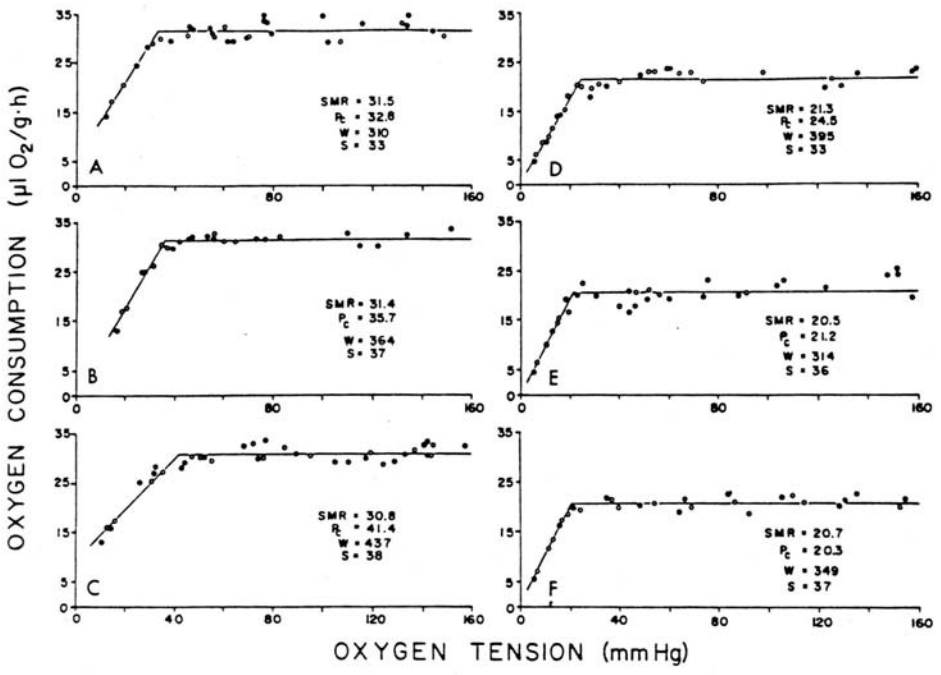

Figure 4. Resting rates of oxygen consumption of toadfish, maintained and tested at 22 ^{o}C, as a function of the ambient pO_2. From Ultsch *et al*. (1981).

Later, a study on two species of sluggish flatfish, the plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*) concluded that the former was a regulator while the latter was an oxygen conformer (Steffensen *et al*., 1982) as shown in Figure 5. In contrast to the sturgeon mentioned above, however, both species of flatfish increased the gill ventilation volume with respect to decreasing oxygen, and the highest oxygen extraction was found at the lowest gill ventilation volume - which was in normoxia as usual.

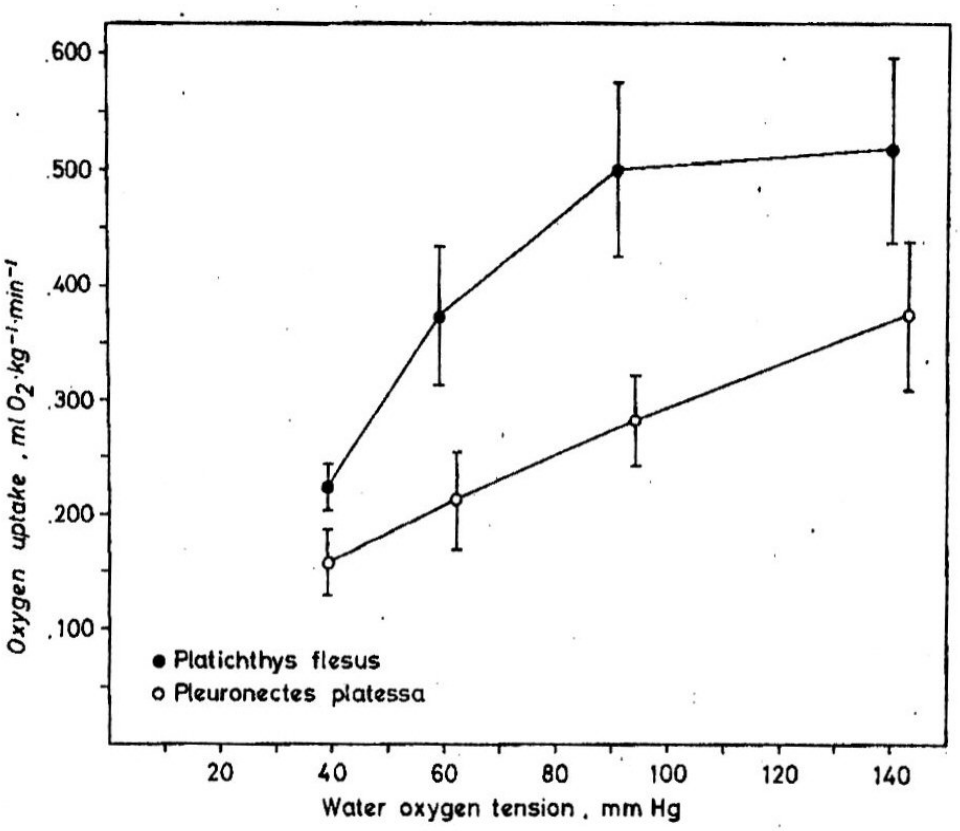

Figure 5. Oxygen uptake across the gills for plaice and flounder at different oxygen tensions. From Steffensen *et al*. (1982).

In 1989 Steffensen described some problems and possible errors with different respirometry methods of aquatic breathers, and how to avoid and correct for them. Only 2 methods, or a combination of these, are normally used – 1) open or flow-through respirometry, or 2) intermittent flow-through respirometry. Ege and Krogh (1914) introduced open or flow-through respirometry. Based on the difference in oxygen content in the water running in and out of the respirometer, and the flow of water through the respirometer, the oxygen consumption could be calculated. Difficulties with correcting for the exponential wash-out if the system came out of steady state, however, is the main problem with this method (Steffensen, 1989). By decreasing the flow of water through the respirometer the oxygen tension inside the respirometer can be forced to decline, and the animal exposed to hypoxia. Unfortunately, however, neither Hall (1929) nor several other investigators at that time considered the dilution factor and affiliated lag-time and the erroneous oxygen consumption values that would be the results (Steffensen, 1989).

Figure 6 shows an example of a somewhat similar erroneous use of flow-through respirometry. The graph on the far left side shows a steady state situation with a constant flow of water through the respirometer and a theoretical fish with constant oxygen consumption. Acutely, the oxygen tension in the inspired water is then changed stepwise, but the expired water will not be in steady state again before more than 50 min later. If oxygen consumption (uncritically) is calculated as the difference in oxygen content of the

in- and out-flowing water multiplied with the flow rate, the interpretation will be that acute hypoxia initially depresses the metabolic rate severely (+ in Fig. 5.). In addition it will be concluded that the fish slowly acclimate to the hypoxic level, and after > 50 min the fish have the same oxygen consumption as they initially had under normoxic conditions. When normoxia is reestablished, the erroneous result for the uncritical experimenter will be that the fish initially has an oxygen consumption of about twice the consumption in steady state in normoxia, and that this is due to an oxygen debt that is paid back during the following 50 min (+).

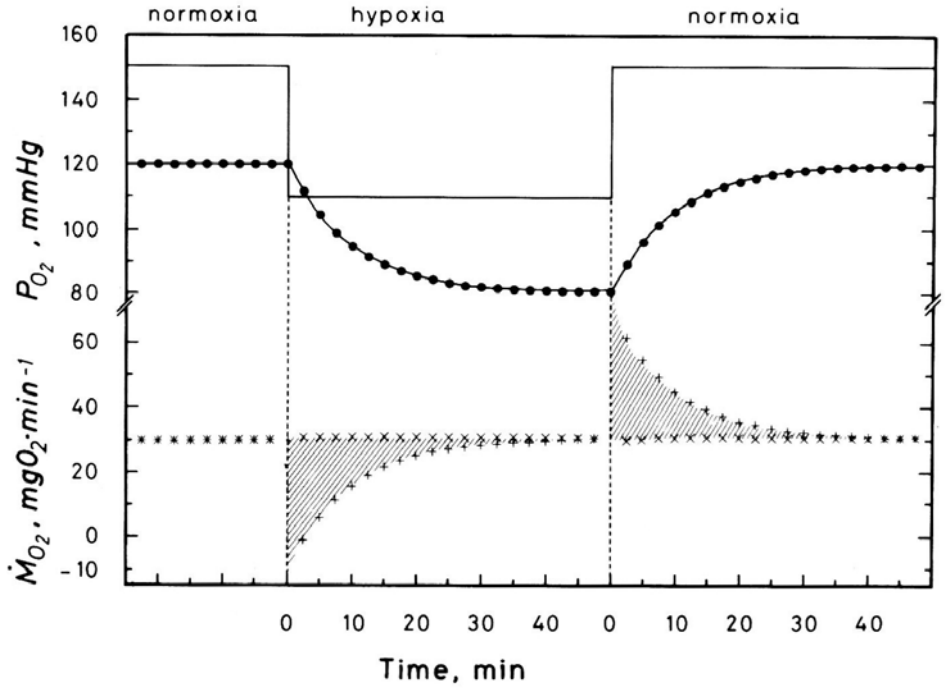

Figure 6. Theoretical example of a flow-through respirometer experiment when exposing a fish to acute hypoxia and return to normoxia. For further details see Steffensen (1989).

A similar use of flow-through respirometry may explain why Hall (1929) reported the toadfish to be a conformer, in contrast to Ultsch *et al*. (1991).

In 1938, van Dam made a modification to the flow-through systems involving a rubber membrane separation of the head and mouth in one compartment and the operculum and the remainder of the fish in another compartment. In the initial experiment oxygen consumption of rainbow trout was studied with respect to hypoxia that was induced by decreasing the inspired water oxygen tension, as shown in Figure 5. The result was that oxygen consumption increased 79 % compared to the normoxic value. This increase was ascribed to added cost of ventilating the gills. Later it has been suggested that a cost of ventilation less than 10 % of the total oxygen consumption (Steffensen, 1985), and the high value found by van Dam can probably be ascribed to improper use of flow- through respirometry.

Theoretically a correction for the wash-out and lag-time can be carried out according to Niimi (1978), and the calculated oxygen consumption (x in Fig. 6) is identical to the theoretical. In practice, however, it often results in quite fluctuating oxygen consumption values due to the exponential correction, and the method can not be recommended.

The flow-through method was also used by Burggren and Randall (1978) when studying the *Acipenser transmontanus*. Steffensen (1989) suggested that the response observed by Burggren and Randall (1978) may have been an experimental artifact affiliated with the problem described above.

Are there any other reasons why some earlier studies reported fish to be oxygen conformers? Spontaneous activity which causes an increase in metabolic rate can also be part of the problem, as pointed out by Ultsch *et al*. (1981), particularly since spontaneous activity often decreases with decreasing partial pressure of oxygen, as shown by Schurmann and Steffensen (1997). The result of spontaneous activity and hypoxia can lead to the T-curve shown above in Figure 1. Handling stress affiliated with the transfer of a fish to the respirometer can also be part of the reason, and can also result in a T-curve.

Overall, most fish species behave as perfect oxygen regulators. Steffensen *et al*'s flatfish experiment (1982) was repeated with intermittent flow-through respirometry to address the idea that the reason they did not behave as oxygen regulators could be ascribed to handling stress causing erroneously high oxygen consumption at normoxia. The results showed that the plaice, *Pleuronectes platessa*, and the dab, *Limanda limada*, are both perfect regulators, as can be seen in Fig. 7. (Søborg *et al*., 1993), and hance Steffensen *et al*. (1982) were wrong.

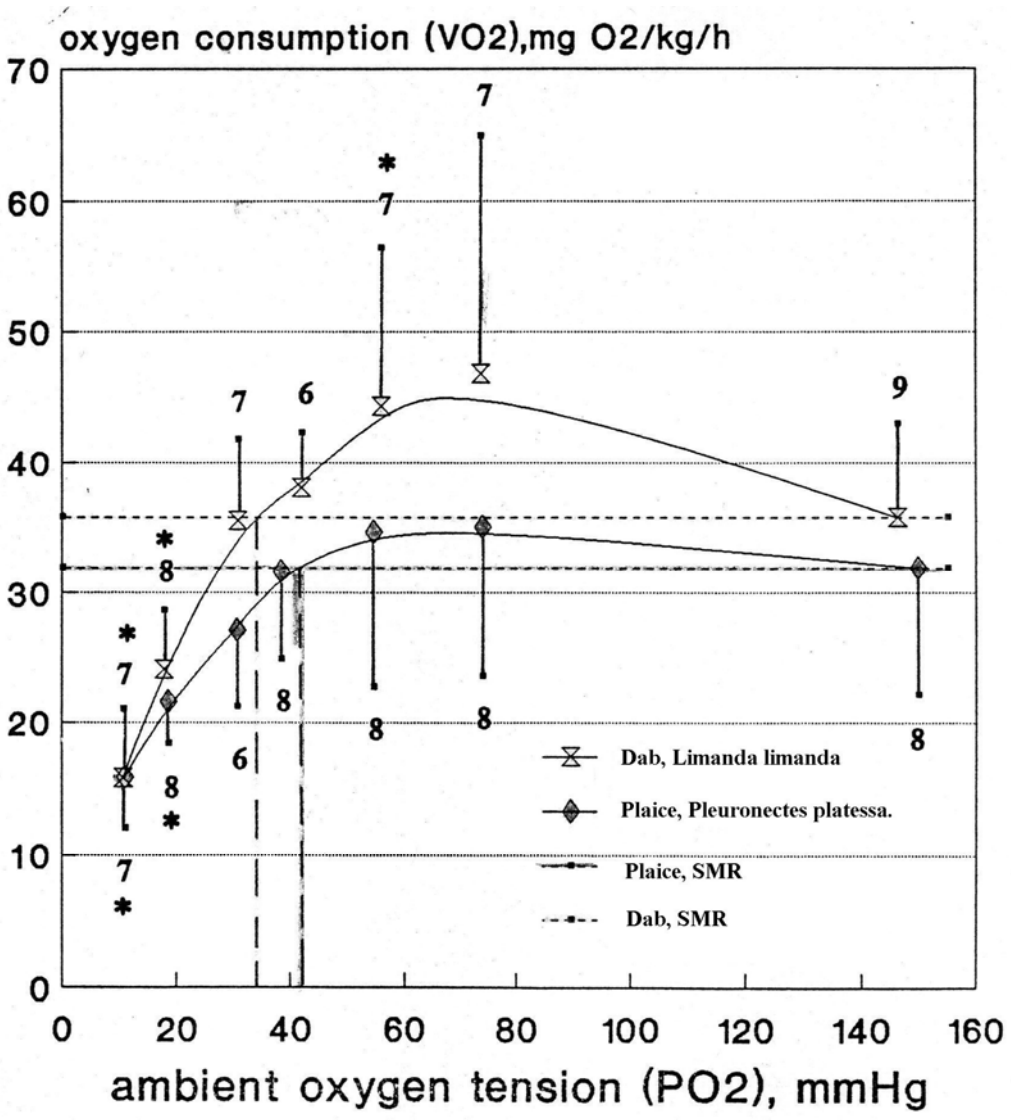

Figure 7. Representative oxygen consumption of 2 species of flatfish exposed to hypoxia. Both species behave as oxygen regulators. At intermediate hypoxia both species have an increased oxygen consumption that probably can be ascribed to a combination of cost of ventilation and spontaneous activity. From Søeborg *et al*. (1993).

Another example of a perfect oxygen regulator is the pikeperch, *Stizostedion lucioperca*, as seen in Figure 8 (Jungersen and Steffensen, submitted). The pikeperch is wide-spread in freshwaters of Northern and central Europe, and inhabits areas that regularly reach severe hypoxia. The critical oxygen partial pressure is approximately 25 mmHg, but this species can tolerate oxygen tensions as low as 10 mmHg for short periods without mortality.

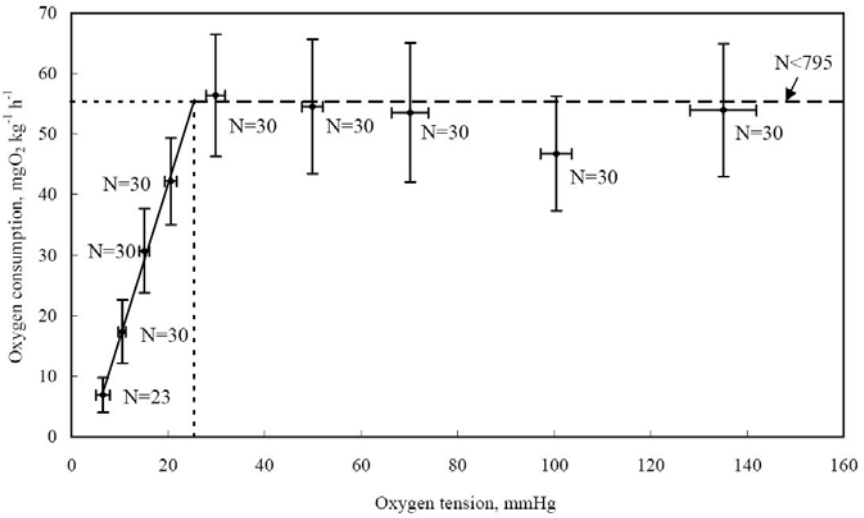

Figure 8. Oxygen consumption of pikeperch, , *Stizostedion lucioperca*, exposed to hypoxia. From Jungersen and Steffensen (Submitted).

Yet another example of a perfect oxygen regulator is the lesser sandell, *Ammodytes tobianus*, shown in Figure 9. This species is abundant in coastal waters in the North Sea, and it is believed to bury in hypoxic or anoxic sediments for long periods, even during the entire winter season. The lesser sandell does not have a swim bladder, and hence has to swim continuously to keep position in the water column. When the sandell isn't swimming it is either lying restless on the seafloor or buried in the sediment.

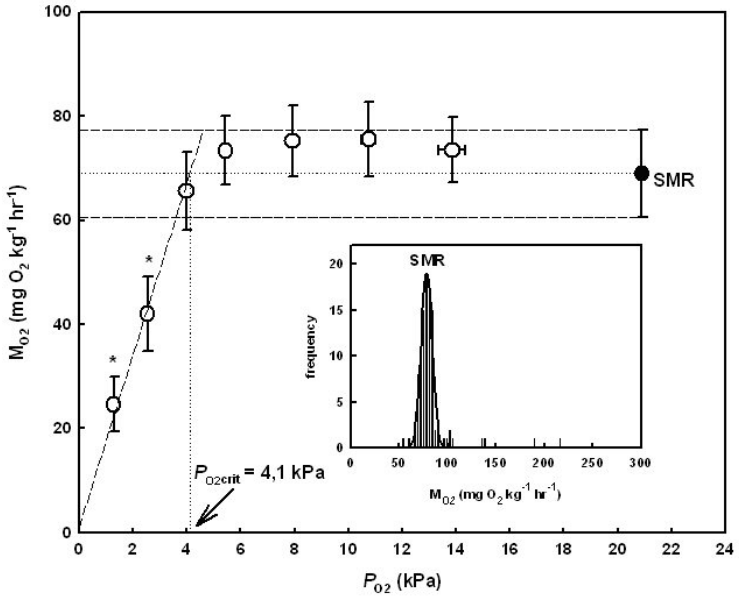

Figure 9. Oxygen consumption of lesser sandell, exposed to hypoxia. From Behrens and Steffensen (Submitted).

What about the sturgeons? Are they conformers as described by Burggren and Randall (1978), who also suggested that this response was an adaptive metabolic depression? A recent study by McKenzie *et al*. (in press) pointed out that it is an unusual behaviour for a sturgeon to be resting as in a respirometer since they normally are cruising around at swimming speeds of approximately 1 body length per second. McKenzie further suggested it could be a result of not being able to swim and ram ventilate. The results showed that resting Adriatic sturgeon behave as oxygen conformers when exposed to hypoxia, and that lactate levels increase while arterial oxygen content decreases in response to progressive hypoxia. While behaving as an oxygen regulator when allowed to swim at the preferred speed, neither lactate nor arterial oxygen content change in response to progressive hypoxia (McKenzie *et al*. in press).

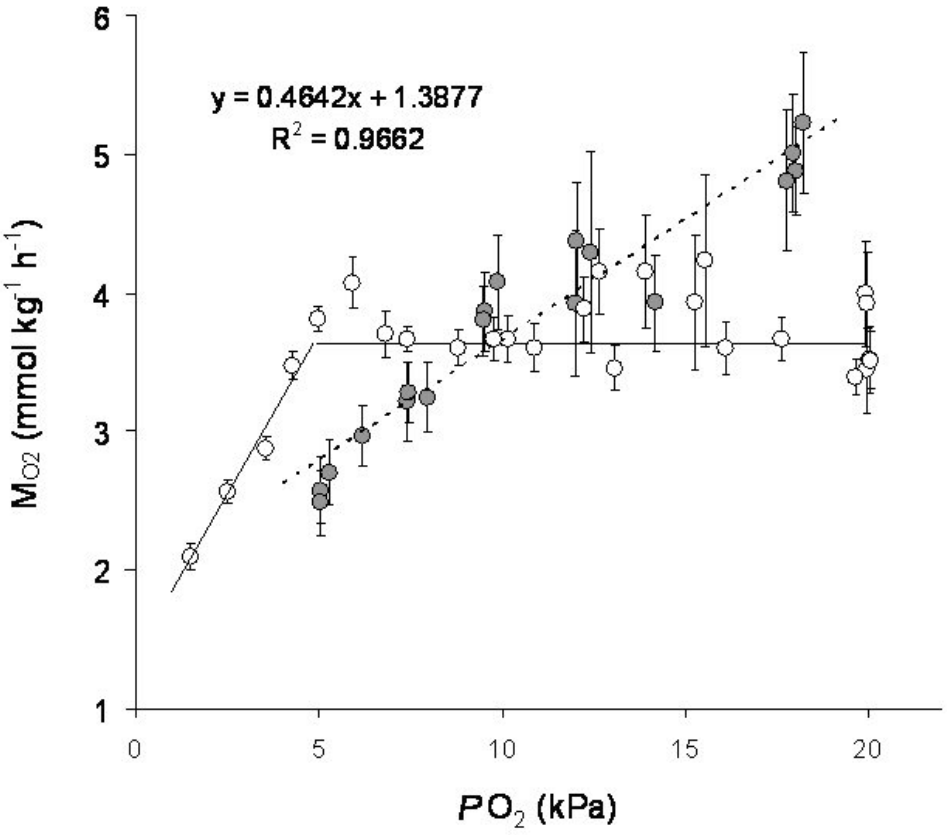

Figure10. Oxygen consumption of resting (filled circles) and swimming (closed circles) Adriatic sturgeon exposed to hypoxia. From McKenzie *et al*. (in press).

Conclusions

In conclusion I believe that most fish species behave as oxygen regulators when exposed to hypoxia within their natural temperature regime, and there are no a priori reasons why any vertebrate with a significant amount of functional respiratory pigment and a reasonable matching of ventilation and perfusion at the gas exchange surface

should not be able to regulate its standard metabolic rate over a wide range of oxygen tensions from normoxia to severe hypoxia. The reason that many investigators have shown T-curves can probably be ascribed to spontaneous activity, handling stress and or erroneous use of flow-through respirometry. It must be accepted, however, that at least some of the sturgeons are oxygen conformers when resting, as described by Burggren and Randall (1978), but may behave as oxygen regulators when allowed to swim at their preferred swimming speed according to McKenzie *et al*. (in press). Are there any other potential oxygen conformers? What about icefish that do not have any red blood cells and no functional respiratory pigment? Experiments in the near future will hopefully solve this question!

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