Phenotypic responses to lifelong hypoxia in cichlids

Rutjes, Hendrikus Antonius

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Promotor	Prof. Dr. M. K. Richardson
Co-promotoren	Dr. F. Witte Dr. G.J.E.E.M van den Thillart
Referenten:	Prof. Dr. L.J. Chapman (McGill University, Canada) Prof. Dr. G. Flik (Radboud Universiteit Nijmegen)
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Contents

CHAPTER 1:

GENERAL INTRODUCTION

H.A. Rutjes, F. Witte and G.J.E.E.M. Van den Thillart

Institute of Biology Leiden, Leiden University
P.O. Box 9516, 2300 RA Leiden, The Netherlands

Lake Victoria and its history

Lake Victoria is by surface the largest tropical lake in the world. With a maximum depth of 70 metres it is relatively shallow compared to the other Great Lakes of Africa (lakes Tanganyika and Malawi). Until the 1980s, the fish fauna was dominated by a species flock of over 500 cichlid species (Greenwood, 1974, Witte and Van Oijen, 1995; Seehausen, 1996). Amongst these species, specialists to virtually every possible food source existed in the lake (Greenwood, 1981; Witte and Van Oijen, 1995; Seehausen, 1996). The lake itself is less than a million years old and the latest data suggest that that a major desiccation event occurred in the late Pleistocene. The lake refilled again about 14.000 years BP (^{14}C 12,400 years). (Johnson *et al*., 1996). Whether the lake was completely dry during this desiccation event or if some remnant waters remained is still unclear (Johnson *et al*., 1996; Fryer, 1997, 2001; Seehausen, 2002). Nagl *et al*., (2000) suggested that the molecular diversity of the Lake Victoria cichlid fauna came into existence 250,000-750,000 years ago. When the ancestral population of the modern species flock entered the lake, when it refilled 14,000 years ago, their molecular polymorphism enabled these fish to rapidly diversify and radiate into the species flock that is known in the present day. It was suggested by Verheyen *et al*. (2003) that all haplotypes known in the modern Lake Victoria are much older than the desiccation event 14,000 years ago and that the major diversification had already occurred before the lake desiccated. The fact is that the cichlid fauna of Lake Victoria must have survived somewhere. Fryer (2001) suggested that a remnant lake or lakes and, in contrast to Seehausen (2001), not remnant streams and rivers have formed the major refuges for the ancestral cichlid species. Recently, in a river north of the Kalahari Desert, Joyce *et al*. (2005) found a riverine cichlid population of which the functional diversity is comparable to that of the functional diversity seen in Lake Victoria. It was concluded that this radiation is a remnant from a lake that desiccated ~2000 years BP and is currently a salt pan. Together, these findings suggest that it is possible for populations of fish to survive desiccation events by using rivers as a refuge.

During the last century, Lake Victoria and its fish fauna have been subject to anthropogenic perturbations leading to major ecological changes. Since the beginning of last century, fishing pressure continuously increased, leading to decreased catches of the tilapiine species (Fryer and Iles, 1972). In the 1950s and 1960s, exotic tilapiine species (Beauchamp, 1958; Welcomme, 1967) and the predatory Nile perch *Lates niloticus* (Arunga, 1981, Welcomme, 1988) were introduced. Initially, populations of these fish did not increase and fisheries were not boosted. In the 1960s a light fishery was developed for the small zooplanktivore cyprinid *Rastrineobola argentea*, and in 1976 trawl fisheries began on haplochromine cichlids, locally affecting cichlid populations. In the beginning of the

1980s, the Nile perch populations boomed, simultaneously with a collapse of the cichlid fauna (Barel *et al*., 1985; Ogutu-Ohwayo, 1990; Witte *et al*., 1992). Populations of *R. argentea* seemed to profit from the decline in cichlid numbers and populations increased considerably (Ogutu-Ohwayo, 1990; Wanink, 1991; Witte *et al*., 1999; Wanink and Witte, 2000b). The occurrence of algae blooms, mainly of cyanobacteria increased in the 1980s. Cyanobacteria replaced the diatoms that previously dominated in Lake Victoria (Verschuren *et al*., 1998). Data derived from sediment cores in the deepest part of the lake revealed that eutrophication, the probable cause of these algae blooms, must already have started between the 1920s and 1930s (Hecky, 1993; Verschuren *et al*., 1998, 2002). The eutrophication of the lake was strongly correlated with the increase of the human population in the region (Verschuren *et al*., 1998, 2002).

Occurence of hypoxia

The story of Lake Victoria is not unique. Human induced perturbations leading to eutrophication, algae blooms, and concomitant large-scale detrimental effects on aquatic life occur worldwide, correlated with the increase in human population (De Jonge *et al*., 2002). Examples of factors leading to eutrophication are run off from agricultural areas, deforestation, industrial discharge, and domestic wastewater. With eutrophication, hypoxia is often introduced in waters where it was uncommon before. This has a dramatic impact on distribution, species richness and densities of zooplankton, crustaceans and fish (Pihl *et al*., 1991, 1992; Pearson and Rosenberg, 1992; Roman *et al*., 1993; Diaz and Rosenberg, 1995; Karlson *et al*., 2002; Wu , 2002). Seasonal or periodic recurrence of hypoxia causes large-scale defaunation through migration and/or mortality. In Lake Victoria, upwelling of hypoxic water, which causes massive fish kills, has increased in frequency (Ochumba, 1990; Ochumba *et al*., 1993; Wanink *et al*., 2001). If possible, fish tend to avoid exposure to low O_2 levels (Wannamaker and Rice, 2000; Wanink *et al*., 2001), but during such upwelling events, fish are exposed to hypoxia suddenly and they are unable to flee or adjust their metabolism (Randall, 1970). In Lake Victoria, chronic hypoxia is nowadays present in much larger areas and for longer periods than before (Kaufman, 1992; Hecky, 1993; Hecky *et al*., 1994; Wanink *et al.*, 2001). In the deep waters of Lake Victoria, severe hypoxia (<1 mg O_2 L^{-1}) was present from October to March at depths of 40 to 54 meters (35% of the lake's bottom area) in 1990-91, whereas this level of hypoxia was observed only below 60 meter in 1960-61 (Hecky *et al*., 1994). In the more shallow Mwanza Gulf (<20m) in the South of the lake, periods of hypoxia became longer and the 1 and 5 mg O_2 L^{-1} isopleths moved upward in the water column between 1979 and 1988 (Wanink *et al*. 2001). This makes the lower part of the water column a less suitable habitat for demersal fish species. Not surprisingly, several researchers have hypothesised that, apart from the introduction of Nile perch, hypoxia was

an important factor in the decline of the haplochromine cichlids in Lake Victoria (Kaufman, 1992; Hecky *et al*., 1994; Verschuren, 2002).

Physiology and hypoxia

The duration of exposure to hypoxia has a large influence on the responses of fish. These responses can be behavioural, physiological, biochemical or anatomical. However, the relation between the duration of hypoxia and the type of response is almost never categorised. Especially physiological responses to hypoxia can be very different depending on the duration of hypoxia. Thus, I distinguish short-term hypoxia from chronic hypoxia. In my definition, short-term hypoxia takes several hours up to several days while chronic hypoxia lasts anywhere between a week and a permanent state of hypoxia.

Physiology of short-term hypoxia

During short-term hypoxia, behavioural and regulatory responses can lead to a decrease in energy consumption, improved O_2 extraction, and an increase of anaerobic metabolism (Van den Thillart and Van Waarde, 1985; Van den Thillart *et al*., 1994; Van Ginneken *et al*., 1995). Fish that are exposed to short-term hypoxia normally react with increased ventilation, reduction of external activity and, aquatic surface respiration (Van den Thillart and Van Waarde, 1985; Van den Thillart *et al*., 1994; Muusze *et al*., 1998; Chapman *et al*., 2002). When exposed to hypoxia suddenly, fish show stress responses correlated with low tolerance. When fish are given time to habituate to the new environment, metabolism can be decreased and stress responses minimised and tolerance is higher (Randall, 1970; Ultsch *et al*., 1981). The ability to tolerate short-term hypoxia is partly dependent on the coping strategy of the animal. From studies on sole, *Solea solea*, (Van den Thillart *et al*., 1994) and rainbow trout, *Oncorhynchus mykiss*, (Van Raaij *et al*., 1996) it is known that animals can either react with tranquil behaviour, or show escape responses. In the latter case, high levels of catecholamines and cortisol and low survival can be observed.

Upon gradually induced short-term hypoxia, energy consumption can be reduced to below the standard metabolic rate in many fish, enabling them to keep metabolism aerobic, which is a determining factor for hypoxia tolerance. At a certain level the metabolic rate exceeds the maximum O_2 extraction and activation of anaerobic metabolism is necessary to meet the total energy demand (Van den Thillart and Van Waarde, 1985; Van den Thillart *et al*., 1994; Van Ginneken *et al*., 1995). As a response to decreased O_2 levels, the perfusion of the gills is increased. This maximises the effective gill surface since all secondary lamellae are optimally perfused, which is not the case during routine activity under normoxia (Hughes, 1972). In *Oreochromis niloticus* the P_{50} of the blood is about 20 mm Hg. Thus, at such low O_2 conditions, we can assume that the blood will be only partially oxygenated in the gills. A common hypoxia response found invertebrates is to increase the

O_2 affinity of the haemoglobin (Hb). In fish, the organic phosphates ATP and GTP are the most important allosteric effectors of Hb, providing a means of rapidly changing the Hb-O_2 affinity (Weber and Jensen, 1988; Weber 1996, 2000; Val, 2000). Hypoxia exposure can already result in a decrease in organic phosphate concentrations within hours, which results in an increase of the Hb-O_2 affinity (Val, 1995; Weber, 1996).

Physiology of Chronic hypoxia

Exposure to chronic hypoxia causes activation of genes, leading to the production of new proteins and tissues (Gracey *et al*., 2000; Zhou *et al*., 2001; Wu *et al.*, 2002). This may result in increases of the O_2 extraction capacity, the anaerobic capacity, and in changes at the tissue level *e.g*. erythropoiesis and angiogenesis. Only few studies have been published on the effects of chronic hypoxia exposure in fish. Experiments with immature as well as adult fish showed that survival is mainly based on the reduction of (aerobic) energy expenditure. In tench, *Tinca tinca*, which were acclimated to 8.8% air saturation (AS) for 6 weeks, a 48% reduction in routine O_2 consumption was found (Johnston & Bernard, 1982a). In addition there was a 43-76% decrease in the perimeter of the capillaries in the muscles and a 60% reduction in volume density of the mitochondria. Stores of glycogen in the muscles and the activity of lactate dehydrogenase, both indicators of the anaerobic capacity, were somewhat increased but not significantly (Johnston & Bernard, 1982b). Experiments with carp, *Cyprinus carpio*, that were acclimated to hypoxia (20% AS, 30 mm Hg) for six weeks, showed about 50% lower O_2 consumption rates than normoxia acclimated (120 mm Hg, 80% AS) carp (Lomholt and Johansen, 1979). In the hypoxia tolerant goldfish, *Carassius auratus*, chronic hypoxia exposure led to depression in protein synthesis in the liver, and elevated enzyme activity that promote conservative use of glycogen stores in the muscles (Van den Thillart and Smit, 1984). Chronic hypoxia exposure of young carp (35 g) resulted in decreases in serum testosterone, estradiol and triiodothyronine. These hormonal changes were associated with retarded gonadal development, and a reduction in spawning success, sperm motility, fertilisation success, hatching rate, and larval survival (Wu *et al*., 2003). In these studies hypoxia lasted only 6-8 weeks and it is quite certain that fish were strongly limited in metabolism by the ambient oxygen concentration. One might wonder whether this type of response enables lifelong survival. Especially in the wild, where animals have to forage for food, defend territories, flee for predators etc. Theoretically, the best adaptation to chronic hypoxia permits unaffected aerobic energy production, and thus a high oxygen extraction capacity under hypoxic conditions.

MORPHOLOGY AND HYPOXIA

Variation in gills

Gills of fish are designed to facilitate optimal gas exchange between water and

blood. At maximal efficiency, all oxygen diffuses from the water into the blood during the water passage between the secondary lamellae. According to Fick's first law of diffusion, the net gas exchange between water and blood is dependent on the concentration difference, which is reduced under hypoxia. Thus, at lower air saturation (AS) levels, more water must be ventilated per unit of time to meet the oxygen demand. At moderately decreased O_2 levels, perfusion of normally not used secondary lamella and a higher ventilation frequency is sufficient to maintain the same O_2 uptake (Hughes, 1972). At a certain AS-level however, increased ventilation rate results in an increase of the speed of water flow over the gills by such a degree that this decreases gas exchange efficiency. In Nile tilapia, *Oreochromis niloticus*, the O_2 uptake efficiency is already decreased at ca. 50% AS, (Fernandes and Rantin, 1994). As Fick's law also dictates that the diffusion rate is dependent on the gill surface, fish exposed to chronic hypoxia would benefit from an increased respiratory surface that allows for high ventilation rates. Hughes (1966) modelled the gills of fish as a series of rectangular channels and predicted the effects of increasing gill surface on water flow and resistance. He concluded that increases in the respiratory surface could be achieved without drastic increases in the resistance by longer filaments and higher secondary lamellae. In contrast, an increase of the respiratory surface by higher frequency of the secondary lamellae or longer secondary lamellae causes large increases in resistance and would therefore be unfavourable.

Size and shape of the gills are similar in fish species that live in comparable habitats or have similar life styles and related O_2 demands (Gray, 1954; Hughes, 1966; 1972; 1973; Palzenberger and Pohla, 1992). Galis and Barel (1980) compared gill dimensions of cichlids from different East African lakes. Within the pharyngeal mollusc crushers of the Lake Victoria cichlids that were included in that study, there was a positive relation between the density of the secondary lamellae and depth at which each species was found. This was explained as an adaptation to the decreased oxygen concentrations at larger depth. The Lake Victoria cichlids *Haplochromis hiatus* and *H. iris*, resemble each other ecologically and morphologically. *H. hiatus* is found between 3 and 9 m depth while *H. iris* is found at a depth of 8-15 m (Hoogerhoud *et al*., 1983). During the rainy season, stratification occurs and O_2 concentrations of 2-3 mg L^{-1} have been observed in the habitat of *H. iris* (Van Oijen *et al*., 1981). The total gill area of this species is 1.6 times greater than that of *H. hiatus* (Hoogerhoud *et al*., 1983). In contrast to the numerous studies that compare interspecific differences in gill size and shape in relation to habitat and mode of life, the phenotypic plasticity of gills in different environments was hardly studied. Phenotypic plasticity is the ability to produce a different phenotype in response to changes in the environment. Phenotypic plasticity of the gill size and shape would enable fish to survive and adapt to a broader range

of habitats and increase fitness during hypoxia.

Anatomical changes under short-term hypoxia

Some highly specialised fish species can alter anatomical features to facilitate oxygen uptake under short-term hypoxia. The Crucian carp *Carassius carassius* has the ability to increase gill size within days (Sollid *et al*., 2003, 2005). Under normoxic conditions, its gill filaments show hardly any protruding secondary lamellae. When the animal is acclimated to hypoxia (6-8% AS, 0.75 ± 0.15 mg L^{-1}) apoptosis of interstitial cells occurs and already existent, normally functional secondary lamellae emerge, thus dramatically increasing the respiratory surface (Sollid *et al*., 2003). In a later study it was shown that this also occurred at higher temperatures in both Crucian carp and goldfish, *Carassius auratus* (Sollid *et al*., 2005). This was attributed to an increased metabolic rate and thus increased oxygen demand. A phenotypic response of a totally different kind is found in the Amazonian fish tambaqui, *Colossoma macroponum*. Under hypoxia this fish is able to extend its lower lip within an hour, enabling it to skim the oxygen-rich surface layers of the water column (Almeida-Val *et al*., 1993). The hypoxia responses in the tambaqui, Crucian carp and goldfish concern fully reversible adaptations occurring under short-term hypoxia.

Anatomical changes at Chronic hypoxia

Relations between gill size and chronic hypoxia have been reported several times within Lake Victoria fish. In the early 1980s, shortly after the Nile perch boomed and, during the period at which chronic hypoxia manifested itself, *R. argentea* populations increased as well. The total number of gill filaments on the first gill arch of *R. argentea* caught in 1988 was 3.6% larger than that of fish that were caught in 1983 (Wanink and Witte, 2000b). However, it is not known whether the differences in filament number were a result of environmentally-induced plasticity or genetic change. The only known study concerning phenotypically induced changes in gills is that of Chapman *et al*. (2000), who raised fry of the non-endemic Lake Victoria cichlid *Pseudocrenilabrus multicolor victoriae* under normoxia (>7.5 mg L^{-1}) and hypoxia (1 mg L^{-1}). At an age of 6 months the HR groups had a 22% larger total gill surface, mainly caused by greater filament length and number (calculated by setting NR fish at 100%). Specimens of the same species from a normoxic and hypoxic habitat showed a 41% difference in gill surface. The fish from the hypoxic habitat showed, in addition to longer filaments, both increases in the surface area of the secondary lamellae as well as in the number of pores.

Consequences and constraints of increasing gill size

Heads of fish are densely packed with muscles, bones and other structures, that are necessary for respiration, vision, feeding and other functions. If, through chronic exposure to hypoxia, it is needed to increase the gill size and stroke volume, extra space may be needed to accommodate the gills and muscles. It was suggested that in fish living at low oxygen concentrations, possible enlargement of the gills could have such dramatic effects that the surrounding structures, and even gross morphology of the head, are affected (Smits *et al*., 1996 b; De Visser and Barel, 2000). Theoretically, creation of extra space may be realised in several ways: (1) use of free space within the head; (2) reduction of surrounding structures; (3) increase of the head volume; (4) a combination of the previous possibilities (Witte *et al*., 1990; Barel, 1993; Smits *et al*., 1996 a, b; Chapman *et al*., 2000). Solutions 2-4 may have a negative impact on the performance of the animal by transformations of anatomically surrounding structures and decreased streamline (Barel, 1993).

De Visser and Barel (2000) found that the shape variance of the head of 73 species of East African cichlids from different lakes was related to anatomical specialisation to different methods for collecting and processing food *e.g.* biting, sucking, mollusc crushing. Additionally, an important part of the inter-specific variation in head shape could be explained by changes in the width of the ventral part of the suspensorium and operculum. Furthermore, Smits *et al.*, (1996b) demonstrated that the same morphological variation could also occur intra-specifically. However, they did not show whether it concerned phenotypic plasticity or genetic variation. Phenotypic enlargement of the width of the ventral part of the suspensorium and operculum would enable cichlids to create extra space within the head. Such changes in the shape of the head may also have a negative impact on the performance of the animal through transformations of anatomically surrounding structures and decreased streamline (Barel, 1993).

Can Lake Victoria cichlids thrive under hypoxia?

Studies on fish that are chronically exposed to hypoxia and that include parameters such as external activity (Johnston and Benard, 1982a; Petersen and Petersen, 1990; van Raaij et al., 1996) oxygen consumption (Lomholt and Johansen, 1979; Johnston and Bernhard, 1982a), enzyme activity (Greaney *et al*., 1980; Johnston and Bernard, 1982b; Van den Thillart and Smit, 1984), reproduction (Wu *et al*., 2003) growth and feeding rate (Chabot and Dutil, 1999; Thetmeyer *et al*., 1999), all show that fish are limited by exposure to chronic hypoxia. In contrast with this, pilot tests with the cichlid *Astatoreochromis alluaudi* showed that young that were recently hatched and released by their mother, could be acclimated to and raised to adulthood at 10% air saturation (AS) levels at 25°C without increased mortality rates (Van den Thillart and Witte, unpublished

data). During the first days at which the juvenile fish were at 10% AS and later on in the experiment, no differences in behaviour were observed between NR and HR fish and they grew to adulthood at the same rate. At adulthood the fish regularly produced nests with normal viable young. These are strong indications that *A. alluaudi* that were raised at 10% AS were, in contrast to the fish in the studies mentioned above, not limited by chronic hypoxia but can even thrive at such an extreme condition.

Aim and overview

Given the trend of increasing occurrence of chronic hypoxia worldwide in the last decades, and the fact that to date, no large system has recovered after persistent hypoxia (Diaz and Rosenberg, 1995), there is a need for more understanding of the effects of chronic hypoxia. The current knowledge on the effects of hypoxia on fish is mainly based on short-term hypoxia studies. Few studies on chronic hypoxia exist, and hypoxia exposure in these studies lasted only several weeks and the fish used were much older than the ones that were used in the pilot study on *A. alluaudi*. Since this pilot study showed that Lake Victoria cichlids can thrive at very low AS levels, this raises questions as to whether the effects of chronic hypoxia are the same in young and adult fish. When exposed to hypoxia from their youth up, the phenotypic responses of cichlids enable them to maintain high metabolic rates This thesis focussus on the phenotypic responses that occur in cichlids that are raised under hypoxic conditions from a post-larval stage to adulthood.

Split brood experiments are very useful in exploring phenotypically plastic responses to hypoxia. In the following 5 research chapters, broods of fish at a post-larval stage were split, after which one half was raised at 80% AS and the other at 10% AS for 1-2 years. Of the adult fish, behavioural, morphological and physiological differences between the NR and HR groups were studied.

In Chapter 2, I describe respirometry experiments that are used to investigate the O_2 consumption of NR and HR tilapia at different AS levels. It is hypothesised that HR fish are less active and have an increased O_2 extraction capacity. HR fish should therefore be able to extract more O_2 at 10% AS than NR fish at the same condition. The adaptive significance of the observed phenotypic responses to chronic hypoxia exposure is discussed. Also an explanation for differences with chronic hypoxia studies of other authors is given.

In Chapter 3, the similar experiments are described, using two mollusc crushing Lake Victoria cichlids *A. alluaudi* and *Haplochromis* (*Labrochromis*) *ishmaeli*. Both species have a similar ecology except that the first is a facultative swamp-dwelling species. It is expected that NR *A. alluaudi* are more hypoxia tolerant than NR *H. ishmaeli*. The hypothesis is tested that HR fish of both species have a higher metabolic rate at 10% AS than NR fish at the same condition. In addition, the hypothesis is tested that HR fish have greater anoxia tolerance than NR fish. In this chapter it

is tried to link the respiratory physiology of NR and HR animals to the modern and paleo-ecology of the animals.

In Chapter 4, a split brood experiment on *H. pyrrhocephalus* is described. The hypothesis is tested that the respiratory surface of HR cichlids is enlarged compared to NR siblings. Measurements on the dimensions of the primary filaments, and secondary lamellae are performed. The functional consequences of the observed differences in gill shape on gas exchange, water flow and resistance are discussed. Also the degree of phenotypic plasticity is compared with inter specific variation in gill shape.

In Chapter 5, it is hypothesised that gill enlargement associated with lifelong hypoxia requires such large internal reorganisations that outer head shape is affected. We expect that the head volume of HR fish is larger than that of NR fish. Broods of cichlids of different phylogenetic lineages, habitats and trophic specialisation, are used. With a three-dimensional model the volume of the oral, suspensorial and opercular compartments are estimated. The observed transformations in head shape are compared with those in other studies and discussed in a functional context.

In Chapter 6, the physiological responses of cichlids exposed to lifelong hypoxia are investigated. Blood and white muscle tissue samples of adults were taken for analysis of physiological parameters for anaerobic metabolism (glycogen and total creatine levels and lactate dehydrogenase activity) and aerobic metabolism (blood haemoglobin concentration, haematocrit, intra-erythrocytic ATP and GTP levels and citrate synthase activity). When comparing NR fish to HR siblings, we hypothesise that first, HR fish are not stressed, second, HR fish have increased O_2 uptake capacity, third, the aerobic capacity of the muscles of HR fish is unaltered, fourth, the P_{50} of the blood is decreased.

In Chapter 7, the results and conclusions are summarised and a general discussion on the results of this thesis is held.

CHAPTER 2:

UNAFFECTED OXYGEN CONSUMPTION UNDER LIFELONG HYPOXIA IN TILAPIA

H.A. Rutjes, F. Witte and G.J.E.E.M. Van den Thillart

Institute of Biology Leiden, Leiden University
P.O. Box 9516, 2300 RA Leiden, The Netherlands

Abstract

Brood of tilapia *(Oreochromis mossambicus* x *O. niloticus)* was split and raised under normoxia (80-90% air saturation) and hypoxia (10% air saturation) at 25.5°C for 10-15 months. The brood survived and grew equally well in both groups. The adult animals were tested for routine metabolic rate and hypoxia tolerance. In a 3-day protocol the O_2 consumption rates were measured under control conditions (respectively 80% or 10% air saturation) and at stepwise decreasing oxygen levels, namely 80, 40, 20, 10 and 5% air saturation. Under control conditions there was no difference in resting, routine or maximum O_2 consumption rate between normoxia- and hypoxia-raised fish. This strongly suggests that the hypoxia-raised fish did not reduce their activity level despite an 8-fold lower oxygen level. At all air saturation levels the O_2 consumption rate of hypoxia-raised fish was higher than that of normoxia-raised siblings, except during the recovery period. This indicates that hypoxia-raised fish had a higher O_2 extraction capacity and were less sensitive to 5% air saturation. In contrast, all literature known on chronic hypoxia, performed on older animals, shows that animals survive by decreasing metabolic needs rather. The unimpaired aerobic metabolism of hypoxia-raised tilapia shows that young cichlids are more plastic and are able to follow a strategy of increasing O_2 extraction capacity with decreasing air saturation levels instead.

Introduction

Van Dam (1938) was the first to publish on the effects of hypoxia on ventilation and O_2 consumption in fish after which many authors followed. Below a critical level, hypoxia limits the oxygen consumption. Oxygen consumption is limited by mainly 3 factors: the oxygen gradient, the oxygen extraction capacity and the oxygen demand. The oxygen gradient is determined by the environment, the oxygen extraction capacity is limited by the gill structure and blood characteristics and the oxygen demand is mainly determined by behaviour. The range of energy demand can be very large. Energy consumption normally varies between standard metabolic rate and at least a 10-fold higher active metabolic rate. When exposed to hypoxia, many fish species are able to reduce energy consumption to below their standard metabolic rate, which is a determining factor for hypoxia tolerance. Fish species differ widely with respect to their ability to cope with hypoxia (see listing in Table 1). The duration of hypoxia exposure has a large influence on the hypoxia responses in fish. However, this relation is almost never categorised. We distinguish short-term hypoxia from chronic hypoxia. Exposure to short-term hypoxia takes several hours up to several days. During this period, behaviour and regulatory changes are important that enable a decrease in O_2 demand, a maximised O_2 uptake, and an increase of anaerobic metabolism (Van den Thillart and Van Waarde, 1985; Van den Thillart *et al*., 1994; Van Ginneken *et al*., 1995). Fish that are exposed to short-term hypoxia normally react with increased ventilation and a reduction in external activity (Van den Thillart and Van Waarde, 1985; Van den Thillart *et al.*, 1994; Muusze *et al.*, 1998). If the metabolic needs exceed the maximum O_2 extraction, suppression of the standard metabolism follows, accompanied by activation of anaerobic metabolism to meet the total energy demand (Van den Thillart and Van Waarde, 1985; Van den Thillart *et al*., 1994; Van Ginneken *et al*., 1995). If hypoxia is induced quickly (acute hypoxia exposure), it generally leads to stress responses resulting in low tolerance. When fish are given time to habituate to the new environment, metabolic rate can be decreased and stress responses avoided, resulting in higher hypoxia tolerance (Randall, 1970; Ultsch *et al.,* 1981). The ability to cope with short-term hypoxia is partly dependent on the coping strategy of the animal. From studies on sole, *Solea solea*, (Van den Thillart *et al.,* 1994) and rainbow trout, *Oncorhynchus mykiss*, (Van Raaij *et al*., 1996) it is known that animals can either react with tranquil behaviour, or show escape responses. In the latter case, high levels of catecholamines and cortisol can be observed and survival is low. During chronic hypoxia, which lasts between a week of exposure and permanent exposure, altered gene

Table 1: *Responses to hypoxia that are commonly found in fish: 1) Avoidance, 2) Increased ventilation activity, 3) Reduction in motor activity, 4) Reduced metabolic rate, 5) Increased anaerobic metabolism.*

Author	Observed hypoxia response	Reduced metabolism at:	Temperature (°C)	Species
Beamish, 1964	2, 3, 4	20% AS 30% AS 32% AS	10 °C, 20 °C 10 °C, 20 °C 10 °C, 15 °C	*Carassius auratus* *Cyprinus carpio* *Salvelinus fontinalis*
Fernandez and Rantin, 1989	2, 4	13% AS	20°C, 25°C	*Oreochromis niloticus*
Lomholt and Johansen, 1979	2, 3, 4	20% AS	?	*Cyprinus carpio*
Muusze *et al.*, 1998	4, 5	20% AS	28 °C	*Astronotus ocellatus*
Petersen and Petersen, 1990	1	Mortality at 15% AS	15 °C	*Pomatoschistus minutus*
Van Ginneken *et al.,* 1997	3, 4, 5	below 15% AS	20 °C	*Oreochromis mossambicus*
Van Raaij *et al.,* 1996	1	Mortality at 25% AS	15 °C	*Oncorhynchus mykiss*
Van den Thillart *et al.*, 1980	4, 5	13% AS	20 °C	*Carassius auratus*
Van den Thillart *et al.*, 1994	1, 2, 3, 4,	12% AS	19 °C	*Solea Solea*

expression can lead to the production of new enzymes, proteins etc (Zhou *et al.*, 2001). An additional distinction must be made between chronic exposure of animals at different life stages. When animals are exposed to hypoxia from a post larval stage until adulthood (lifelong), ontogenetic changes and large differences in growth may influence hypoxia responses. Only few studies have been published on the effects of chronic hypoxia exposure. Experiments with immature as well as adult fish, showed that survival is mainly based on the reduction of (aerobic) energy expenditure by decreasing the total growth, gonadal growth and external activity (Lomholt and Johansen, 1979; Van den Thillart *et al.*, 1980; Johnston and Bernard, 1982a, 1982b; Zhou *et al.*,

2000; Wu *et al.*, 2003). In the hypoxia tolerant goldfish, *Carassius auratus*, chronic hypoxia exposure resulted in depressed protein synthesis in the liver, and elevated enzyme activity that promote conservative use of glycogen stores in the muscles (Van den Thillart and Smit, 1984). Indubitably, the metabolism of the fishes in these studies was strongly limited by the ambient oxygen concentration. One might wonder whether this type of response enables lifelong survival. Theoretically, the best adaptation to chronic hypoxia should permit unaffected aerobic energy production, and thus a high oxygen extraction capacity under hypoxic conditions.

The capacity of fish to adapt to environmental challenges might well be related to age dependent plasticity of the animal. Pilot tests with recently hatched young of the cichlid *Astatoreochromis alluaudi* showed that these fish could acclimate to 10% air saturation (AS) levels at 25°C (Van den Thillart and Witte, unpublished data). The juvenile *A. alluaudi* were able to grow to adulthood at the same rate as their normoxia-raised siblings. In addition, hypoxia-raised (2 years) *A. alluaudi* from a later experiment, had an 80% enlarged gill area and 30% higher haemoglobin and haematocrit levels (Chapter 4). This indicates that phenotypic plasticity plays an important role in surviving chronic hypoxia and that young cichlids are more plastic than adults. We hypothesised that two major adaptive responses occur in fish under lifelong hypoxia. First, hypoxia-raised fish should have reduced routine activity levels, allowing more energy for growth. This is in fact a reallocation of the available energy. Second, hypoxia-raised fish should have an increased oxygen extraction capacity compared to normoxia-raised siblings. Both responses *i.e.* reduced activity and improved O_2 extraction, should become evident from respirometry experiments. From the routine O_2 consumption rates, an impression of the energy consumption at the control conditions (*e.g.* the AS level at which fish were raised) can be obtained, while the response to stepwise decreasing hypoxia provides an impression of the oxygen extraction capacity. In this study we conducted a split brood experiment on tilapia that were raised under 10% AS and 80-90% AS. When animals were adult, oxygen consumption was measured under the conditions that the animals were raised at, as well as under progressive hypoxia.

Material and Methods

Animals and conditioning

A commercial strain of *Oreochromis mossambicus* (hybridized with *Oreochromis niloticus*) was used. Of one brood with young measuring 0.5-1 cm standard length (SL), that were just released by the mother (three weeks after fertilisation) six groups of 30 animals

were selected randomly. Three groups were raised at normoxia (80-90% air saturation; AS) and three at hypoxia (10% AS). For the latter, the air saturation level of the water was lowered stepwise from normoxia to 40%, 30%, 20%, 15%, 12% and 10% in four weeks time. All animals were kept at a temperature of 25.5 °C and a day-night cycle of 12-12 hrs. They were fed with cyclops, Duplarin (Dupla Aquaristik GmbH) and from about 5 cm SL onwards with commercial 4.5 mm tilapia pellets (Trouw Nutritions BV). After 6 months the number of animals in the aquaria was reduced to 15 by removing the largest and smallest individuals. After 10-15 months respirometry experiments were performed on 8 normoxia-raised (NR) and 7 hypoxia-raised (HR) tilapia, from one group each. At an age of 15 months, all animals were killed and used for other projects. Of two NR and HR groups the length and weight was recorded for determining the length-weight relationship.

Conditioning

The fish were raised in tanks of the same dimensions (45x50x50 cm). The glass tanks contained an extra compartment from where water was pumped into the animal compartment to ensure fast mixing with the inflow of hypoxic water. Hypoxia was reached by a continuous inflow of degassed water (6-9% AS) at a rate of 1-2 L min^{-1}. A stainless steel plate that was placed 3 cm below the water level prevented oxygen uptake from the air by the fish, as well as by the circulating water. The oxygen level of the water was regulated by Applikon biocontrollers (ADI 1030) equipped with polarographic oxygen sensors (Applikon ZZ71202AP10) switching solenoid valves in line with air diffusers. Thus, air was bubbled automatically through the water in the extra compartment, when the oxygen level was below the setpoint. This way the oxygen level in the animal chambers could be kept constant. Water in the normoxia and hypoxia tanks was continuously refreshed from the same biological filter system. Before flowing into the hypoxia tanks the water was degassed by a vacuum system as described by Van den Thillart and Smit (1984).

Respirometer set up

Open flow respirometers were used for oxygen consumption measurements (Figure 1). Per experiment, individual fish were placed in 4.2-liter rectangular flow chambers with a transparent lid, allowing observations during the experiment. Grids were placed at the inflow and outflow of the chamber to achieve a low equally distributed water flow through the whole flow chamber such that the animal would not or hardly drift away by the flow. With a polarographic oxygen sensor connected to a biocontroller (Metrohm PH-signal amplifiers, E561), the AS level was kept constant. When the AS level dropped

below the desired value, the biocontroller activated a solenoid valve allowing inflow of air-saturated water. The water volume was measured with flowmeters (Rhodes, "lowflo" transmitters). Per 2 minutes a computer recorded the total added water and the concentration of the circulating water with software designed by the electronics service department of the Institute of Biology Leiden. A 150-litre tank that was filled with water, from the system in which the fish were raised, was used as a reservoir and continuously aerated and irradiated with UV light.

From the concentration difference and the amount of water used to keep the AS level constant, the O_2 consumption could be calculated according to:

$V_{O2} = v\ (c_{in}\text{-}c_{out})$ mg $O_2\ h^{-1}$
(Van den Thillart and Verbeek, 1991)

V_{O2} = O_2 consumption of the fish in mg $O_2\ h^{-1}$
v = Flow rate in L h^{-1}
c_{in} = O_2 concentration of in-flowing water in mg L^{-1}
c_{out} = O_2 concentration of out-flowing water in mg L^{-1}

Both c_{in} as well as c_{out} remained constant throughout the experiment

Respirometry protocol
To obtain a stable metabolic rate of the fish, they were not fed for 36-48 hours prior to the respirometry experiment. Oxygen consumption rates were measured under control conditions during 36 hours, after which all fish were exposed to the same experimental protocol (Table 2). The control conditions were 80% AS for the NR fish and 10% AS for the HR fish. The fish were kept at the same day-night cycle as they were raised under. To prevent disturbance of the fish by the presence of the experimenter, light at daytime was reduced to about 3 lux by covering the transparent lid. Observations at daytime showed that the animals regularly moved around. After

Table 2: *The experimental animals and respirometry protocols that they were exposed to. For this study, normoxia-raised or hypoxia-raised fish from the same brood were used. They were kept at the acclimation level (Control; respectively 80% and 10% air saturation for normoxia and hypoxia-raised fish) for 24 hours, followed by stepwise changes in AS level as indicated. The AS level decreased about 10% per hour in between subsequent lower AS levels. After the desired AS level was reached, the animals were kept at that level for four hours. Number of fish (N), weight in grams ± standard deviation (SD).*

	N	Weight ±SD	Hours of exposure at each AS level:						
			Control	80%	40%	20%	10%	5%	Recovery
Normoxia-raised	8	91.9 ±43.8	24 (80%)	4	4	4	4	4	1 (80%)
Hypoxia-raised	7	73.4 ±41.2	24 (10%)	4	4	4	4	4	1 (10%)

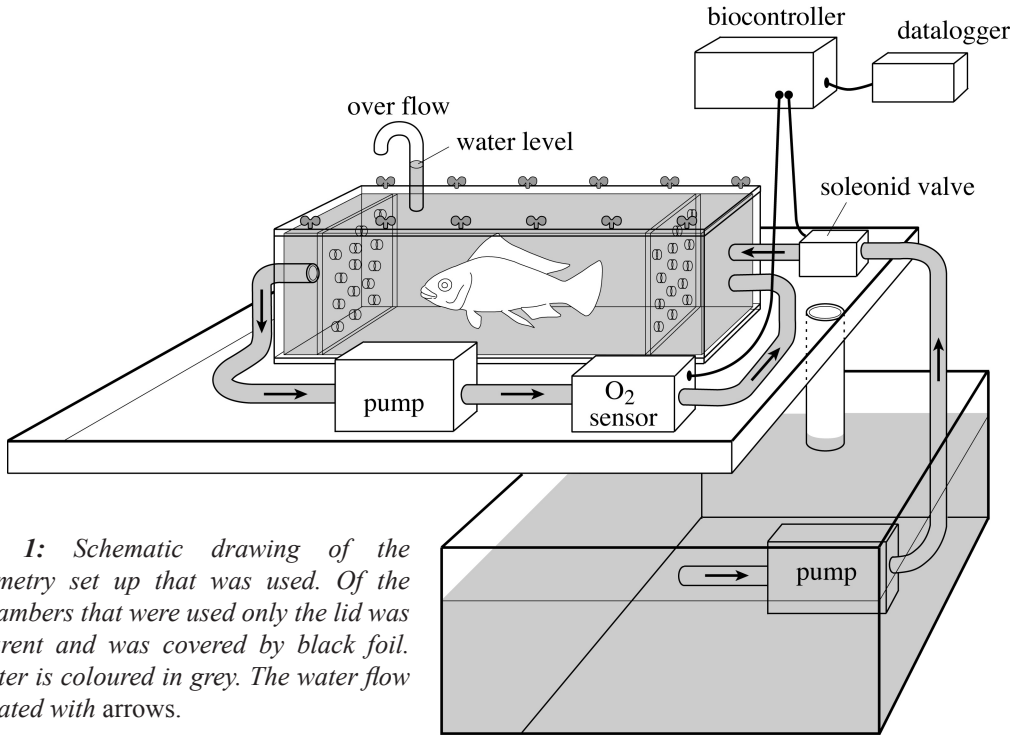

Figure 1: *Schematic drawing of the respirometry set up that was used. Of the flow chambers that were used only the lid was transparent and was covered by black foil. The water is coloured in grey. The water flow is indicated with* arrows.

each experiment a blank experiment was done without fish to determine the oxygen consumption caused by micro-organisms. This varied between 0.17 and 0.25 mg O_2 L^{-1} h^{-1}, which was below 15% of the total consumption and was used to correct the data. Occasionally, video recordings, and remote observations with video equipment, were made during the respirometry experiments.

Data analysis

The O_2 consumption per fish was corrected for weight differences according to Van den Thillart & Kesbeke (1978):

$$M_{100} = M\ 100^{0.8} / W^{0.8}$$

Where M_{100} is the metabolic rate for a fish of 100 grams, M is the measured metabolic rate and W is the weight of the fish. The exponent 0.8 appears to be the same for different fish species (Basu, 1959; Beamish, 1964; Duthie, 1982; Yamamoto 1991), including *Oreochromis niloticus* (Yamamoto, 1992), and at

different temperatures. During the experiments oxygen consumption was recorded every 2 minutes resulting in a large number of data for each AS level. A frequency distribution was made for each oxygen level of which the 5-, 50-, and 95-percentile was determined. These 3 percentiles represent the resting, routine, and the voluntary active level (Van den Thillart *et al.*, 1994). The voluntary active level should not be mistaken for the active level as measured at forced activity in, for instance, swimming experiments. In our respirometer set up, the animals were only able to swim for about 2 body lengths. The difference between the 5- and 95-percentile was considered as the scope for routine activity (Van den Thillart *et al*., 1994). Statistics were performed with the program SPSS for Windows version 10.0. To determine whether the oxygen consumption rates differed between NR and HR animals, ANCOVA was performed on the consumption rates under control conditions using weight as a covariable. On the O_2 consumption data at the 80%, 40%, 20%, 10%, and 5% AS level repeated measures ANOVA was performed to determine whether the oxygen consumption patterns were different between NR and HR animals. To test whether the oxygen consumption rates differed within the NR and within the HR group, separate repeated measures ANOVA were performed on all subsequent AS levels for the NR and HR group.

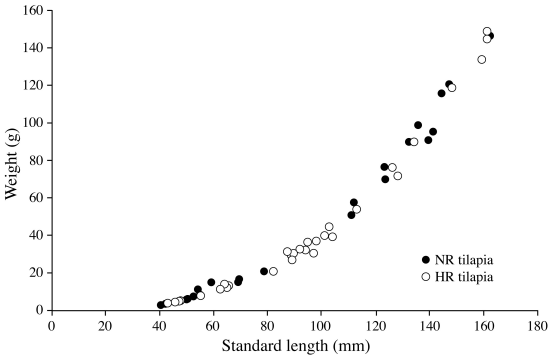

Figure 2: *Standard length against weight of harvested tilapia. Data from NR and HR siblings in neighbouring aquaria were included.*

Results

General observations

During the conditioning experiment, standard length was regularly measured of all NR and HR animals. The data showed no notable differences in growth between the NR and HR animals. Mean length of animals in the 6 groups differed only slightly and overlapped between NR and HR groups. The relation between length and weight of NR and HR animals was very similar (Figure 2). No deaths occurred in the HR group, in the NR group 2 animals died due to fighting.

Observations of the growing fish during the conditioning experiment showed that the HR tilapia were less aggressive than the NR animals. During feeding the NR animals were also very gluttonous while HR animals would wait and took more time to finish eating the same

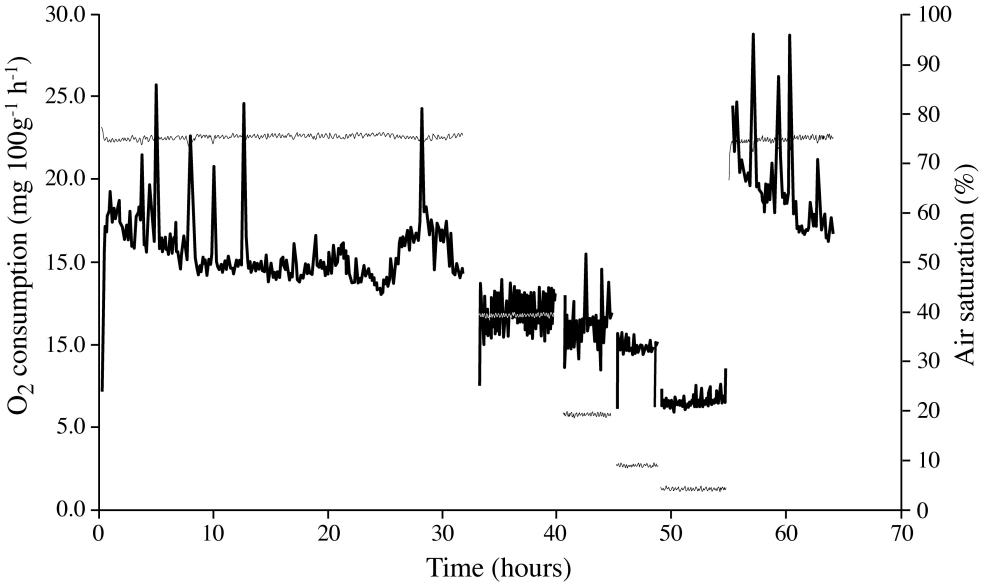

Figure 3: *Oxygen consumption of a tilapia in a typical respirometry experiment. Oxygen (fat line) consumption was measured of individual tilapias at the conditions they were raised at and during a series of stepwise decreasing AS levels (thin line, for protocol see Table 2). The protocol used is given in Table 2. For the particular experiment shown here, a normoxia-raised tilapia weighing 98.6 grams was used.*

amount of food. Observations during the respirometry experiments with a camera, showed that under control conditions, all animals (NR as well as HR fish) usually hovered above the bottom and displayed regular swimming activity. In contrast during the hypoxia protocol, NR and HR animals were virtually motionless at 10 and 5% AS. No fin movements were observed and animals usually leaned on a fin or against the wall of the flow chamber. From time to time the animals became active for a short period at a low level. No quantification was made of the activity.

A typical respirometry experiment is shown in figure 3. The oxygen consumption was measured every 2 minutes, thus yielding about 1500 data points for each experiment. The oxygen consumption rates, corrected to the metabolic weight of a fish of 100g, showed large variations, most likely due to changes in activity of the animal. The highest oxygen consumption for this experiment was 29.02 and the lowest 5.27 mg O_2 100g^{-1} h^{-1}. During the control period in the respirometry experiments, the fish would sit on the bottom and were immobile from time to time. It is likely that the metabolic rates at these moments

are close to the standard metabolic rate. During the stepwise lowering of the oxygen level (to respectively 40, 20, 10, 5% AS) the range between low and high oxygen consumption became smaller. The mean oxygen consumption rates at those levels were 11.05, 10.23, 8.78 and 6.43 mg O_2 $100g^{-1}$ h^{-1} respectively. Obviously, the standard metabolic rate became depressed during hypoxia exposure. During recovery the oxygen consumption was much higher than during the initial acclimation phase and declined slowly during this period. Both observations indicate oxygen debt incurred during the exposure to 10 and 5% AS. This phenomenon did not occur in all fish.

Following the protocol shown in Table 2, eight complete respirometry experiments were carried out with NR fish, and seven with HR fish. From the frequency distributions the 5-, 50-, and 95-percentile values were calculated and summarised in Table 3.

Oxygen consumption patterns within groups

In the NR group oxygen consumption levels decreased with decreasing AS levels (Table 3, Figure 4). At 40% AS the 50-percentile values were already significantly lower than under control conditions. A significant decrease in 50- and 95-percentile values was found when AS levels changed from 20% to 10% AS. Also at 10% and 5% AS, the 5-, 50-, and 95-percentiles were all significantly lower than under control conditions. Decreasing the AS level to 5% AS resulted in a further but non-significant decrease of the 5- and 50-percentiles in comparison to the 10% AS level before. The 95-percentile was significantly lower than at the 10% AS level before. At 5% AS, the average of the 95-percentile values decreased even to below the 5-percentile that was measured under control conditions. When the AS level was increased from 5% AS to 80% AS in the recovery period, all percentile-values showed a large significant increase compared to the 5% AS period (Table 3).

To expose both test groups to the same experimental protocol, the AS level in the HR group was changed from 10% AS to 80% AS (Table 2, 3). This caused a significant increase in the 5- and 50-percentile values. Subsequent decrease of the oxygen level to 40% AS, resulted in oxygen consumption that was not significantly different from that under control conditions. The 50- and 95-percentile values at 10% AS were significantly lower than under control conditions and 20% AS. At 5%, AS but also during the 1-hour recovery period afterwards, all percentile values were significantly lower than under control conditions.

When comparing subsequent levels, the O_2 consumption at the 5- and 50-percentile during the 40% AS period, was significantly lower than during the 80% AS level before. At 10% AS, both the 50-

Table 3: *Oxygen consumption rates (mg O_2 $100g^{-1}$ h^{-1}) of normoxia- and hypoxia-raised tilapia (means ± standard deviation) at the 5-, 50- and 95-percentile of each of the different conditions. Fish were exposed to the acclimation level for 24 hours (Control), after which both normoxia- and hypoxia-raised fish were exposed to a series of decreasing air saturation levels. The percentiles were calculated from the frequency distributions of the data per condition. The scope for activity was calculated as the difference between 5- and 95-percentile.*
Repeated measures ANOVA was used to calculate whether O_2 consumption rates differed significantly between different conditions. a = significantly different to the control period at the $p<0.005$. A = significantly different to the control period at $p<0.001$. b = significantly different to the previous AS-level at the $p<0.005$. B = significantly different to the previous AS-level at $p<0.001$.

	Normoxia-raised tilapia (n=8)			
	5-Percentile	**50-Percentile**	**95-Percentile**	**Scope**
Control (80% AS)	11.5± 42.82	13.61± 2.18	16.81± 3.45	5.27± 2.06
80% AS	11.73± 2.73	12.92± 2.53	15.72± 2.99	3.98± 1.24
40% AS	9.76± 2.04	11.46± 1.07 **a**	13.64± 1.91	3.88± 2.56
20% AS	9.24± 2.15	11.00± 1.05 **a**	13.77± 1.40	4.53± 2.91
10% AS	9.12± 0.82 **a**	10.27± 1.07 **A**, **B**	12.21± 1.25 **A**, **b**	3.10± 1.00 **a**
5% AS	8.03± 1.73 **a**	9.48± 1.52 **A**	10.72± 1.96 **A**, **b**	2.68± 2.33 **A**
Recovery (80% AS)	12.41± 2.60 **B**	13.17± 2.47 **b**	15.65± 4.38 **b**	3.25± 2.03 **a**
	Hypoxia-raised tilapia (n=7)			
	5-Percentile	**50-Percentile**	**95-Percentile**	**Scope**
Control (10% AS)	11.88± 1.30	13.98± 2.01	15.94± 2.10	4.06± 1.95
80% AS	14.51± 1.45 **A**	16.18± 2.02 **A**	17.43± 1.26	2.92± 0.76 **a**
40% AS	11.81± 1.69 **B**	13.85± 1.64 **B**	16.63± 2.17	4.81± 3.16
20% AS	11.60± 1.48	13.76± 2.33	15.89± 2.28	4.29± 2.20
10% AS	10.44± 1.20	11.57± 1.67 **a**, **b**	12.91± 2.62 **a**, **b**	2.47± 1.64 **A**, **b**
5% AS	9.90± 1.26 **a**, **b**	10.94± 1.73 **a**, **b**	12.13± 1.95 **A**	2.23± 1.16 **A**
Recovery (10% AS)	10.31± 1.05 **A**	11.83± 1.67 **A**	13.71± 2.11 **a**, **b**	3.40± 1.77 **b**

as well as the 95-percentile values were significantly lower than at 20% AS. At 5% AS the 5- and 50-percentile values were significantly lower than at 10% AS. In the 1-hour recovery period only the 95-percentile value showed a slight but significant increase compared to the level before (Table 3).

Oxygen consumption patterns between groups

At control conditions, the oxygen consumption rates of the NR and HR animals were remarkably similar (Figure 4). ANCOVA performed on the 5-, 50-, and 95-percentile values under control conditions, using the weight of the animals as a covariable, showed no significant differences between NR and HR animals (p= 0.799, 0.760 and 0.694 respectively). At 80% AS, the oxygen consumption rates increased markedly in the HR animals and did not change in the NR animals (Table 3). Repeated measures ANOVA on the 80%, 40%, 20% 10% and 5% AS periods showed that the oxygen consumption rates (5-, 50- and 95-percentiles) of the HR animals were significantly higher than those of the NR group (p= 0.013, 0.006, and 0.039 respectively).

Scope for routine activity

In the NR group, the scope for activity was significantly smaller at 10% AS, 5% AS and during the recovery period than under control conditions (Table 3). No significant differences were found in the scope for activity between subsequent AS-levels.

When the AS levels increased from 10% AS to 80% in the HR group, the

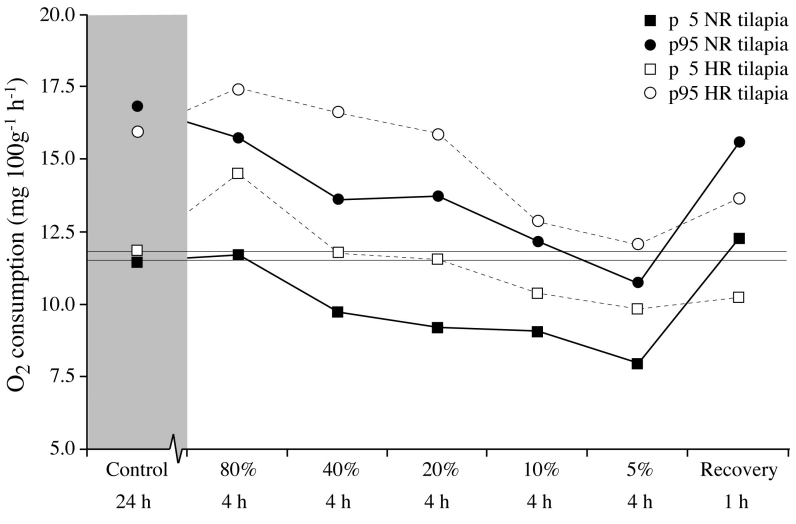

Figure 4:
The averaged O_2 consumption patterns of normoxia and hypoxia-raised tilapia measured at the acclimation level (Control, respectively 80% and 10% air saturation for normoxia and hypoxia-raised fish) and a series of decreasing AS levels. For the protocol and animals used, see table 2. Of the frequency distribution of the data per condition, the 5-percentile and 95-percentiles are given. HR tilapia showed higher consumption rates at all experimental conditions.

scope for activity decreased significantly. This was mainly caused by the significant increase in the 5-percentile value (Table 3). As in the NR group, at 10% AS and 5% AS the scope for activity was significantly lower than under control conditions. When AS-levels decreased from 20% AS to 10% AS, the scope for activity decreased significantly. In the recovery period, the scope increased significantly compared to the 5% AS-level.

ANCOVA performed on the scope for activity under control conditions, and using the weight of the animals as a covariable, showed no significant difference in the scope for activity ($p=0.302$, Figure 4) between NR and HR tilapia. Repeated measures ANOVA on the 80%, 40%, 20%, 10% and 5% AS periods showed no significant difference in the scope for activity between the NR and HR animals ($p= 0.299$).

Discussion

In our study we tried to minimise stress by covering the respirometer chamber with black foil and leaving the fish to habituate to the experimental set up. Observations on external activity showed no indications of stress *e.g.* fast pectoral fin movements or fleeing attempts, when disturbed.

When measuring O_2 consumption in fish, the set up and experimental procedures will affect the behaviour of the fish and its responses to hypoxia. Three methods to measure the oxygen consumption of fish are widely used: method 1, measurement of the oxygen decline in a closed respirometer chamber (Beamish, 1964), method 2, measurement of the O_2 concentration difference between in- and outflow at continuous flow through (Lomholt & Johansen, 1979, Fernandez & Rantin, 1989), method 3, measurement of the oxygen suppletion necessary to keep the oxygen levels at a constant value (Van den Thillart *et al.*, 1994, Muusze *et al.*, 1998). Method 1 is the easiest to perform, requires little equipment, and is most often used. The disadvantage is that it provides compounded results as several factors act at the same time. First, stress responses normally occur due to a change in condition, which increases the oxygen demand. Second, normal behaviour shows random changes in oxygen consumption rates, causing variation in consumption. This would require repetition of the experiment or extended observation at the same oxygen level. Third, when the oxygen level is changed, an animal needs time to reset its circulation/ventilation system, which takes about one hour (Randall, 1970). When using method 2, O_2 conditions are already much better controllable. However, the accuracy of measurement is dependent on the difference between O_2 inflow and outflow. This difference also depends on the activity level of the animal. Thus, the animal may encounter a variation of oxygen conditions just by increasing its

activity. Method 3 which was applied in the present study, is the least disturbing for the fish, as environmental conditions do not change. It allows measuring the oxygen consumption rates at constant oxygen levels regardless the activity of the animal.

Several studies showed that fish are more tolerant to hypoxia if they are given sufficiently long periods of time to habituate to changed AS levels (Ott, *et al.*, 1980; Ultsch *et al.*, 1981). Fernandez and Rantin (1989), who used method 2 (flow through), argued that they found critical oxygen levels (where O_2 uptake is limited by the O_2 availability) in Nile tilapia (*Oreochromis niloticus*) that were 50% lower than those of other authors, due to the difference in the methods and protocol. In our study, animals were acclimated in the respirometer set up for 24 hours at the AS level at which they were raised, followed by a series of stepwise decreasing AS levels that each lasted 4 hours. In between subsequent AS levels it took 10-15 minutes to decrease the AS level by 10%. During this period, O_2 consumption did not show large shifts that would indicate stress-induced metabolic rate changes. Nor did we see any disturbed behaviour that indicates additional stress.

The routine metabolic rate (50-percentile) of the NR tilapia in our study was approximately 13 mg O_2 $100g^{-1}$ h^{-1}. Studies on two other cichlids, Nile tilapia (*Oreochromis niloticus*) and the South American cichlid acara-açu (*Astronotus ocellatus*), showed similar consumption rates of 11.7 and 12.0 mg O_2 $100g^{-1}$ h^{-1} (Fernandez and Rantin, 1989; Muusze *et al*., 1998). For comparison, the data of the former authors were scaled to fish of 100 grams and corrected for the metabolic weight difference (see Materials and Methods). Critical O_2 concentration for

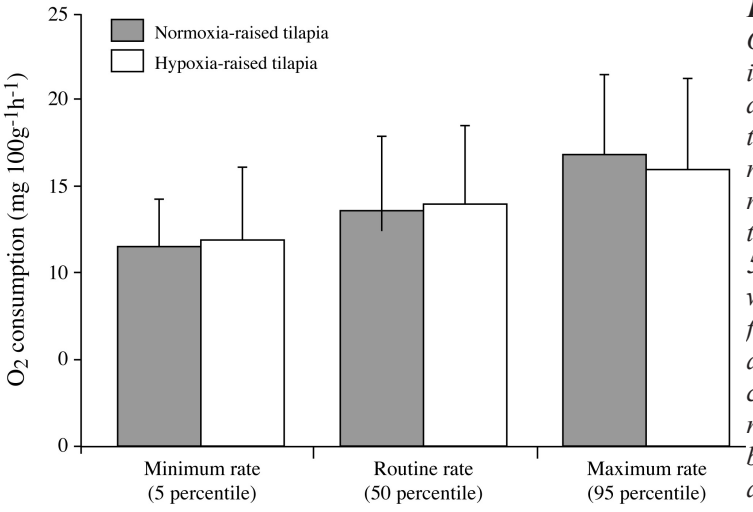

Figure 5:
Oxygen consumption of individual NR and HR tilapia during 24 hours of exposure to the AS level that they were raised under. NR tilapias were raised under 80% AS and HR tilapia under 10% AS. The 5-, 50-, and 95-percentile were calculated from the frequency distribution and averaged. Oxygen consumption rates showed no significant differences between NR and HR fish in any of the three percentile values.

Nile tilapia was estimated to be 18 mm Hg, which corresponds to 11.6% AS (Fernandez and Rantin, 1989). In the NR fish from our study, no significant changes in O_2 consumption occurred in the 5- and 95-percentile at 20% AS, while at 10% AS, O_2 consumption was significantly decreased at the 5-, 50- and 95-percentile. Most probably the critical oxygen level can be found between 20% AS and 10% AS.

Under control conditions, during the respirometry experiments, the NR and HR animals showed very similar oxygen consumption rates (Figure 4, 5) and behaviour, in spite of an 8-fold difference in oxygen level. The scope for activity was not significantly different. This shows that the HR fish were well acclimatised. When exposed to hypoxia, already at 40% AS fish from the NR group decreased O_2 consumption rates to below that of the control period (Table 3, Figure 4). Also, NR fish did not survive exposure to 10% AS for more than 12 hours. Compared to control levels, HR tilapia increased their oxygen consumption when exposed to 80% AS. A subsequent lowering of the AS levels (from 80% to 5%), resulted in a concomitant decrease of O_2 consumption rates. However, at all levels the oxygen consumption rate was higher than that of the NR fish. Thus, HR tilapia must have a markedly increased oxygen extraction capacity that compensates for a reduced O_2 ambient concentration.

Mechanisms of adaptation

A possible strategy for surviving chronic hypoxia would be to invest less energy in total growth and gonads. We expected a reduction in growth of HR tilapia in order to conserve energy for maintaining a normal scope for external activity. Remarkably, the growth in standard length, as well as the length-weight relationship, was the same for both groups (Figure 2). Therefore, we can conclude that there was no reduction in growth rate. It must be noted, however, that this experiment was not designed to be a growth study. Daily food intake per fish was not regulated and the total amount of food that was administered in each aquarium daily was not properly standardised.

Data from other authors on growth of fish under hypoxic conditions confirm, as well as contradict our results. A study done on Atlantic menhaden (*Brevoortia tyrannus*) and spot (*Leiostomus xanthurus*) showed that AS-levels must approach lethal values to impair growth of juveniles (Mcnatt and Rice, 2004). In contrast to the 10% AS level used in our experiment, the lowest levels used by Mcnatt and Rice were 19% AS. Exposure of sea bass (*Dicentrarchus labrax* L.) of 40-90 grams to 40% AS already reduced food uptake, growth and condition factor (Thetmeyer *et al.*, 1999). Experiments on older but still immature carp (30g) that were exposed to 1 mg O_2 L^{-1} for twelve weeks, showed decreased serum levels of testosterone, estradiol,

and triiodothyroxine. These hormonal changes were associated with retarded growth, reduced gonadal development in both sexes, and in reduced spawning success, sperm motility, fertilisation success, hatching rate and larval survival (Wu *et al.*, 2003). In our study however, both NR as well as HR tilapia frequently started to produce nests with viable young at the same age, suggesting unchanged reproduction capacities.

In order to keep O_2 extraction stable under hypoxic conditions, a greatly enlarged ventilation activity is required, which may increase the costs of breathing considerably (Van Dam, 1938; Beamish, 1964; Schuman and Piiper, 1966; Rantin *et al.*, 1992; Fernandez and Rantin, 1994). In Nile tilapia, the metabolic cost of breathing was estimated to be 3% of total consumed oxygen at 90% AS and 28.5% when AS levels decreased to 21%, while O_2 extraction efficiency dropped from 83% to 61% (Fernandez and Rantin, 1994). As suggested by Beamish (1964), improved O_2 uptake efficiency, and therefore a decrease in costs, would enable fish to live under hypoxic conditions permanently. Indeed, hypoxia-acclimated carp had an increased efficiency compared to normoxia-acclimated carp Lomholt and Johansen (1979). There is evidence that the O_2 extraction efficiency of HR tilapia in our study is higher than that of NR tilapia at 10% AS. High oxygen extraction efficiency can be achieved by increasing the contact area between water and respiratory surface, and by higher haemoglobin concentrations in the blood. This has been observed in wild cichlids that live in hypoxic environments (Galis and Barel, 1980; Chapman *et al*., 2000, 2002; Witte *et al*., 2000), as well as in animals raised under hypoxia in the laboratory (Weber and Wells, 1989; Chapman *et al.*, 2000). Such improved oxygen extraction is likely to be crucial for hypoxia adaptation as it reduces the costs of ventilation.

Adaptivity in young and adult fish

Ross (2000) mentioned that the high tolerance to hypoxia of tilapia in general, has led many to assume that tilapia will grow and thrive in such conditions. He stated that, "there is a great deal of hard evidence to the contrary and they will not tolerate low DO in the long-term, nor will growth, feeding, digestion or reproduction be normal in these circumstances." In our experiments, however, routine O_2 consumption levels of HR tilapia were unaffected by life at 10% AS, and fish grew well and produced nests regularly. The literature dealing with chronic hypoxia exposure shows that, in contrast to the tilapia in our study, adult carp (*Cyprinus carpio*) and tench (*Tinca tinca*) survive chronic hypoxia by decreasing oxygen demand (Lomholt and Johansen, 1979; Johnston and Bernard, 1982a; Zhou *et al.*, 2000; Wu, 2003). Carp that were acclimated to hypoxia (20% AS) for six weeks, showed about 50% lower O_2 consumption

rates than normoxia-acclimated carp under control conditions (Lomholt and Johansen, 1979). Experiments with tench that were acclimated to 8.5% AS for six weeks showed a 48% reduction in routine O_2 consumption (Johnston and Bernard, 1982a). The reduction in consumption rate can partly be explained by depressed external activity. In addition, the density of mitochondria and capillaries in the muscles had decreased, together with an increase in glycolytic capacity in liver as well as in slow muscles (Johnston and Bernard, 1982a, b). This indicates that hypoxia-acclimated tench are not capable of the same aerobic activity as normoxia-acclimated animals and anaerobic capacity is more important under hypoxia. In humans that were exposed to long term hypoxia in mountain expeditions, also a decrease in the mitochondria content of the muscles was found (reviewed by Hoppeler *et al.*, 2003). These responses to hypoxia in tench and humans are accompanied with a decrease in aerobic capacity, which is in contrast with the observed response of HR tilapia in this study. An obvious explanation for the differences in oxygen consumption in hypoxia-acclimated fish, between our study and the studies of Lomholt and Johansen (1979), and that of Johnston and Bernard (1982), is that the tilapia in the present study were exposed to lifelong hypoxia, starting shortly after they were released by the mother. During this period, which lasted 10-15 months, the tilapia grew a thousand-fold larger while in other studies there was virtually no growth. Theoretically, different relations between growth of an animal and adaptivity of anatomy and physiology to changed environments can exist (Witte *et al.*, 1990). The plasticity of shape and size of a structure *e.g.* the cardiovascular system, gills and respiratory system might well be dependent on ontogenetic stage. Indeed, measurements on the gills of NR and HR cichlids (Chapter 4), revealed large differences in gill shape and respiratory surface. Consequently, it would be more difficult to change the relative shape of these structures if animals do not grow, and conceivable phenotypic responses to chronic hypoxia are likely to be stronger and/or different in very young fish. Possibly, as a consequence of a decreased plasticity in adult fish, they must revert to an alternative strategy *viz.* reducing energetic needs, whereas very young cichlids can maintain normal metabolic rates by increasing their oxygen extraction capacity.

CHAPTER 3:

METABOLISM OF HYPOXIA-RAISED LAKE VICTORIA CICHLIDS: IS A NORMAL LIFE CYCLE POSSIBLE UNDER LIFELONG HYPOXIA?

H. A. Rutjes, F. Witte, G.J.E.E.M. Van den Thillart

Institute of Biology Leiden, Leiden University
P.O. Box 9516, 2300 RA Leiden, The Netherlands

Abstract

Broods of *Astatoreochromis alluaudi* and *Haplochromis ishmaeli* were split and exposed to normoxia (80-90% air saturation) and hypoxia (10% air saturation) for up to 21 months. The broods survived and grew equally well in both treatment groups. In a 3-day protocol the O_2 consumption rates were measured under control conditions (respectively 80% or 10% air saturation) at stepwise decreasing oxygen levels, and at anoxia. Normoxia-raised fish of both species were able to maintain high oxygen consumption rates until 10% air saturation. The critical O_2 level of *A. alluaudi* and *H. ishmaeli* was about 5% AS. This is lower than of any other fish known. Hypoxia-raised fish were able to tolerate anoxia significantly longer than normoxia-raised siblings. At all air saturation levels the O_2 consumption rate of hypoxia-raised fish was higher than that of normoxia-raised siblings, except during the recovery period. This indicates that hypoxia-raised fish had an increased O_2 extraction capacity. In contrast, all literature known on chronic hypoxia, performed on older animals, shows that animals survive by decreasing metabolic needs. The unimpaired aerobic metabolism of hypoxia-raised *A. alluaudi* and *H. ishmaeli* shows that young cichlids are more plastic and are able to follow a strategy increasing O_2 extraction capacity with decreasing air saturation levels instead. We pose that chronic hypoxia does not have a direct negative effect on the life cycle of cichlids when exposed from an early life stage onwards. The high hypoxia tolerance of *H. ishmaeli* possibly helped their survival and dispersion during the desiccation of the lake, when the water level of the lake was very low and hypoxic habitats were abundant.

Introduction

In Lake Victoria, the largest tropical lake in the world by surface, several lake-wide events have dramatically changed the ecosystem at all trophic levels. They comprise an increase of the human population, concomitant increase of land use and deforestation of the shore areas (Hecky and Bugenyi, 1992; Verschuren *et al.*, 2002), increased fisheries (reviewed by Witte *et al.*, 2005), introduction of Nile perch and Nile tilapia (Ogutu-Ohwayo, 1990) and introduction of the water hyacinth (Njuguna 1991). An important consequence is presence of chronic hypoxia in larger areas and for longer periods than before (Hecky, 1993; Hecky *et al.*, 1994, Wanink, 2001). In the deep waters of Lake Victoria, severe hypoxia (<1 mg O_2 L^{-1}) was present from October to March at depths of 40 to 54 meter (35% of the lake's bottom area) in 1990-91, whereas this level of hypoxia was measured only below 60 meter in 1960-61 (Hecky *et al.*, 1994). In the more shallow Mwanza Gulf (<20m) in the South of the lake, between 1979 and 1988, periods of hypoxia became longer and the 1 and 5 mg O_2 L^{-1} isopleths moved upward in the water column (Wanink *et al.* (2001). This makes the makes the lower part of the water column a less suitable habitat while the majority of the fish species in Lake Victoria are dependent on this habitat. Wanink *et al.* (2001) found that the small cyprinid *Rastrineobola argentea* occurs only above the oxycline during periods of hypoxia. It was argued that the presence of an oxycline limits *R. argentea* in reaching it's feeding areas near the bottom. In addition to increased long term and chronic hypoxia, the upwelling of hypoxic water, which caused massive fish kills, has increased in frequency (Ochumba, 1990; Ochumba *et al.*, 1993, Wanink *et al.*, 2001). If possible, fish tend to avoid exposure to low O_2 levels (Wannamaker and Rice, 2000; Wanink *et al.*, 2001) but during such upwelling events, fish are suddenly exposed to hypoxia, and are unable to flee or adjust their metabolism. During more gradually induced hypoxia, many fish species are able to reduce energy consumption to below their standard metabolic rate, which is a determining factor for hypoxia tolerance. When unable to sufficiently decrease metabolic rate, the oxygen demand exceeds the oxygen uptake capacity. To compensate, the energy needed is produced through anaerobic pathways and fish eventually die of acidification and depletion of substrates (Van den Thillart and Van Waarde, 1985). Some researchers have hypothesised that, apart from the introduction of Nile perch, hypoxia was an important factor in the decline of the haplochromine cichlids in Lake Victoria (Kaufman, 1992; Hecky *et al.*, 1994, Verschuren, 2002).

The duration of hypoxia exposure has much influence on the hypoxia responses in fish. However, this relation is almost never categorised. We

distinguish between short-term hypoxia and chronic hypoxia. Exposure to short-term hypoxia takes several hours up to several days. During this period, behaviour and regulatory processes are important that enable a decrease in O_2 demand, a maximised O_2 uptake, and an increase of anaerobic metabolism (Van den Thillart and Van Waarde, 1985; Van den Thillart *et al*., 1994; Van Ginneken *et al*., 1995). Fish that are exposed to short-term hypoxia normally react with increased ventilation, a reduction in external activity and, if possible, aquatic surface respiration (Van den Thillart and Van Waarde, 1985; Van den Thillart *et al.*, 1994; Muusze *et al.*, 1998 Chapman *et al.*, 2002). If the metabolic needs exceed the maximum O_2 extraction, suppression of the standard metabolism follows, accompanied by activation of anaerobic metabolism to meet the total energy demand (Van den Thillart and Van Waarde, 1985; Van den Thillart *et al*., 1994; Van Ginneken *et al*., 1995). Acute hypoxia exposure generally leads to stress responses and low tolerance. When fish are given time to habituate to the new environment, metabolic rate can be decreased and stress responses avoided, resulting in higher hypoxia tolerance (Randall, 1970; Ultsch *et al.,* 1981). During chronic hypoxia, which lasts between a week of exposure and a permanent state of hypoxia, changed gene activity leads to the production of new enzymes, proteins etc. (Gracey *et al.*, 2000; Zhou *et al.*, 2001). This will result in alterations of the O_2 extraction capacity, the anaerobic capacity, and even, changes in anatomy. A distinction must be made between chronic exposure of animals at different life stages. When animals are exposed to hypoxia from a post larval stage until adulthood (lifelong), ontogenetic changes and large differences in growth may influence hypoxia responses. Only few studies have been published on the effects of chronic hypoxia exposure. Experiments with immature as well as adult fish, showed that survival is mainly based on the reduction of (aerobic) energy expenditure by decreasing the total growth, gonadal growth and external activity (Lomholt and Johansen, 1979; Van den Thillart *et al.*, 1980; Johnston and Bernard, 1982a, 1982b; Zhou *et al.*, 2000; Wu *et al.*, 2003). In the hypoxia tolerant goldfish, *Carassius auratus*, chronic hypoxia exposure led to depression in protein synthesis in the liver, and elevated enzyme activity that promote conservative use of glycogen stores in the muscles (Van den Thillart and Smit, 1984). Indubitably the metabolism of the fish in these studies was strongly limited by the ambient oxygen concentration. One might wonder whether this type of response enables lifelong survival. Theoretically, the best adaptation to chronic hypoxia permits uninhibited aerobic energy production and thus, a high oxygen extraction capacity under hypoxic conditions.

The capacity of fish to adapt to

environmental challenges might well be related to age dependent plasticity of the animal. Pilot tests with recently hatched young of the cichlid *Astatoreochromis alluaudi* showed that these fish could acclimate to 10% air saturation (AS) levels at 25°C (Van den Thillart and Witte, unpublished data). The juvenile *A. alluaudi* were able to grow to adulthood at the same rate as their normoxia-raised siblings. In addition, hypoxia-raised (2 years) *A. alluaudi* from a later experiment, had an 80% enlarged gill area and 30% higher haemoglobin and haematocrit levels (Chapter 4 and 6). This indicates that phenotypic plasticity plays an important role in surviving chronic hypoxia and that young cichlids are more plastic than adults are.

We hypothesised that, in contrast to the findings of the above-mentioned authors, hypoxia-raised cichlids survive by improved aerobic capacities when subjected to lifelong hypoxia at a very young age. In this study we split broods of two species of cichlids from Lake Victoria and raised them at 10% or 80-90% air saturation for 1.5-2 years. The two species used, are ecologically and morphologically very similar but differ in their phylogeny and the natural range of O_2 conditions that they live at. While *Astatoreochromis alluaudi* occurs in both well-oxygenated as well as hypoxic water, *Haplochromis* (*Labrochromis*) *ishmaeli* is restricted to a well-oxygenated habitat only. Therefore, the latter is likely to be less hypoxia tolerant. On the adult fish, respirometry experiments were performed to test whether animals had an increased oxygen consumption capacity.

Material and Methods

Animals and conditioning

The mollusc crushing cichlids *Astatoreochromis alluaudi* and *Haplochromis* (*Labrochromis*) *ishmaeli* were used (Table 1). *A. alluaudi* is not endemic to Lake Victoria. It also occurs in Lakes Nabugabo, Edward-George and many small lakes around Lake Victoria (Greenwood, 1959, 1965, 1973; Hoogerhoud, 1986). According to morphological as well as molecular studies, *A. alluaudi* is from a different phylogenetic lineage than all other Lake Victoria haplochromines (Greenwood, 1959; Sage *et al.*, 1984; Meyer, 1990; Nagl *et al.*, 2000; Seehausen *et al.*, 2003). The habitat of *A. alluaudi* includes well-oxygenated streams as well as hypoxic swamps and a variety of bottom types (Greenwood, 1974; Witte, 1981). In Lake Victoria, it is rarely found deeper than 20 meters. The maximum SL is 160 mm (Greenwood, 1959).

H. ishmaeli is endemic to Lake Victoria. Though Lake Victoria haplochromines used to be considered as a monophyletic group, recent research suggests that this may not be the case (Nagl *et al.*, 2000; Seehausen *et al.*, 2003; Verheyen *et al.*, 2003). Regardless of taxonomic debates, in contrast to *A.*

Table 1: *The fish and respirometry protocols that were used. The number of fish (N) and weight in grams (W) ± standard deviation (SD) are given. Animals from one brood were normoxia raised or hypoxia raised. For respirometry experiment and anoxia experiments separate broods were used. At the beginning of each experiment, animals were acclimated to the air saturation level (AS) they were raised at (Control, 80% AS or 10% AS) for 24 hours, followed by stepwise changes in AS level as indicated. In the anoxia experiments, animals were exposed to anoxia after the 5% AS period for as long as they could maintain equilibrium.*

			Hours of exposure:						
Respirometry groups	**N**	**W±SD**	**Control**	**80%**	**40%**	**20%**	**10%**	**5%**	**Recovery**
NR *A. alluaudi*	6	17.2±2.3	24 (80%)	4	4	4	4	4	8 (80%)
HR *A. alluaudi*	6	14.0±2.2	24 (10%)	4	4	4	4	4	8 (10%)
NR *H. ishmaeli*	6	9.9±3.1	24 (80%)	4	4	4	4	4	8 (80%)
HR *H. ishmaeli*	6	10.3±4.3	24 (10%)	4	4	4	4	4	8 (10%)
Anoxia groups	**N**	**W±SD**	**Control**	**80%**	**40%**	**20%**	**10%**	**5%**	**Anoxia**
NR *A. alluaudi*	6	19.0±3.0	24 (80%)	2	2	2	2	2	variable
HR *A. alluaudi*	6	21.8±6.3	24 (10%)	2	2	2	2	2	variable
NR *H. ishmaeli*	9	18.8±3.4	24 (80%)	2	2	2	2	2	variable
HR *H. ishmaeli*	5	16.0±3.5	24 (10%)	2	2	2	2	2	variable

alluaudi, H. ishmaeli can be considered as a member of the Lake Victoria super-flock. The animals used in the present study were offspring of fish collected in the southern part of the lake, where their distribution was virtually restricted to well oxygenated water of less than 6 meters deep with sand bottoms (Witte, 1981).

All animals that were used, were offspring of only a few animals that were caught in 1984 in Mwanza Gulf of Lake Victoria, and bred since for 15-20 generations in our laboratory. In addition to using split-brood experiments, genetic diversity is very small amongst the individuals of each species used. This drastically narrows the possibility that differences in results between the NR and HR siblings can be attributed to genetic differences. However, the results from these experiments are not necessarily representative for each species as a whole.

Of *A. alluaudi* as well as *H. ishmaeli*, two broods were selected. Of both species, one brood was used for respirometry and one for anoxia experiments. At about four weeks after fertilisation, when the fish had a standard length (SL) of *ca.* 1.5-cm, each brood was randomly split into a normoxia and a hypoxia group. Normoxia groups were raised at 80-90% air saturation (AS) and the hypoxia groups at 10% AS. For the latter, the AS level of the water was

lowered stepwise from 80-90% to 40%, 30%, 20%, 15%, 12% and 10% in four weeks time. All animals were kept at a temperature of 25.5 °C and a day-night cycle of 12-12 hrs. The fish were given a diverse diet of flake food, frozen midge larvae, frozen zooplankton, and a mixture of pulverized shrimps, mussels and flake food. The SL of the fish was measured regularly. After 17 months, when the fish had reached a sufficient weight to properly measure O_2 consumption (above 10 grams) experiments were performed over a period of 4 months. Occasional deaths that occurred during growth were caused by fighting with dominant males. All fish were raised in tanks of 45x50x50 cm. The glass tanks contained an extra compartment from where water was pumped into the animal compartment to ensure fast mixing with the inflow of hypoxic filtered water. Hypoxia was reached by a continuous inflow of degassed water (6-9% AS) at a rate of 1-2 L min^{-1}. A stainless steel plate, laying 3 cm below the water level, prevented oxygen uptake from the air by the fish as well as by the circulating water. The oxygen level of the water was regulated by Applikon biocontrollers (ADI 1030) equipped with polarographic oxygen sensors (Applikon ZZ71202AP10), switching solenoid valves in line with air diffusers. Thus, air was bubbled automatically through the water in the extra compartment, when the oxygen level was below the setpoint. This way the oxygen level in the animal chambers could be regulated with an accuracy of 2%. Water in the normoxia and hypoxia tanks was continuously refreshed from the same biological filter system. Before flowing into the hypoxia tanks the water was degassed by a vacuum system as described by Van den Thillart and Smit (1984).

Respirometer set up

Open flow respirometers were used for oxygen consumption measurements (for figure, see Chapter 2). Per experiment, individual fish were placed in 4.2-litre rectangular flow chambers with a transparent lid, allowing observations during the experiment. Grids were placed at the inflow and outflow of the chamber to reach low semi-laminar water, flow such that the animal could maintain its position without having to swim. With a polarographic oxygen sensor connected to a biocontroller (Metrohm PH-signal amplifiers, E561), the AS level was kept constant. When the AS level dropped below the desired value, the biocontroller activated a solenoid valve allowing inflow of air saturated water. The water volume was measured with flowmeters (Rhodes, "lowflo" transmitters). Every two minutes a computer recorded the total amount of added water and the O_2 level in the flow chamber by means of software designed by the electronics service department of the Institute of Biology Leiden. A 150-litre tank filled with water, from the system in which the fish were raised, was used as a reservoir

and continuously aerated and irradiated with UV light.
From the O_2 concentration difference and the amount of water used to keep the AS level constant, the O_2 consumption was calculated according to:

$V_{O2} = v (c_{in}-c_{out})$ mg O_2 h^{-1}
(Van den Thillart and Verbeek, 1991)

V_{O2} = O_2 consumption of the fish in mg O_2 h^{-1}
v = Flow rate in L h^{-1}
c_{in} = O_2 concentration of in-flowing water in mg L^{-1}
c_{out} = O_2 concentration of out-flowing water in mg L^{-1}

Both c_{in} as well as c_{out} remained constant throughout the experiment.

Respirometry protocol
To obtain a stable metabolic rate, the fish were not fed for 36-48 hours prior to the respirometry experiment. Oxygen consumption rates were measured at control conditions for 36 hours after which all fish were exposed to the same experimental protocol (Table 1). The control conditions were 80% AS for the NR fish and 10% AS for the HR fish. The fish were kept at the same light cycle as they were raised at. To prevent disturbance of the fish by the presence of the experimenter, light at daytime was reduced to about 3 lux by covering the transparent lid. Observations at daytime showed that the animals regularly moved around. After each experiment a blank experiment was done without fish to determine the oxygen consumption caused by micro-organisms. This varied between 0.17 and 0.25 mg O_2 L^{-1} h^{-1}, which was below 15% of the total consumption and was used to correct the data. Occasionally, video recordings and remote observations, using video equipment were made during the respirometry experiments. Two to four respirometry set-ups were used at the same time. Care was taken that the use of set-ups was alternated for NR and HR fish and NR and HR fish were tested in alternating sequence. Fish were not used more than once for a respirometry experiment.

To test anoxia tolerance, individual fish were tested while using the same equipment as in the respirometry experiments but the protocols were different (Table 1). In NR fish, the exposure times to each AS level were reduced to two hours. In the HR groups, the AS level was directly lowered from 10% AS to 5% at the same moment as in the NR group. At 09:00 hours, the setpoint of the AS level was set to 0%. The fish consumed the remaining O_2. By measuring the AS level with a pen recorder, the moment was determined at which the water was depleted from oxygen. From 5.0% AS to 0.0% AS oxygen was consumed at a rather constant rate. The time that each fish could spend at anoxia was defined as the time between the moment the AS level

was 0.0 % and the time that the fish was exhausted and could not longer maintain nor regain its equilibrium. Directly before this moment, violent swimming and breathing activity was observed in all cases. Anoxia tolerance was tested with 6 NR and 6 HR *A. alluaudi*, 6 NR and 5 HR *H. ishmaeli*.

Data analysis

The O_2 consumption per fish was corrected for weight differences according to Van den Thillart and Kesbeke (1978):

$$M_{100} = M\ 100^{0.8} / W^{0.8}$$

Where M_{100} is the metabolic rate for a fish of 100 grams, M is the measured metabolic rate and W is the weight of the fish. The exponent 0.8 appears to be the same for different fish species, 0including cichlids and at different temperatures (Basu, 1959; Beamish, 1964a; Duthie, 1982; Yamamoto 1991). During the experiments oxygen consumption was recorded every 2 minutes resulting in a large number of data for each AS level. A frequency distribution was made for each oxygen level of which the 5-, 50-, and 95-percentile was determined. These 3 percentiles represent the resting, routine and voluntary active levels (Van den Thillart *et al.*, 1994). The voluntary active level should not be mistaken for the active level as measured at forced activity in, for instance, swimming experiments. In our respirometer set up, the animals were only able to swim for about 3-4 body lengths.

The 5-, 50- and 95-percentile values were analysed with the software program SPSS for Windows, version 10.0. To determine whether the oxygen consumption rates differed between normoxia-raised (NR) and hypoxia-raised (HR) animals, ANCOVA was performed on the consumption rates at control conditions using weight as a covariable. On the 80%, 40%, 20%, 10%, and 5% AS level repeated measures ANOVA was performed to determine whether the oxygen consumption patterns were different between NR and HR animals. To test whether the O_2 consumption rates differed within the NR group and within the HR group, separate repeated measures ANOVA were performed on all subsequent AS levels for the NR and HR group. Data from the anoxia tolerance experiments were analysed with ANCOVA and ANOVA to see whether weight, species or HR and NR differences explained variation in the time that animals could spend at 0% AS.

Results

Growth

For respirometry experiments two groups of 21 *A. alluaudi* and two groups of 11 *H. ishmaeli* were raised. After respectively 53 and 43 weeks, several deaths occurred due to fighting within a

short period of time, leaving 8 NR and 13 HR *A. alluaudi* and 10 NR and 8 HR *H. ishmaeli*. In both *A. alluaudi* groups, the dominant male was removed. At that moment, standard lengths, including dominant males (SL in mm ± standard deviation) of NR and HR *A. alluaudi* were respectively 57.8 ± 8.13 and 55.3 ± 4.37. The SL of NR and HR *H. ishmaeli* were 55.5 ± 2.4 and 56.4 ± 3.4. Standard lengths were not significantly different between NR and HR animals (Independent t-test, p=0.530 for *A. alluaudi* and p=0.257 for *H. ishmaeli*). In the groups that were used for anoxia, neither the SL nor the weight of the fish was recorded regularly.

Oxygen consumption within groups

The 5-, 50-, and 95-percentile values and scope for routine activity are summarised in Table 2 for *A. alluaudi* and in Table 3 for *H. ishmaeli*.

NR *A. alluaudi*: Oxygen consumption of NR *A. alluaudi* was hardly influenced by ambient O_2 concentration (Table 2, Figure 2). At 10% AS, the 95-percentile value was significantly increased. At 5% AS the 95-percentile was significantly lower than the 95-percentile at the previous AS level (10% AS) but not different from control conditions. In the recovery period the 5-, 50- and 95-percentile values were all significantly higher than in the control period. In the NR *A. alluaudi*, during the four hours at

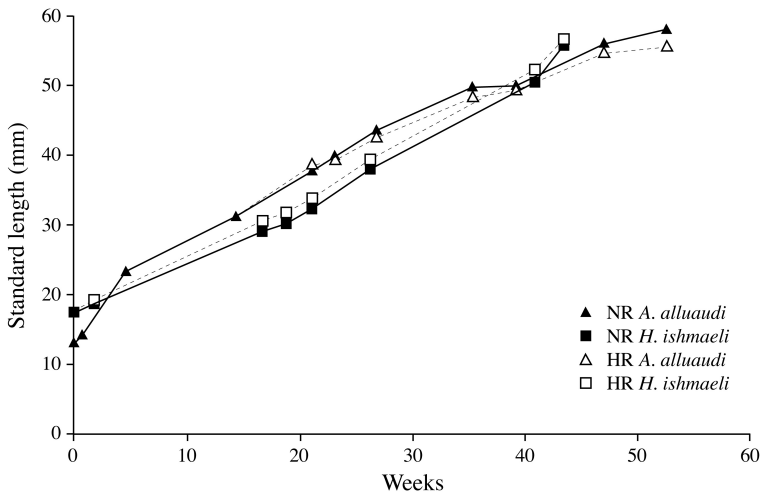

Figure 1: *Growth of normoxia-raised (NR) and hypoxia-raised (HR)* A. alluaudi *and* H. ishmaeli*, which were used in the respirometry experiments. The x-axis represents the time that animals were in the experiment. NR animals were raised at 80-90% air saturation (AS) and HR animals at 10% AS. After respectively 53 and 43 weeks several deaths occurred due to fighting within a short period of time and growth could not be compared between NR and HR groups anymore. At that moment the average standard length of the* A. alluaudi *was respectively 57.8 cm and 55.3 cm and the standard length of the* H. ishmaeli *was 55.5 cm and 56.4 cm. Standard lengths were not significantly different between NR and HR animals (Independent t-test, p=0.530 and p=0.257).*

80% AS after the control period (also at 80% AS), the scope for routine activity was significantly lower than during the control period. At all other AS levels, the scope for routine activity was not significantly different from that at control conditions. When comparing subsequent AS levels though, the scope for activity was significantly smaller at 5% AS than at the 10% AS level before.

HR *A. alluaudi*: Fish in this group responded with a significant increase in 5-, and 50-percentile values when the AS level was increased from 10% AS (Control period) to 80% AS (Table 2, Figure 2). Although the average increase in the 95-percentile was large, it was not statistically significant. At 40% AS, all percentile values were significantly increased. When the AS level was decreased further to 20% AS, only the 5-percentile was higher than that of the control period. At 10% AS and 5% AS, percentile values were not significantly different from those of the control period, while during recovery all percentiles were

Table 2: *Oxygen consumption rates (mg O_2 $100g^{-1}$ h^{-1}) of normoxia- and hypoxia- raised* A. alluaudi ± *standard deviation at the 5-, 50- and 95-percentile at each air saturation (AS) level. The percentiles were calculated from the frequency distributions of the data per condition. Repeated measures ANOVA was used to calculate whether O_2 consumption rates differed significantly between different conditions. a = significantly different to the control period at the p<0.005. A = significantly different to the control period at p<0.001. b = significantly different to the previous AS-level at the p<0.005. B = significantly different to the previous AS-level at p<0.001.*

	Normoxia raised *A. alluaudi* (n=6)			
	5 percentile	**50 percentile**	**95 percentile**	**Scope**
Control (80% AS)	12.30± 4.79	16.38± 5.78	19.66± 6.30	7.36± 3.08
80% AS	12.38± 4.61	15.25± 4.86	17.80± 5.78	5.42± 2.48 **a**
40% AS	14.07± 6.29	16.54± 3.20	20.56± 5.32	6.49± 4.39
20% AS	13.80± 4.73	16.49± 2.88	21.93± 5.02	8.13± 3.75
10% AS	13.76± 3.35	15.92± 2.68	23.62± 5.70 **a**	9.85± 6.06
5% AS	12.20± 2.85	13.78± 3.89	17.73± 4.49 **b**	5.53± 2.48 **b**
Recovery (80%AS)	17.49± 5.66 **A**	19.43± 6.58 **a**	22.65± 6.75 **a**	5.16± 2.47
	Hypoxia raised *A. alluaudi (n=7)*			
	5 percentile	**50 percentile**	**95 percentile**	**Scope**
Control (10% AS)	16.64± 3.76	19.99± 2.99	26.30± 2.19	9.67± 3.19
80% AS	24.07± 3.09 **a**	29.11± 7.61 **a**	34.61± 10.95	10.55± 8.05
40% AS	20.18± 5.71 **a**	25.91± 7.09 **a**	33.33± 8.32 **a**	13.15± 3.57
20% AS	18.58± 4.56 **a, b**	21.72± 4.28 **b**	36.67± 18.37	10.23± 3.59
10% AS	17.39± 4.19	20.83± 3.72	29.38± 4.88	11.98± 4.32
5% AS	15.98± 4.45 **b**	19.28± 3.63 **b**	24.99± 3.30 **b**	9.01± 3.92 **b**
Recovery (10% AS)	15.90± 3.92 **a**	18.37± 3.38 **a**	23.32± 1.21 **a**	7.42± 3.82

Table 3: *Oxygen consumption rates (mg O_2 $100g^{-1}$ h^{-1}) of normoxia- and hypoxia-raised* H. ishmaeli ± *standard deviation at the 5-, 50- and 95-percentile at each air saturation (AS) level. The percentiles were calculated from the frequency distributions of the data per condition. Repeated measures ANOVA was used to calculate whether O_2 consumption rates differed significantly between different conditions. a = significantly different to the control period at the p<0.005. A = significantly different to the control period at p<0.001. b = significantly different to the previous AS-level at the p<0.005. B = significantly different to the previous AS-level at p<0.001.*

	Normoria raised *H. ishmaeli* (N=6)			
	5 percentile	**50 percentile**	**95 percentile**	**Scope**
Control (80% AS)	10.05± 3.23	13.80± 3.19	16.71± 3.78	5.76± 1.76
80% AS	10.05± 4.08	13.40± 3.41	15.57± 4.18	4.55± 2.62
40% AS	13.71± 2.68 **A**	15.95± 3.35 **b**	19.26± 5.73 **b**	5.17± 3.44
20% AS	13.23± 2.31 **a**	15.25± 3.10	22.98± 6.91	9.17± 6.32
10% AS	13.20± 1.97 **a**	15.40± 2.96	19.00± 6.41	5.69± 4.59
5% AS	9.51± 2.14 **B**	11.51± 2.67 **B**	13.94± 4.58 **B**	4.19± 2.70 **b**
Recovery (80% AS)	18.02± 4.20 **A**,**B**	20.28± 4.12 **b**	22.48± 4.09 **b**	4.36± 1.90 **a**
	Hypoxia raised *H. ishmaeli* (N=6)			
	5 percentile	**50 percentile**	**95 percentile**	**Scope**
Control (10% AS)	11.80± 1.48	13.59± 1.99	16.24± 2.83	4.44± 4.37
80% AS	14.25± 3.42 **a**	15.35± 3.65 **a**	17.31± 4.01	3.07± 3.78
40% AS	13.53± 2.37 **a**	15.11± 2.61 **a**	17.42± 3.36 **a**	3.89± 4.04
20% AS	12.83± 1.47 **a**	15.90± 2.28 **a**	25.88± 11.41 **a,b**	13.05± 8.49
10% AS	12.6± 2.25	15.08± 2.42	23.68± 11.09	11.01± 9.59
5% AS	11.78± 1.74 **b**	13.46± 2.21 **B**	16.39± 4.30 **b**	4.61± 4.41 **b**
Recovery (10% AS)	11.26± 1.50	12.60± 1.87 **A**	14.73± 3.02 **a**	3.47± 2.93 **a**

significantly lower than at the control level. When comparing subsequent levels, the 5- and 50-percentile showed a significant decrease when the AS level was lowered from 40% AS to 20% AS. At 5% AS, all percentile values were significantly lower than at the 10% AS level before. They were, however, not significantly different from consumption levels in the control period. In the HR *A. alluaudi,* the scope for routine activity remained relatively constant throughout the experiment. Only at 5% AS a small but significant decrease in the scope for activity was found compared to the 10% AS level before.

NR *H. ishmaeli*: In the NR *H. ishmaeli*, 5-percentile values at 40%, 20% and 10% AS were significantly higher than at control conditions (Table 3, Figure 2). The 50- and 95-percentiles did not significantly change at these AS levels. During the first hour of recovery, the 5-percentile was significantly increased by about 80% compared to the 5-percentile value at control conditions.

The average 50- and 95-percentile values at the recovery period were much higher than at control conditions but with a large standard deviation. Thus, the difference between recovery and control conditions was non-significant. When comparing subsequent AS levels, at 40% AS, the 50- and 95-percentile values were significantly increased compared to the 80% AS level before. At 5% AS, the 5-, 50- and 95-percentile values were significantly lower than at the 10% AS level before, but not when compared to the control period. During recovery, all percentile values showed a large and significant increase compared to the 5% AS. In the NR *H. ishmaeli*, the scope for routine activity did not vary much over the different AS levels. At 5% AS, the scope for routine activity was significantly lower than at the 10% AS level before. During the recovery period, the scope for routine activity was significantly lower than at control conditions.

HR *H. ishmaeli*: In the HR *H. ishmaeli*, the 5- and 50-percentiles were significantly increased at 80% AS compared to the 10% AS level in the control period (Table 3, Figure 2). At 40% AS and 20% AS, all percentiles were significantly elevated compared to the control period. At 10% and 5% AS no significant differences were found with percentiles from the control period. As in the HR *A. alluaudi*, in the recovery period, the 50- and 95-percentile values were significantly lower than in the control period. When subsequent AS levels were compared, the 95-percentile showed a large and significant increase when the AS level was decreased from 40% AS to 20% AS. The averages for the 95-percentiles at 20% and 10% AS showed a large standard deviation, caused by two fish of which one showed a temporal increase in O_2 consumption at 20% AS, and the other fish at 10% AS. When removing the data of these fish, significant differences would remain the same *i.e.* the increase in consumption rate at 20% AS would still be significant compared to the control period, as well as to the previous AS level. At 5% AS, the 5-, 50- and 95-percentile values showed a significant decrease compared to the 10% AS level before, but not compared to the control period. Compared to the control period, the scope for activity in the HR *H. ishmaeli* showed a large but non-significant increase at 20% and 10% AS. This was caused by increased activity of one fish at 20% AS and a different fish at 10% AS. The scope for activity at 5% AS was significantly lower than at the 10% AS level before. Remarkably, during the recovery period, the scope for activity dropped further and was significantly lower than the scope at control conditions.

Oxygen consumption between groups

Repeated measures ANOVA on the 80%, 40%, 20%, 10% and 5% AS periods show that the 5-, 50-, and 95-percentile values of the HR *A. alluaudi* were

significantly higher (p=0.031, 0.008, 0.003) than those of NR siblings. At control conditions (respectively 80% and 10% AS for NR and HR fish), the scope for routine activity was not significantly different between NR and HR fish.

Repeated measures ANOVA on data of *H. ishmaeli* showed no significant difference between the 5-, 50- and 95-percentile values of NR and HR fish (p=0.517, 0.573, 0.174). The scope for routine activity at control conditions was not significantly different between NR and HR *H. ishmaeli*.

Anoxia

An ANCOVA with treatment group (NR and HR) and species (*A. alluaudi* and *H. ishmaeli*) as fixed factors and weight as a cofactor showed no relation between the time that animals could spend at anoxia and their weight. Additionally, HR animals were able to spend significantly more time at anoxia than NR animals ($p<0.001$, Figure 3). Three of the NR *H. ishmaeli* even lost equilibrium before they reached 0% AS. In addition, the *A. alluaudi* could spend significantly more time at anoxia than *H. ishmaeli* ($p<0.001$, Figure 3). At control conditions both NR as well as HR animals would hover above the bottom, showing regular swimming activity. At anoxia the animals generally ceased all movements and would sit on the bottom of the respirometer chamber leaning on one fin or against the wall of the respirometer chamber, thus keeping the body upright. Respiration was reduced to minute opercular movements. Every 5-15 minutes the animals would become active again and move around in the respirometer chamber, after which often the same position would be assumed at the same place. Though seemingly lethargic, on several occasions fish started to swim when they noticed movement made by the observer. The *A. alluaudi* would keep still for much longer in between activity bouts than *H. ishmaeli*. Behaviour of HR *A. alluaudi* was very typical and different from NR siblings. The four HR animals that could spend most time at anoxia did not lean against the walls of the respirometer chamber, like the rest did, but lied flat on the bottom, showing only eye movements. This behaviour was not seen in NR animals. The difference between fish lying flat on the bottom deliberately and fish doing so involuntarily due to exhaustion, was that the first did not panic and showed no escape behaviour or violent breathing activity. All four fish that lied flat on the bottom deliberately, regained upright positions for some time before getting exhausted. The total amount of time that animals spent swimming or sitting on the bottom, was not measured, but seemed a good predictor of how long fish would tolerate anoxia. Animals that moved relatively much could not stay at anoxia as long as more passive animals.

When getting closer to the moment that the animals lost equilibrium, the fish generally showed more frequent bouts

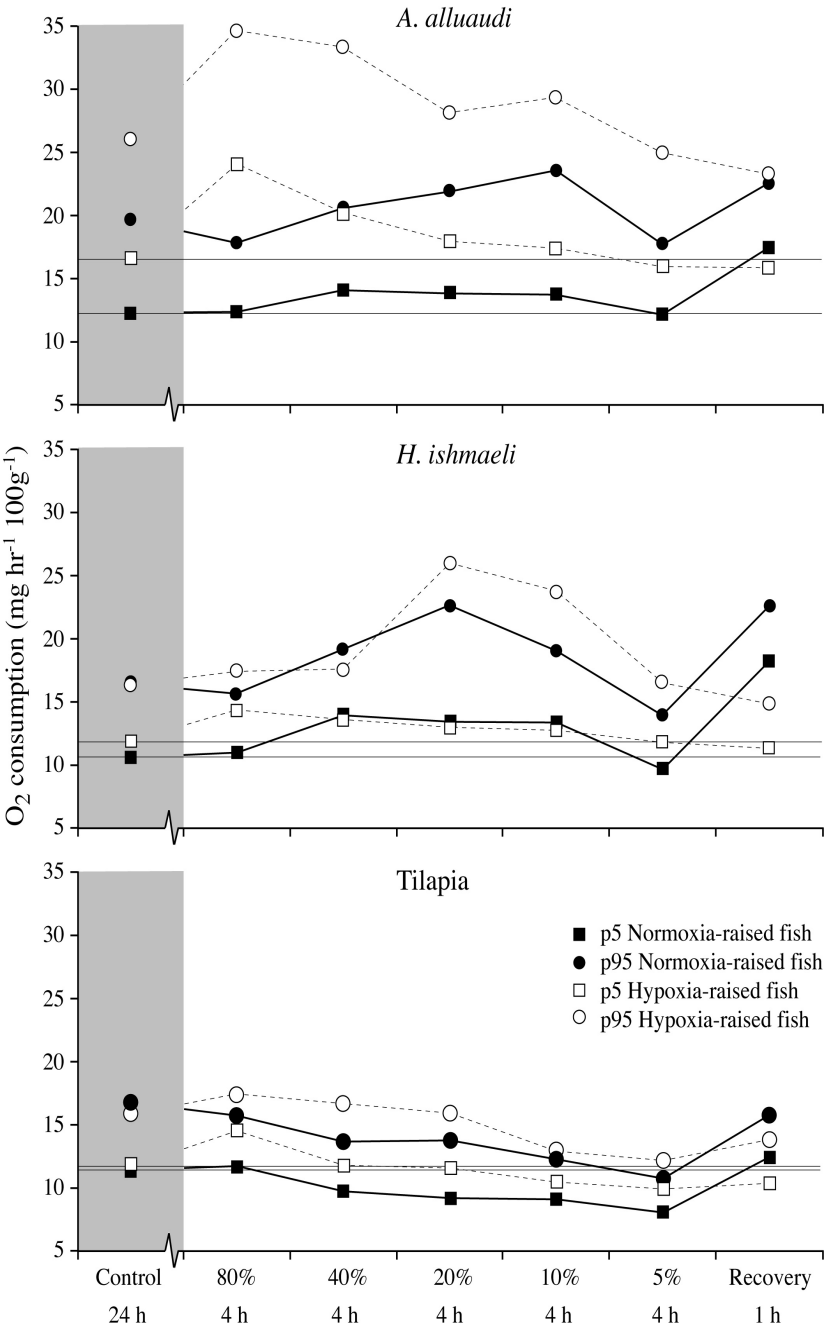

Figure 2: *The averaged O_2 consumption patterns of NR and HR* A. alluaudi, H. ishmaeli *and tilapia under control and experimental conditions. Of the frequency distribution of the data per condition, the 5-percentile and 95-percentiles are given. Horizontal lines represent the standard metabolic rate of both groups (5-percentile at control conditions).*

of activity and seemed to be seeking for exits actively. Before exhaustion and involuntarily loss of equilibrium, all fish panicked and would shoot through the respirometer chamber after which they sank to the bottom, accompanied by increasingly violent breathing activity. This was repeated several times, after which the animals did not attempt to swim nor did they remain upright. After the animals had lost equilibrium and the AS level was raised again, the *A. alluaudi* took 5-15 minutes to regain equilibrium while the *H. ishmaeli* regained equilibrium in seconds.

Discussion

Experimental design

When using several treatment groups it is impossible to keep all factors other than the treatment identical. To rule out the influence of uncontrollable factors the use of replications is important. In the present study, individuals all of each treatment group per brood were raised in the same aquarium (pseudo replication) Thus, it should be considered that theoretitally, differences in hypoxia tolerance between NR and HR groups could be caused by factors other than the difference in AS level that they were raised at. However, for several reasons we believe that this is very unlikely. First, we used a randomly split brood with little genetic variation. The parental animals were offspring from a breeding stock that was kept in our laboratory since 1986. This stock originated from only three females that were imported in our laboratory. Second, while the difference in AS level between NR and HR fish was a factor eight, care was taken that other environmental differences were kept at a minimum. All groups were raised in water from the same filter system in the

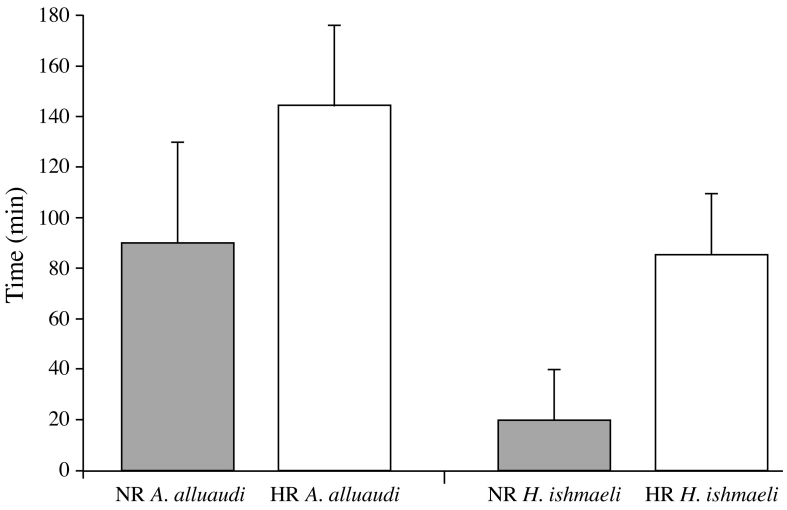

Figure 3: *Anoxia tolerance of NR and HR fish. ANCOVA showed no effects of weight*. A. alluaudi *could spend significantly more time under anoxia than* H. ishmaeli *(MANOVA, P=0.000). In addition HR animals could spend more time under anoxia than NR animals (MANOVA, p=0.000).*

same climate-room and in identically built aquaria. Third, data of experiments on other species of cichlids, showed similar effects of chronic hypoxia. These experiments can be regarded as replications when considering only the effects of lifelong hypoxia irrespective of species differences. Fourth, we formulated a prediction about the direction of the differences between NR and HR siblings and the differences found correspond with our expectations.

Growth

In the *A. alluaudi* and *H. ishmaeli*, two to nine deaths in each tank occurred due to fighting after 53 and 43 weeks respectively. At that time males became territorial and females carrying batches of eggs in their mouth were found. The deaths of fish caused unequal biomass in the tanks. To save other animals from being killed, the dominant males were removed. Naturally, the average biomass was influenced by mutations in the amount of fish in each tank. This made changes in average SL no longer for determination of the effect of hypoxia on growth of the fish. At the moment that animals started reproducing, the average standard lengths were not significantly different between NR and HR animals. However, within each group, there were considerable differences in growth rate, which seemed to be related to social status. Since growth differences between NR and HR fish were small and non-significant, we conclude that HR animals at 10% AS were able to grow at the same rate as their NR siblings at 80% AS. Similar observations were made in split-brood experiments with tilapia crossbreeds (*Oreochromis niloticus* X *Oreochromis mossambicus*, further referred to as tilapia; See also Chapter 2).

Literature on the effects of hypoxia on growth is somewhat controversial. Exposure of Atlantic cod, *Gadus morhua*, to 65% AS (10° C), and of European sea bass, *Dicentrarchus labrax*, to 40% AS (22° C), resulted in a reduction of food intake. In accordance with this, also growth rates decreased (Chabot and Dutil, 1999; Thetmeyer *et al.*, 1999). In the present study, the amount of food administered each time, was estimated by eye and approximately the same per animal. No data on food intake were collected though. A study done on juveniles of Atlantic menhaden, *Brevoortia tyrannus*, and spot, *Leiostomus xanthurus*, showed that AS levels must approach lethally low values *e.g.* 19% AS, to negatively affect the growth rate of juveniles (McNatt and Rice, 2004). It must be noted that in the mentioned growth studies, the animals were not as small as in our study and experiments lasted only several weeks. Experiments on older but still immature carp (30g) that were exposed to 1 mg O_2 L^{-1} for twelve weeks, showed decreased serum levels of testosterone, estradiol, and triiodothyroxine. These hormonal changes were associated with retarded

growth, reduced gonadal development in both sexes, and in reduced spawning success, sperm motility, fertilisation success, hatching rate and larval survival (Wu *et al.*, 2003).

Effect of hypoxia on oxygen consumption of normoxia-raised animals

In response to a reduction of the AS level, NR *H. ishmaeli* showed an increased minimum O_2 consumption until an AS level as low as 10% (Figure 2). Elevation of O_2 consumption of fish at hypoxic conditions was already described by Beamish (1964b) and explained by increased costs of respiration. Below the critical AS level, oxygen extraction becomes dependent on the AS level, thus resulting in decreased O_2 consumption and activity levels.

Similar equipment and methods as in the present study were used for studying the hypoxia tolerance of the sole, *Solea solea*, (Van den Thillart *et al.*, 1994), the South American cichlid acara-açu, *Astronotus ocellatus*, (Muusze *et al.*, 1998) and tilapia (Figure 2, see also Chapter 2). A significant reduction in metabolic rate was observed in these animals between 20% and 10% AS (Figure 4), which is generally considered to be a normal response to hypoxia. In contrast, NR *A. alluaudi* and *H. ishmaeli* are able to maintain high O_2 consumption rates at 10% AS (Figure 4). When exposed to 10% AS, NR *A. alluaudi* and NR *H. ishmaeli* showed elevations rather than reductions in O_2 consumption compared to control conditions (Figure 2; Table 2, 3). Only 2 out of 6 NR fish in both NR *A. alluaudi* and NR *H. ishmaeli* showed a clear reduction of O_2 consumption with decreasing AS level. Clearly, the critical O_2 level was not reached yet at 10% AS. We believe that the critical O_2 level for NR *A. alluaudi* and *H. ishmaeli* is close to 5% AS for two reasons. First, a clear decrease in O_2 consumption rates was observed when AS levels decreased from 10% AS to 5% AS. In addition, the O_2 consumption levels and scope for activity at 5% AS, are the lowest in the whole respirometry experiment for both species. Second, in the recovery period a considerable increase in consumption rates was found in NR groups of both species, indicating that at 5% AS the fish had produced energy through anaerobic metabolism (Figure 2). It can be assumed that an increase in anaerobic metabolism occurs when aerobic energy production is limited and below energy demand. Thus, we can consider the activation of anaerobic metabolism at 5% AS, as indirect evidence that the critical AS level was reached at ca. 5% AS. To our knowledge, such low critical oxygen levels are the lowest of any fish species of which O_2 consumption data are known.

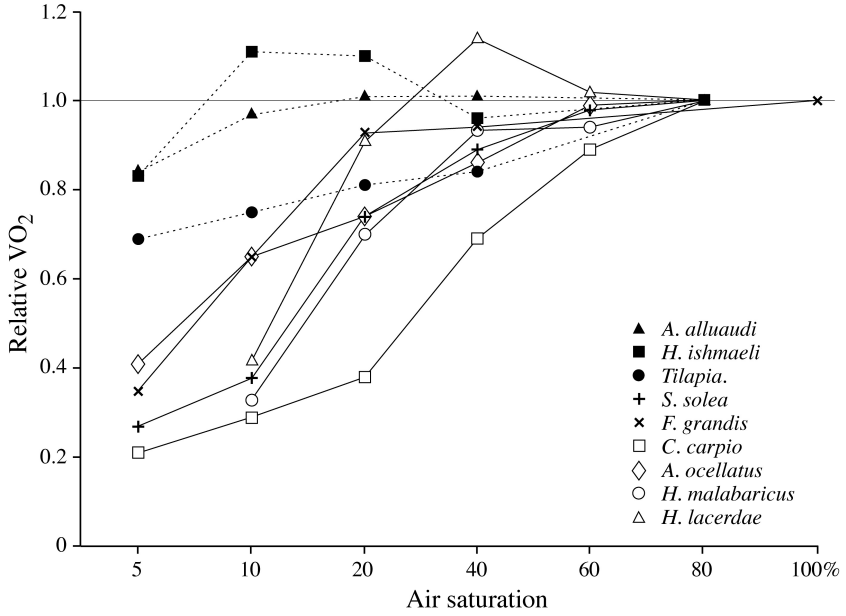

Figure 4: *Relative O_2 consumption at decreasing AS-levels for different species.* A. alluaudi *and* H. ishmaeli *and tilapia were able to maintain high O_2 consumption rates in comparison to other species. References for the data used: Astatoreochromis alluaudi, this study;* Haplochromis ishmaeli*, this study; tilapia, Chapter 2;* Solea solea*, Van den Thillart et al., 1994;* Cyprinus Carpio*, Lomholt and Johansen, 1979;* Fundulus grandis*, Virani and Rees, 2000;* Astronotus ocellatus *Muusze et al., 1998;* Hoplias malabaricus*, Rantin et al., 1992;* Hoplias lacerdae*, Rantin et al., 1992.*

Oxygen consumption of hypoxia-raised fish is not reduced

In spite of lifelong exposure to 10% AS, HR animals showed the same or higher O_2 consumption rates at 10% AS than NR siblings at 80% AS (Figure 2). Similar results were found in tilapia and *Haplochromis (Yssichromis) pyrrhocephalus* (For tilapia, see Figure 2, the data on *H. pyrrhocephalus* are unpublished). This suggests a more general phenotypic response to lifelong hypoxia *i.e.* an increase in oxygen uptake capacity.

In contrast, experiments with carp, *Cyprinus carpio*, and tench, *Tinca tinca*, show that these fish survive by decreasing oxygen demand (Lomholt and Johansen, 1979; Johnston and Bernhard, 1982). Correlated effects were found that suggest a strategy of reducing energetic needs, such as: retarded growth, decreased reproductive capacity, decreased densities of mitochondria in white muscles, and down-regulation of gene activity (Wu, 2003; Zhou *et al.*, 2000, 2001; Bagowski, unpublished).

An explanation for the differences

in oxygen consumption in hypoxia-acclimated fish, between our study and that of Lomholt and Johansen (1979), Johnston and Bernhard (1982a, b), Wu *et al.* (2003), and Zhou *et al.* (2000, 2001), is that the fish in the present study were exposed to lifelong hypoxia, starting shortly after they were released by the mother. During this period, which lasted up to 21 months, the fish grew roughly 250 times larger, while in other studies there was virtually no growth. Theoretically, different relations between growth of an animal and adaptability of anatomy and physiology to changed environments can exist (Witte *et al.*, 1990). The plasticity of shape and size of a structure *e.g.* the cardiovascular system, gills and respiratory system might well be dependent on ontogenetic stage. Measurements on the gills of NR and HR cichlids (Chapter 4) revealed plasticity in gill shape and respiratory surface, that for some parameters even exceeded differences between species of mollusc crushers living at different AS levels in Lake Victoria (Galis and Barel, 1980). Changing the relative shape of structures in animals that are environmentally challenged seems easier in animals that grow several orders of magnitude than in animals that hardly grow. Consequently, phenotypic responses to chronic hypoxia are likely to be stronger and/or different when fish are exposed to hypoxia from their youth up. Possibly, as a consequence of a decreased plasticity in adult fish, they must revert to an alternative strategy *viz.* reducing energetic needs, whereas very young cichlids can maintain normal metabolic rates by increasing their oxygen extraction capacity. To test this hypothesis, we exposed adult *H. ishmaeli* to 10% AS for 6 months. The first three weeks, fish in the hypoxia group ceased most swimming activity and hardly fed. Activity gradually increased over time but remained much more passive as compared to the normoxia group. Due to a sudden infection with gill parasites (*Lernaea* spp.), the experiments had to be terminated untimely.

Anoxia tolerance

Hypoxia-raised animals were able to spend significantly more time at anoxia than NR animals (Figure 3). This shows that, apart from the *aerobic* metabolism that was discussed above, either the *anaerobic* capacity and/or the ability to depress metabolic rate was increased in HR fish. The *A. alluaudi* were more tolerant to anoxia than *H. ishmaeli*, indicating that the anaerobic capacity was developed less in the latter. This conforms to expectations based on the range of natural habitats in which both species are found. They include swamps and mud bottoms in *A. alluaudi* (Greenwood, 1974; Witte, 1981) but not in *H. ishmaeli* (Witte, 1981, Van Oijen *et al.*, 1981).

Van den Thillart *et al.* (1980) found that acclimation of goldfish to 17-20 mm Hg (~8% AS) for 2 months

resulted in a significant increase of creatine phosphate and glycogen of respectively 35% and 84%. Creatine phosphate and glycogen can be used for ATP production anaerobically. Thus, if stores of both compounds are increased, fish can potentially tolerate anoxia longer. Observations on the behaviour during anoxia showed very clearly that a reduction of movement activity is related to the amount of time that can be spent during anoxia. Though movement activity was not quantified, *A. alluaudi* was clearly more passive at anoxia than *H. ishmaeli* and could spend more time at anoxia (Figure 3). In addition, the animals that could spend most time at anoxia lied flat on the bottom, instead of maintaining an upright position, thus saving more energy.

Hypoxia, a factor of importance in species extinction?

The fish used in this study were bred in the laboratory for 15- 20 generations and were permanently kept under stable high-oxygen conditions, making it possible that selection occurred for this environment. Under wild conditions, O_2 conditions are less stable and occasional or periodic occurrence of hypoxia may be a selective force favouring higher plasticity or tolerance for hypoxia than demonstrated in this study.

In *A. alluaudi*, high hypoxia tolerance was to be expected. This species occurs in a wide variety of habitats, including swamps and wetlands, which are low oxygen environments (Greenwood, 1974). The *H. ishmaeli* that were used, however, were progeny of fish caught in the Mwanza Gulf, in the south of Lake Victoria. There, they were only found over shallow sandy bottoms, where O_2 conditions are predominantly normoxic (Witte, 1981, Van Oijen *et al.*, 1981).

Gel electrophoresis of the blood of *H. ishmaeli* from this study, showed that NR and HR animals had different iso-haemoglobins in their blood (Chapter 6). Such clear-cut phenotypic response of the haemoglobin system was never found before. In HR *A. alluaudi* and tilapia, no such response of the haemoglobin system was found.

The ability of *H. ishmaeli* to maintain high O_2 consumption rates during hypoxia and their large phenotypic responses in the haemoglobin components may have a historical explanation. A study done by Chapman *et al.* (1995), that included *H. ishmaeli*, showed that Lake Victoria cichlids from shallow waters were able to tolerate acute hypoxia up to three times longer than ecologically similar Lake Tanganyika species from well-oxygenated rocky habitats. They suggested that high tolerance to hypoxia "may have contributed to the widespread distribution of many species in the Lake Victoria basin because it would have facilitated their dispersal through extensive papyrus swamps and permitted broader habitat use". Johnson *et al.* (1996) suggested that Lake Victoria had dried up about 14,000 years

ago. However, this was disputed in later articles, which argued that most likely remnant waters with extensive swamps remained in the Lake Victoria basin that served as fish refuges (Fryer, 1997, 2001). During this desiccation event, and during the time that the Lake Victoria basin refilled again, swamps, that are known to contain mainly hypoxic areas, would have made up a major part of the available habitats for the Lake Victoria fish fauna. Consequently, fish must have been frequently exposed to hypoxic conditions and high tolerance to hypoxia would indeed have been beneficial for survival. Possibly the high hypoxia tolerance of *H. ishmaeli*, demonstrated in the present study, is a relict from the desiccation event 14,000 years ago.

During the past decades, the majority of the cichlid species in Lake Victoria, including *H. ishmaeli, H. piceatus,* and *H. (Yssichromis) pyrrhocephalus*, were heavily affected by the ecological changes in Lake Victoria (Witte *et al.*, 1992). Explanations that were given for this decline were: over-fishing (Marten, 1979; Witte and Goudswaard, 1985; reviewed by Witte *et al.*, 2005), predation by Nile perch (Ugutu-Ohwayo, 1990; Kaufman, 1992; Witte *et al.*, 1992), water transparency (Seehausen *et al.*, 1997; Witte *et al.*, 2005) and hypoxia (Kaufman, 1992; Hecky *et al.*, 1994; Verschuren *et al*., 2002). In our laboratory, however, all cichlids that were raised at 10% AS (*A. alluaudi*, *H. ishmaeli H. pyrrhocephalus*, *H. piceatus* and tilapia), showed normal growth and unimpaired O_2 consumption. The present study is the first that provides concrete evidence that Lake Victoria cichlids can not only survive chronically low oxygen concentrations but, slow habituation can result in developmental responses that permits cichlids to thrive under hypoxia. Still, hypoxia could have a direct effect on the development of cichlid embryos that affects survival (Witte *et al.*, 2005; H.A. Rutjes, F. Witte and G.J.E.E.M. van den Thillart, pers obs.) Although relatively quick exposure to hypoxic events *e.g.* during upwelling or shifts of oxyclines certainly have large effects on Lake Victoria's fish fauna this study indicates that the direct effect of hypoxia on the decline of the haplochromines in Lake Victoria may be far less destructive than previously assumed.

CHAPTER 4:

DRAMATIC INCREASE IN GILL SURFACE OF A HYPOXIA-RAISED LAKE VICTORIA CICHLID

H.A. Rutjes, C. Senadheera, G.J.E.E.M. van den Thillart, F. Witte

Institute of Biology Leiden, Leiden University
P.O. Box 9516, 2300 RA Leiden, The Netherlands

Abstract

We predicted that cichlids exposed to chronic hypoxia grow larger gills than normoxia-acclimated siblings. Experiments were performed with a split brood of *Haplochromis (Yssichromis) pyrrhocephalus* raised under normoxia (80-90% air saturation) and hypoxia (10% air saturation). After conditioning for 22 months O_2 consumption under normoxic and hypoxic conditions was measured. After that, the fish were killed and the dimensions of the right hand side of the third gill arch were measured. With light microscopy, the number and length of the primary filaments was determined. With scanning electron microscopy, the dimensions of the secondary lamellae were measured. Of the hypoxia-raised fish, the filament length, secondary lamella length and height were dramatically enlarged resulting in a total increase of the respiratory surface of the measured gill arch of 80%. This difference between siblings is larger than the inter-specific differences seen between related species. The range in the frequency of the secondary lamellae in normoxia and hypoxia raised fish, was the same as between species of cichlids living in normoxic and hypoxic habitats. The number of filaments in cichlid species from hypoxic habitats was considerably larger than that of species from normoxic habitats. Such differences in filament number were not found in hypoxia-raised *H. pyrrhocephalus*. This study shows that gills of fish are extremely plastic and that the size and shape are to a large extent determined by environmental cues.

Introduction

In the last century, occurrence of chronic hypoxia caused by human-induced perturbations became increasingly common in water bodies worldwide (Wu, 2002; Reddy, 2005). This is also the case in Lake Victoria, where during the past decades, O_2 concentrations decreased considerably due to eutrophication (Ochumba and Kibaraa, 1989; Kaufman, 1992; Hecky *et al.*, 1994; Wanink *et al.*, 2001; Witte *et al.*, 2005). In 1979-80, at a 14 m-deep sampling station in the Mwanza Gulf, hypoxic conditions (<3 mg L^{-1}) were present near the bottom for several days only during the long rainy season (van Oijen *et al.*, 1981). However, during the rainy season in 1987-88, at the same sampling station more severe hypoxia (<1 mg L^{-1}) was present for several months. Moreover, hypoxic water layers reached higher into the water column (Wanink *et al.*, 2001).

Studies on several species of Lake Victoria cichlids showed that non-acclimated adult fish which are gradually exposed to 10% air saturation (ca. 0.8 mg L^{-1}) within a few hours, survive less than one day (Rutjes, unpublished). This is normal for most teleosts (Van den Thillart and Van Waarde, 1985). Broods of cichlids that were exposed to increasing levels of hypoxia over several weeks could, however, be raised under 10% air saturation (AS). The fish grew well and even reproduced (Chapters 2 and 3). One adaptive response found in hypoxia-raised (HR) fish, was a considerable enlargement of the oxygen uptake capacity (Chapters 2 and 3). In this way, HR fish were capable of the same routine O_2 consumption rate under 10% AS as normoxia-raised (NR) siblings were under 80% AS. This indicates that phenotypic plasticity must play an important role in survival under lifelong hypoxia.

In order to extract the same amount of oxygen under hypoxic conditions, the ventilation frequency and the volume per breath stroke of HR fish should be dramatically increased. This was indeed observed in all species of cichlids that were raised under hypoxia until now (Chapters 2 and 3). The high ventilation activity and the low O_2 yield per amount of water under hypoxia, pose different demands on the gills than under normoxia, when ventilation activity is lower and the O_2 yield is high. Normoxia-acclimated fish that are exposed to hypoxia show a considerable decrease of oxygen uptake efficiency, resulting in high cost of respiration (Schumann and Piiper, 1966; Lomholt and Johansen, 1979; Fernandes and Rantin, 1994). This suggests structural changes of the gills of HR fish that accommodate efficient O_2 uptake under hypoxic conditions. Hughes (1966, 1973) concludes that at a high water flow per unit of time a larger respiratory surface is required to maintain high gas exchange efficiency. There are several ways in which the respiratory surface can be enlarged. It would be most

favourable to increase the gill surface area without increasing water resistance of the gills, which would result in higher cost of ventilation. Hughes (1966) used a model that represented the gill filaments and secondary lamellae as a series of rectangular channels, and predicted effects of changes in dimensional parameters of the gills on water flow. Increases in gill surface area that had the least effect on the total flow were an increase in total filament number, an increase in filament length and an increase in height of the secondary lamellae. In contrast, an increase of the respiratory surface by higher frequency of the secondary lamellae or longer secondary lamellae would cause relatively large increases in resistance and would therefore be unfavourable.

It is well known that there is a vast inter-specific variation in size and shape of teleost gills. This variation in shape and size seems to be correlated with variation in habitat, life style and O_2 demand (Gray, 1954; Hughes, 1966; 1972; 1973; Palzenberger and Pohla, 1992). Evidence for this was also found within African cichlids (Galis and Barel, 1980). The Lake Victoria cichlids *Haplochromis (Gaurochromis) hiatus* and *H. (Gaurochromis) iris*, resemble each other ecologically and morphologically. *H. hiatus* was found between 3 and 9 m depth while *H. iris* was found at a depth of 8-15 m (Hoogerhoud *et al*., 1983). During the rainy season, stratification occured and O_2 concentrations of 2-3 mg l^{-1} have been observed in the habitat of *H. iris* (Van Oijen *et al*., 1981). The total gill area of this species is 1.6 times larger than that of *H. hiatus*, mainly due to longer and more primary filaments (Hoogerhoud *et al*., 1983).

In contrast to the numerous studies that compare inter-specific differences in gill size and shape in relation to habitat and mode of life, little is known about the role of phenotypic plasticity of the gills (*e.g.* Chapman *et al*., 2000; Sollid *et al*., 2004). The ability to phenotypically enlarge the respiratory surface when exposed to chronic hypoxia would enable for a more efficient O_2 uptake in a fluctuating environment. In the present study we test the hypothesis that HR individuals of the Lake Victoria cichlid *Haplochromis (Yssichromis) pyrrhocephalus* have a strongly increased O_2 uptake capacity and have enlarged gills.

Materials and Methods

Raising and sampling of the animals

In this study, we used the cichlid *H. pyrrhocephalus*, which belongs to the Lake Victoria super-flock. This zooplanktivore reaches a maximum standard length (SL) of about 73 mm (Witte and Witte-Maas, 1987). *H. pyrrhocephalus* is found over muddy bottoms between 3 and 21 m deep, where hypoxia (< 3 ppm, ~35% air saturation) occasionally occurred near

the bottom during the rainy season in the 1980s, but is now present for longer periods and further from the bottom (van Oijen *et al.*, 1981; Wanink *et al.*, 2001). The species is partly pelagic and used to feed on zooplankton near the bottom during daytime and on *Chaoborus* larvae near the surface at night (Witte and Witte-Maas 1987; Goldschmidt *et al.*, 1990). A number of brooding females of *H. pyrrhocephalus* was caught by J.H. Wanink in the Mwanza Gulf in 1987. The breeding stock in our laboratory was based on the offspring of these brooding females. In 1999, of brooding females of this breeding stock, one brood was selected when they measured about 1.5 cm SL, one to two weeks after the young were released by their mothers. The nest was split and the fish were raised in 100-litre aquaria in the same climate room. Thus, genetic diversity was small and treatment of the fish prior to the experiment was the same. When the fish were 4-6 months old, the air saturation (AS) level of the water of the HR group was lowered stepwise to 10% AS in four weeks. The water in the aquarium of the normoxia raised group was kept under 80-90 % air saturation. Circulating water was made hypoxic in a vacuum equilibration column in which dissolved air in the water equilibrated at 6-9 kPa. The 100-L aquaria were covered with a metal plate that was placed a few centimetres below the water surface to restrict O_2 exchange with the surface and to prevent aquatic surface respiration of the fish. The AS-level was constantly monitored (ADI 1030 biocontroller, Applikon, equipped with polarographic oxygen sensors) and adjusted to 10% AS via a solenoid valve in line with an air stone. The fish were kept at a temperature of 25.5 °C and a light-dark regime of 12-12 hrs. They were given a diverse diet of flake food, frozen midge larvae, frozen zooplankton, and a mixture of pulverized shrimps, mussels and flake food. Before adulthood, survival approximated 100% in both groups.

Respirometry Experiments

About 22 months after the brood was split, respirometry experiments were carried out using similar equipment as described in Chapter 2. Oxygen consumption was measured for 36 h under the AS-level that where they were raised at (80% AS for NR fish and 10% AS for HR fish). Afterwards they were exposed to a series of decreasing AS-levels (80%, 40%, 20%, 10% and 5% AS). The same protocols were used as in Chapter 2. The flow chambers of 4.2 L that were used in Chapter 2 were designed for fish of a minimum weight of 10 grams. The absolute O_2 consumption of *H. pyrrhocephalus* is lower than that of the animals used in Chapter 2 since they reach a maximum weight of only 4-5 grams. In order to detect smaller changes in the O_2 concentration due to consumption of the animals, smaller flow-chambers of 1.6 L were designed. While *H. pyrrhocephalus* survived the

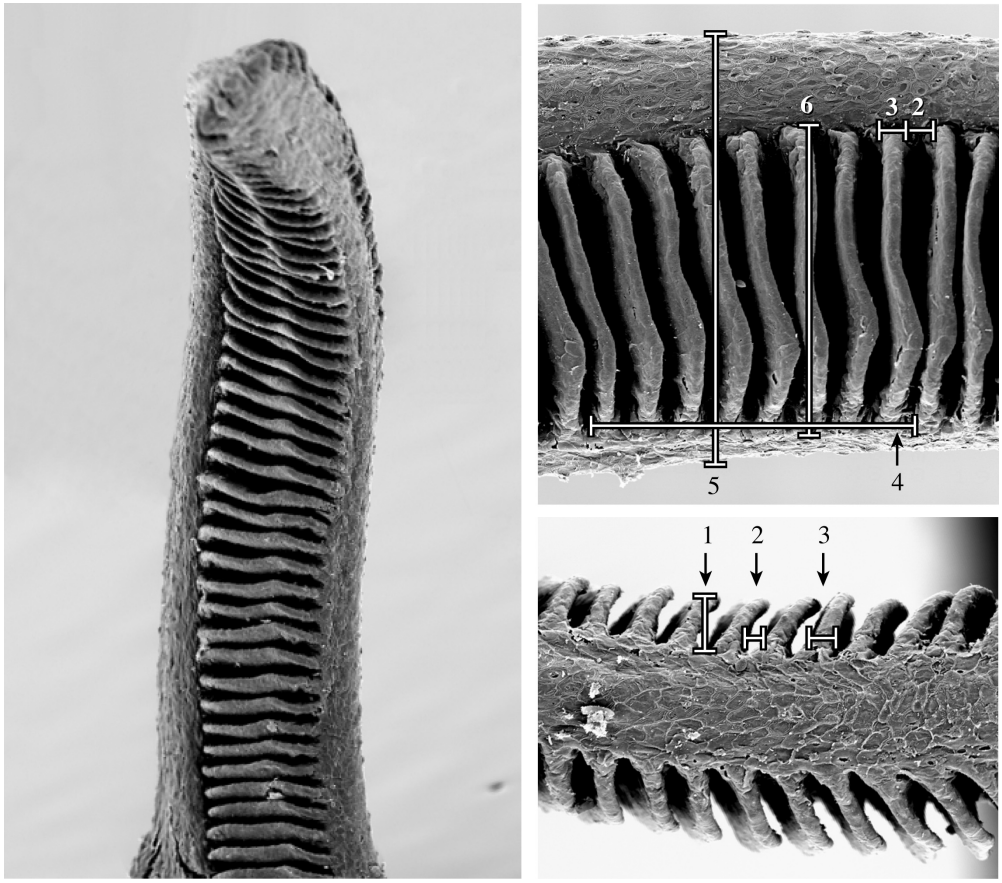

Figure 1: *Measures that were taken with light microscopy and scanning electron microscopy (SEM) of filaments on the lateral hemibranch of the third gill arch of H. pyrrhocephalus. The photograph left above shows the four positions where filaments were selected for measurement of the densities of the secondary lamellae. These filaments were subsequently dissected and used for scanning electron microscopy (SEM; left below). The two close ups on the right show the measurements that were taken from SEM photographs. 1= secondary lamella height, 2= distance between secondary lamellae, 3= thickness of lamellae, 4= frequency of secondary lamella, 5= width of primary filament, 6= length of secondary lamella.*

respirometry procedure under normoxia, they died when they were subjected to the entire protocol in the flow chambers of 1.6 L under 5% AS or during recovery. In contrast, other haplochromines of similar size survived. A possible explanation is the additional stress due to confinement of *H. pyrrhocephalus* (which is normally pelagic) in a very small space. When using flow chambers of 16L *H. pyrrhocephalus* did survive the whole protocol under hypoxia. To have enough biomass, we chose to do two group experiments with the 16L flow chambers using each time the complete NR and HR group (respectively 9 and 7 animals). The O_2 consumption per fish was corrected for weight differences using a modification of the formula used by Van den Thillart and Kesbeke (1978):

$$M_{100} = M\ 100^{0.8} / (W_1^{0.8} + W_2^{0.8} + \ldots + W_n^{0.8})$$

Where m100 is the metabolic rate of a fish of 100 grams, M is the measured metabolic rate and W is the weight of each fish in the experiment. After respirometry, the animals were killed with an overdose of anaesthetic (300 mg L^{-1} tricainemethanesulfonate, Finquel) and stored in 3.6% formaldehyde (buffered with Borax) at room temperature. After 12 months the animals were transferred to 70% ethanol for further storage.

Measurements

Of six NR and seven HR fish, the gill arches on the right side of the head were removed separately under a dissection microscope and stored in 70% alcohol for further processing. With a digital camera (Nikon Coolpix) that was mounted on a dissection microscope, photographs were made of the lateral hemibranchs of the four gill arches, together with a plastic bar of a known size for calibration. From these photographs the number of filaments was counted, and the length of filaments was measured, using the program Imagetool V3.0 (Texas University Health Science Centre). To determine the average filament length per hemibranch, at least 15 filaments were measured of each hemibranch. The filaments that were measured were determined by dividing the total number per hemibranch by 15 and rounding it off to the nearest integer below. Thus, rendering 15-18 filaments to be measured that were equally divided over the whole hemibranch. Comparison of the average filament length of several hemibranchs based on 15-18 filaments with averages of the same hemibranchs based on measurement of all filaments showed differences of 2-3%.

At the highest magnification of the dissection microscope (63X), the density of secondary lamellae on the filaments was determined at four positions of each lateral hemibranch (Figure 1). These filaments were selected by dividing the gill arch into five sections of an

equal number of filaments. At the same positions, filaments were then dissected and the dimensions of the secondary lamellae and of the channels in between were measured.

As the maximum magnification of the dissection microscope (63 X) did not allow detailed measurements of the dimensions of the secondary lamellae, a Scanning Electron Microscope (SEM) was used. These allowed us to make sharp images of a filament in three directions, to study shape differences and to measure the surface areas of the secondary lamellae with high precision (Figure 1). A disadvantage of using both SEM and light microscopy is that the preparation procedures for SEM include complete dehydration (see below). Possibly, extra shrinkage of the gills occurred compared to light microscopy samples that were stored in 70% ethanol. Since the SEM procedure is very time consuming, only the lateral hemibranch of the third gill arch was used. The outer two gill arches, one and four, were not used since they have a different shape. In addition, their shape could possibly be affected by alterations in adjacent structures. Apart from this, the choice for the lateral hemibranch of gill arch three was arbitrary.

To make the filaments stiffer, they were treated with a 1% osmium tetroxide solution (in phosphate buffer) after dissection. Afterwards, the alcohol and water in the filaments were replaced in a series of increasing acetone concentrations leaving them for critical point drying. The 100% acetone was replaced by CO_2 (Baltec CPD 030 critical point dryer). Subsequently, the CO_2 was evaporated, leaving the dehydrated filaments. The filaments were then glued on stubs with silver glue and, sputter coated with gold (Polaron Equipment Ltd, SEM coating unit E5100). A scanning electron microscope (Jeol JSM-6400) was used for photographing the filaments.

Images were made of the middle of each filament and perpendicular to all four sides of the filament (Figure 1). From these images, the maximum height, length and thickness of the lamellae, interlamellar distance and filament width were measured. Since no SEM images could be taken perpendicular to single lamellae, the surface area was calculated from the height and length measurements of the secondary lamellae. The shape of the secondary lamellae resembled a rectangle with a slightly dome shaped top, rather than an ellipse. The ‘dome’ was sometimes more pointed. To calculate the surface area of one side of a single secondary lamella, the length and maximum height of each lamella were multiplied. This gave a slight overestimation of the surface area. As the lamella shape was the same between groups, measuring errors due to the method used, were the same for both groups. Thus, the relative difference between groups remains the same. Per filament the above mentioned parameters

were measured in at least 6 secondary lamellae on each side of the filament. Average sizes per filament e.g. position were used for further calculations. The total respiratory surface (total surface area of the secondary lamellae) of a complete hemibranch was calculated as:

Surface area = 2F L 2A

Where F is total filament length (number of filaments times average length); L is lamellar frequency; A is area per secondary lamella. The total filament length was multiplied by two since lamellae are found on both sides of each filament. The area per secondary lamella was multiplied by two since both sides of each lamella are used for oxygen exchange.

Statistics

All data were analysed with the software program SPSS V10.0 (SPSS Inc. Chicago, Il.) for Windows. For Analysis of Covariance, the data were linearised by ln-transformation. With ANCOVA, differences between NR and HR fish were investigated while using the position at the hemibranch (a to d, see Figure 1) as a factor and fish weight as a cofactor. Parameter estimates were used to back calculate the sizes of the parameters that were measured. All parameters were corrected along common regression lines to that of a fish of the common mean weight of all experimental animals used (4.9 grams). Both the raw data as well as the residuals from the variance analysis were normally distributed.

Results

Respirometry

During respirometry experiments with the NR group, one animal died after 1 hour in the recovery period. The experiment was terminated at that moment. Of the data, minimum (5 percentile), median (50 percentile) and maximum (95 percentile) O_2 consumption was calculated as described in Chapter 2.

At the acclimation level (Control, respectively 80% AS and 10% AS), O_2 consumption of the HR group was approximately 30% higher than that of the NR group (Figure 2). When increasing the AS-level from 10% during the acclimation period to 80%, the HR *H. pyrrhocephalus* reacted with a very large increase in O_2 consumption (Figure 2). At subsequent decreasing AS-levels, the O_2 consumption gradually decreased. The 5-percentile, which approximates the minimum O_2 consumption at the acclimation level (Chapters 2 and 3) was taken as the standard metabolic rate (SMR). Even during exposure to 5% AS the O_2 consumption of the HR group did not fall below the SMR.

In the NR group, there was an increase in O_2 consumption when the AS-level was decreased to 40%. When lowering the O_2 concentration to 20% and below, consumption rates dropped to below the standard metabolic rate at normoxia. At

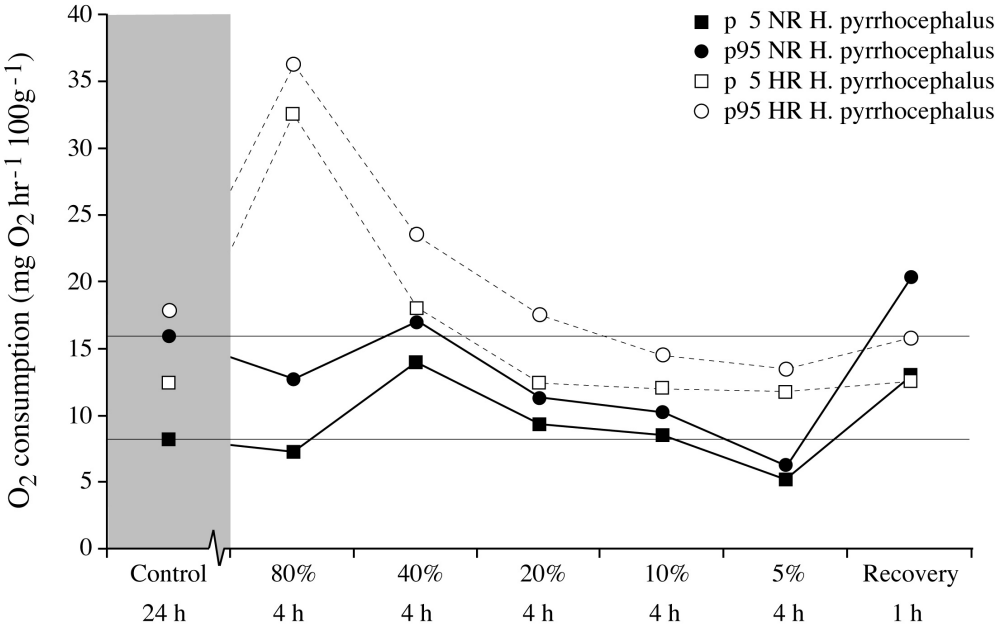

Figure 2: *The averaged O_2 consumption patterns of the normoxia- (n=7) and hypoxia-raised (n=9)* H. pyrrhocephalus *group, measured at the acclimation level (Control, respectively 80% and 10% air saturation for normoxia and hypoxia-raised fish) and a series of decreasing AS levels, followed by recovery. Of the frequency distribution of the data per condition, the 5-percentile and 95-percentiles are given.*

5% AS, the O_2 consumption of the NR group at the 5- and 95-percentile was between 8.4 and 15.3 mg O_2 h^{-1} $100g^{-1}$ at 80% AS and between 5.1 and 6.2 mg O_2 h^{-1} $100g^{-1}$ at 5% AS, meaning a reduction in O_2 consumption by about 40%.

Filaments

The weight of NR fish was 5.6g ±0.6g standard deviation. That of HR fish was 4.3g ±0.5g standard deviation. The weight of NR fish was significantly larger than of HR siblings (t-test, p=0.000). All parameters were size dependent. Since the length and weight of the fish hardly overlapped between groups and since size dependency creates a large standard deviation in the mean values, ANCOVA, was used to correct measurement values along a common mean regression line to a fish of a common mean weight (4.9 grams). The normalised data are presented in Table 1.

The number of filaments was not significantly different between NR and HR fish (ANCOVA p=0.448). The filament length of HR animals was 26.9% larger than that of NR siblings

Table 1: *Morphometric data on gills of* H. pyrrhocephalus *that were raised at normoxic (N=6) and hypoxic (N=7) conditions. Filament length and Lamella density 1 and 2 were measured using a light microscope. The rest was measured using a scanning electron microscope. Filament length and Lamella density 1 were determined on all 4 lateral hemibranchs. Lamella density 2 was, just like the rest of the measurements, determined on the lateral hemibranch of the third gill arch. The values given are estimates based on ANCOVA modelling. The variation of the residuals was normally distributed and hence estimates are reliable approximations of the data. Values are interpolations to a mean weight of 4.9 grams. Where values did not differ significantly ($p<0.05$) between normoxia raised (NR) and hypoxia raised (HR) fish, values were estimated to be the same. Pos= position on the hemibranch (See Figure 1).*

	mean change	**Pos1**		**Pos 2**		**Pos 3**		**Pos 4**	
		NR	HR	NR	HR	NR	HR	NR	HR
Filament length (mm)	+26.9%	1.2	1.5	1.6	2.0	1.5	1.9	0.9	1.2
Lamellar length (µm)	+37.7%	131.6	181.3	159.0	219.0	155.3	213.8	118.8	163.5
Lam. height (µm)	+9.2%	49.7	49.8	51.9	63.4	47.3	55.1	42.9	41.3
Lamellar area (μm^2)	+58.8%	6205	9854	8709	13830	7541	11975	4680	7432
Filament width (µm)	+31.4%	178.3	220.5	222.2	269.3	195.7	267.4	147.5	220.2
Lam. density 1 (no. mm^{-1})	-9.6%	31.0	28.3	30.5	27.8	31.5	28.8	33.1	30.2
Lam. density 2 (no. mm^{-1})	-4.3%	31.6	30.2	30.5	29.1	29.2	28.0	29.8	28.5
Lam. dist (µm)	0% not-sign	20.0	20.0	19.8	19.8	19.0	19.0	17.8	17.8
Lam. thickness (µm)	12.9%	5.8	6.5	6.8	7.7	6.0	6.8	5.4	6.1

(ANCOVA p=0.000, Table 1). Filament length varied between the four positions (Figure 1) and was dependent on the weight of the fish. The filament width of HR animals was on average 31.4% larger than of NR siblings (ANCOVA p=0.000, Table 1). From caudal (position a) to rostral (position d) on the hemibranch, the filament width increased with respectively 23%, 21%, 37% and 49%.

Secondary lamellae

The secondary lamellae density as measured over all four lateral hemibranchs, was negatively correlated with the fish weight (Spearman, correlation coefficient = 0.286, p=0.040) as well as with the length of the filament (Spearman, correlation coefficient = -0.585, p=0.000). There was no significant effect of filament length on any of the other measurements that were taken. The density of secondary lamellae over all four hemibranchs was 9.6% lower in HR than in NR siblings. When measured over the third gill arch only, the difference was 4.3%.

In the HR fish, the length of the secondary lamellae was 37.7% larger than in NR siblings (p=0.000, Table 1). The ratio between the filament width and secondary lamellae length was not significantly different between NR and HR fish (respectively 1.3 and 1.26).

The height of the secondary lamellae was on average 9.2% larger in HR fish (p=0.024). The increase found in HR fish varied considerably over the four positions (*e.g.* 0.1%, 22% 16% and -4%).

The distance between secondary lamellae was not significantly different between NR and HR *H. pyrrhocephalus* (p=0.291). It was negatively correlated with the weight and varied over the four positions. The thickness of the secondary lamella was on average 12.9% larger in HR *H. pyrrhocephalus* (p=0.001). An ANCOVA on the measurements at all four positions per hemibranch and using both filament length and weight of the fish as a covariable, showed that the density of the secondary lamellae was significantly lower by about one lamella per mm (p=0.000, Table 1).

Gill surface

With ANCOVA, we tested for a difference in total gill surface area of the third gill arch between NR and HR fish, using weight as a covariable. The gill surface area of gill arch 3 was dependent on weight and significantly larger in HR than in NR fish (ANCOVA, p=0.027 and 0.001). Estimates were 105 mm^2 for NR and 190 mm^2 for HR fish, meaning that the gill area of gill arch 3 of HR fish was 80% larger than that of NR siblings. Apart from the size difference, the gills of HR fish were much darker than those of NR siblings. In fresh non-preserved animals, dissected gills of HR fish were coloured deeply red while dissected gills of NR siblings were very pale.

Discussion

Experimental design

When using several treatment groups it is impossible to keep all factors other than the treatment identical. To rule out the influence of uncontrollable factors the use of replications is important. In the present study, individuals all of each treatment group per brood were raised in the same aquarium (pseudo replication) Thus, it should be considered that theoretically, differences in gill morphology between NR and HR groups could be caused by factors other than the difference in AS level. However, for several reasons we believe that this is unlikely. First, we used a randomly split brood with little genetic variation. The parental animals were offspring from a breeding stock that was kept in our laboratory since 1987. This stock originated from only three brooding females that were imported in our laboratory. Second, while the difference in AS level between NR and HR fish was a factor eight, care was taken that other environmental differences were kept at a minimum. All groups were raised in water from the same filter system in the same climate-room and in identically built aquaria. Third, data of experiments on other species of cichlids, showed similar effects of chronic hypoxia. These experiments can be regarded as

replications when considering only the effects of lifelong hypoxia irrespective of species differences. Fourth, we formulated a prediction about the direction of the differences between NR and HR siblings and the differences found correspond with our expectations.

Respirometry

The objective of performing respirometry experiments was to show that HR *H. pyrrhocephalus* were able to remain at the same routine O_2 consumption level at 10% AS as the NR siblings at 80% AS. The NR group that was exposed to hypoxia showed a considerable depression of metabolic rate at 10% AS. One animal even died after hypoxia exposure, indicating that NR fish are only temporarily able to cope with such conditions. In contrast, the routine O_2 consumption of HR fish at 10% AS was even higher than that of NR fish at 80% AS. This indicates that phenotypic responses occurred, ensuring maintenance of routine O_2 consumption at 10% AS. Similar phenotypic responses were seen in three other species of cichlids (Chapters 2 and 3) showing that early exposure to hypoxia induces strong adaptive responses in cichlids.

Effects of changed gill dimensions

The difficulty in interpreting the functionality of the observed differences in gill dimensions is the lack of a good theoretical basis. In the past, several authors have made attempts to model flow of blood and water through gills (Hughes, 1966; Jones *et al.*, 1970; Smith and Johnson, 1977; Matsuda and Sakai, 1999). However, since there are many parameters determining the dynamics in O_2 diffusion and flow of blood and water through the gills (*e.g.* Figure 3), a comprehensive model is still not available. Based on two models we tried to assess what the effects of the observed anatomical changes in gill dimension were on gas exchange. For each it is assumed that the other parameters remain constant unless stated otherwise.

At 10% AS, O_2 extraction is performed at conditions that pose very different demands on the gills than at normoxia. As HR fish at 10% AS had the same routine O_2 consumption as NR siblings had at 80% AS, they would have needed about an eight-fold increase in water ventilation to supply the gills with enough O_2. An assumption is that the O_2 extraction efficiency was just as high. Thus, the morphology of the gills must have allowed for a greater water flow, which is dependent on the resistance of the gills. Laminar (non-turbulent) flow of fluids through small circular channels is described by the Hagen-Poisuille equation:

$$q = \frac{(p1-p2)\ \pi \cdot r^4}{\eta \cdot 8 \cdot l}$$

Where q (flow in ml $pore^{-1}$ sec^{-1}) is dependent on the pressure difference between both sides of the tube in (p1-

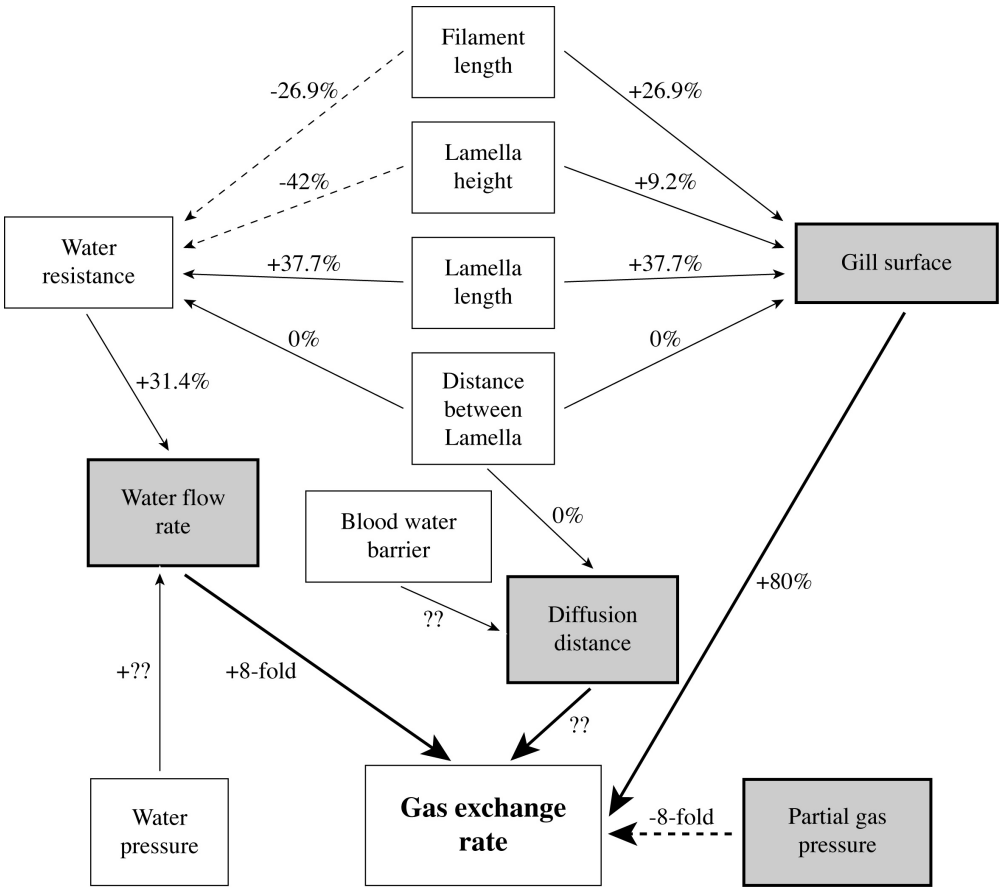

Figure 3: *The most important parameters that affect gas exchange rate in the gills. Gas exchange principally is dependent on sufficient supply of oxygen. As the partial gas pressure at 10% air saturation was eight-fold decreased compared to 80% AS, the same increase in water flow rate is necessary to supply the gills with enough O_2. Altering the dimensions of the gills can reduce resistance, the diffusion distance and gill surface area, which in turn affect the gas exchange rate.*

p2), the diameter of the canal (r), the viscosity of the fluid (η) and the length of the canal (l). Sufficient O_2 exchange is dependent on other factors than the mere availability of O_2 in the gills alone (Figure 3). Fick's first law of diffusion describes the gas exchange process:

$$J_{net} = D\ A\ \Delta PO_2/\Delta\chi$$

According to Fick's law the total O_2 flux from water to blood (J_{net}) is directly proportional to the surface area (A), the difference in partial O_2 pressure (ΔPO_2)

and the inverse of the diffusion distance ($\Delta\chi$).

When applying the two equations above to fish gills, it becomes clear that certain alterations in dimensions of the gills play a central role in increasing both gas exchange and the water flow through the gills (Figure 3). In the section below we discuss the effects of the observed differences in gill dimensions on water resistance and gas exchange.

Gill resistance and water flow

When applying the Hagen-Poiseuille equation to water flow through gills, flow rate can be enlarged by increasing the pressure difference or decreasing the resistance of the respiratory channels. The latter can be realised by changing the dimensions of the gills *e.g.* increasing the cross-sectional area, decreasing the length and increasing the number of respiratory channels.

Since secondary lamella height was increased by 9.2% (Table 1), the cross-sectional area of each respiratory channel was larger which reduced resistance of each channel. This change may seem small but according to the Hagen-Poiseuille equation the positive effect on the flow is exponential, resulting in a 42% increase in flow per pore. However, the Hagen-Poiseuille equation can only be used semi-quantitatively in this case because the respiratory channels are not tubes but resemble a rectangular shape. According to Mortensen *et al.* (2005) this shape difference has a slight effect on resistance due to the larger circumference and alters the flow regime through a channel. According to Bendib and Tabeling (2001) the flow through a tube with a circular cross-section and a rectangular cross-section resemble each other closely as long as the ratio between height and width is smaller than 4.5. In this study, the ratio was about 2.5. Although the secondary lamella thickness increased by 12.9%, a significant decrease of the respiratory channel width did not occur since the frequency of secondary lamellae decreased as well (Table 1). In this way, a decrease of the cross-sectional area of the respiratory channels that would drastically increase their resistance was avoided.

The HR fish had 37.7% longer secondary lamellae than NR siblings, thereby not only increasing the gill size, but also increasing the resistance and negatively affecting the water flow by the same amount. Interestingly, this more or less compensates for the increased flow rate that was realised by the increased height of the secondary lamellae (Figure 3).

In the HR fish, total filament length increased by 26.9%, thereby increasing the total cross-sectional area of all respiratory channels together, which decreases resistance. The increase in number of channels can be calculated by multiplying the length of the filaments (increased by 26.9%) and frequency of channels (decreased by 9.6%).

The total reduction of resistance of the gills seems to be realised by mainly the increased number of respiratory channels and not so much by the changed shape. The concomitant increase in water flow does not nearly resemble the eight-fold difference in water flow necessary to supply HR fish with enough O_2. Therefore, in HR fish undoubtedly the pressure difference over the gills must have increased and water flow speed were considerably larger to allow for the required increase in total flow rate.

Oxygen diffusion

In HR fish at 10% AS, the O_2 flux (J_{net}) was the same as in NR fish at 80% AS. However, ΔPO_2 was reduced eight-fold in HR fish. A logical prediction would be that HR fish maintained the same O_2 flow through an increase of the surface area and a decrease of the diffusion distance. In the HR fish from the present study, indeed an increase in respiratory surface was realised in several ways. First, 26.9% longer primary filaments resulted in an increase in the total number of secondary lamellae. Second, the secondary lamellae had a 58.8% larger surface caused by an increase in both height as well as length. The total respiratory surface of the HR *H. pyrrhocephalus* from this study was thus enlarged by as much as 80%. Under normal conditions, much of the arterial blood is shunted through the marginal vessels of the gill filaments and NR fish perfuse only a fraction of the total amount of secondary lamellae during routine activity (Randall, 1970). Thus, the difference in the respiratory surface that was actually used between NR and HR *H. pyrrhocephalus* is probably larger than 80%.

The diffusion distance is dependent on the width of the respiratory channel and the thickness of the water-blood barrier. As discussed in the previous section, the width of the respiratory channels did not change significantly. The thickness of the blood-water barrier was not measured in this study. The speed of O_2 exchange is inversely proportional to the diffusion distance. According to Fick's law, a reduction of the water-blood barrier thickness would result, just like an enlarged gill surface, in a larger O_2 exchange rate. Between species, the width of the water-blood barrier can vary between 0.6 µm in the tuna *Katsuwonis pelamis* and 11 µm in the dogfish *Sciliorhinus canicula* (Hughes, 1972). However, little is known about adaptive phenotypic responses of the water-blood barrier. HR fish were capable of the same O_2 consumption as NR fish at 80% AS, and the increase in respiratory surface alone compensates only partly for the reduced O_2 flux over the gills at 10% AS. Therefore, we expect that in HR *H. pyrrhocephalus* a significant reduction in the water-blood barrier occurred, which contributes to the ability of maintaining high O_2 extraction at 10% AS.

Efficiency and respiratory cost

Although O_2 exchange is increased by an enlarged respiratory surface, the way this is realised has a large effect on gas exchange efficiency and therefore the cost of respiration. Smith and Johnson (1977) showed that O_2 saturation of the blood does not change much at a wide range of water flow speeds and secondary lamella shapes, but the percentage of oxygen that is extracted from the water is easily reduced by a high water flow speed. For most fish species, high water flow speeds through the respiratory channels that result in low O_2 extraction efficiency must be avoided. For buccal ventilators, which need to generate a ventilatory water flow by active pumping movement, a relatively high proportion of the total energy budget is spent on ventilation, which makes high efficiency and low gill resistance necessary. The cost of ventilation, relative to the total O_2 consumption is 3-18% in *Oreochromis niloticus* (Fernandes and Rantin, 1994), 10-43% in tench, *Tinca tinca* (Schumann and Piiper, 1966), and 10-25% in trout, *Oncorhynchus mykiss* (not swimming, Fernandez and Rantin, 1994). In contrast, the tuna *Katsuwomis pelamis*, which is a ram-ventilator, uses only 1% of its total energy expenditure for respiration (Muir and Hughes, 1969).

Under the demands that cost of respiration must be low, the O_2 extraction efficiency must remain high. The extraction efficiency is dependent on the amount of time that water remains in contact with the gills. As shown in (Figure 3), water flow rates must have increased considerably in HR fish, resulting in a reduced extraction efficiency caused by a shorter time during which water and gills are in contact and O_2 exchange occurs. However, secondary lamellae were 37.7% longer, resulting in an equal increase in the contact time. In summary, the longer secondary lamellae result in larger resistance to water but also in an improved O_2 extraction efficiency.

Apart from parameters that alter water flow through the gills, there are indications that cardiovascular responses occurred. The increased thickness of the secondary lamellae in HR fish suggests an increased diameter of the vascular space. Muir and Brown (1971) stated that "vessel diameter determines, considerably, the thickness of the secondary lamellae". In addition, they showed that in several teleosts, the diameter of the vascular space within the secondary lamellae was correlated with the length of the secondary lamellae. They argued that this compensated for the increased pressure drop between beginning and end of a secondary lamella that is caused by enlarged resistance of a longer secondary lamella. In accordance, in the HR *H. pyrrhocephalus* from this study, the increased thickness of the secondary lamellae was observed concomitantly with an increase of lamellar length. As also argued by Muir and Brown (1971), an increase in diameter of the vascular space requires

a larger cardiac output. Pilot studies on tilapia and *Haplochromis (Astatotilapia) piceatus* showed that this is indeed the case in HR fish.

Comparison with other species

Chapman *et al.* (2000) performed a split-brood experiment with the cichlid *Pseudocrenilabrus multicolor victoriae* that were laboratory-raised at normoxia (7.5 mg L^{-1}) or hypoxia (1 mg L^{-1}). Additionally, they compared two populations caught in the wild in a normoxic and hypoxic habitat. In the laboratory group, the respiratory surface of the HR fish was (calculated by setting NR groups at 100%) 22% larger than that of NR siblings mainly caused by longer filaments. The respiratory surface of fish caught in the hypoxic habitat was 41% larger than that of fish caught in the normoxic habitat. This was realised through longer filaments and larger secondary lamellae. Chapman *et al.* (2000) argued that the discrepancy in the hypoxia related difference between fish caught from the wild and the split-brood experiment could be attributed to inherited changes in addition to phenotypic plasticity in fish from the wild, allowing for larger differences and different responses.

In comparison, in our study, the respiratory surface of the third gill arch of HR animals was 80% larger than that of NR fish. This was realised trough longer filaments and larger secondary lamellae. Irrespective of the differences in hypoxia exposure, phenotypic responses alone can result in even larger differences in gill size than shown in wild-caught *P. multicolor*. The considerable phenotypic plasticity that was found in the gills of *H. pyrrhocephalus*, raises the question whether inter-specific differences in gill size between related species are a result genetic differences, phenotypic plasticity or a combination of the two. As also by Chapman *et al.* (2000), observed phenotypes that are initially a result of phenotypic plasticity, may even lead to genetic diversification in populations that remain in a stable different environment. Galis and Barel (1980) found a positive correlation between the density of the secondary lamellae of a number of mollusc-crushing Lake Victoria cichlids and the depth at which each species was found. This was explained as an adaptation to the decreased oxygen concentrations at larger depth. Data were collected from one specimen per species. They observed densities of the secondary lamellae ranging between 23 and 27. This is similar to the intra-specific variation found in the *H. pyrrhocephalus* in the present study which is 29-34 when measured in the same way as the study of Galis and Barel (1980). In contrast, the variation in filament number in the mollusc crushers from the study of Galis and Barel was larger than the variation in *H. pyrrhocephalus* in the present study. Similar results as in the mollusc crushers were found in a study on *Haplochromis iris* and *H. hiatus*, two closely resembling Lake Victoria cichlids. The

deeper-living *H. iris* had a 1.6 times greater gill surface (Hoogerhoud *et al.*, 1983). The difference in the density of secondary lamellae falls within the range seen in NR and HR *H. pyrrhocephalus*. However, the difference in the number of filaments between *H. iris* and *H. hiatus* was considerably larger than the range that was seen in *H. pyrrhocephalus*.

Plasticity and its ecological consequences

Thus far we have raised nests of five species of African cichlids at normoxia and hypoxia (Rutjes *et al.*, unpublished). In all five species, hypoxia was survived very well if animals were exposed to these conditions from their youth onwards. In HR fish of *A. alluaudi*, *H. (Labrochromis) ishmaeli*, *H. piceatus*, and a crossbreed between *Oreochromis niloticus* and *O. mossambicus*, the same large increases in respiratory surface were found as in the HR *H. pyrrhocephalus* in the present study (Unpublished data). The increase in total gill surface is most probably a key factor that enables these fish to have O_2 consumption rates at 10% AS that are just as high as that of NR siblings at 80% AS (Chapters 2 and 3). The extreme phenotypic plasticity of these fish likely enables them to survive a broad range of O_2 concentrations and successfully exploit hypoxic environments comparable to the chronically decreased AS-levels that are nowadays present in Lake Victoria.

Literature shows that the gill size and shape of fish varies considerably between species from different habitats and with different life styles (Gray, 1954; Hughes, 1966, 1973; De Jager and Dekker, 1975; Galis and Barel, 1980; Palzenberger and Pohla, 1992). We should be vary careful when interpreting these differences since the present study shows that phenotypic plasticity alone can result in an equally large variation in gill shape.

CHAPTER 5:

A DISCRIMINATING SHAPE FACTOR AMONG AFRICAN CICHLIDS CAN BE INDUCED PHENOTYPICALLY

H. A. Rutjes, M.P. de Zeeuw, G.J.E.E.M. van den Thillart, F. Witte

Institute of Biology Leiden, Leiden University
P.O. Box 9516, 2300 RA Leiden, The Netherlands

Abstract

A massive enlargement of the gill surface proved to be an important factor in the hypoxia survival of young cichlids. As heads of cichlids are densely packed with structures related to both feeding and breathing, we hypothesised that gill enlargement requires such large internal reorganisations that outer head shape is affected. We used a three-dimensional model to describe changes in the outer head shape of cichlids. The model estimates the dimensions of the oral, suspensorial and opercular compartments in the head. Broods of cichlids of different phylogenetic lineages, habitats and trophic specialisation, were split and raised at either 10% or 80-90% air saturation until adulthood. They comprised two endemic Lake Victoria haplochromine species, a non-endemic haplochromine and a tilapia species. In spite of the above-mentioned differences between the species that were used, all hypoxia raised groups showed similar volume enlargements. Volume increases were most prominent in the ventral suspensorial and ventral opercular sub-compartments. A relation with the enlarged gills of hypoxia raised fish is likely, as the gills are mainly located in these compartments. Differences in ventral width are found in other studies comprising a wide variety of genotypic and phenotypic variations. The present study shows that such variation in the ventral width is conceivable by phenotypic plasticity alone. That head shape is to a large extent phenotypically plastic could be an important factor explaining the vast morphological diversity that is found in East African cichlids.

Introduction

Many fish may encounter abnormally low ambient oxygen concentrations (hypoxia) at some stage during their life. There are several mechanisms to cope with *short* periods of hypoxia, *e.g.* aquatic surface respiration, metabolic depression, and migration to areas with higher O_2 concentrations (Lomholt and Johansen, 1978; Van den Thillart and Van Waarde, 1985; Verheyen *et al.*, 1994; Almeida-Val *et al.*, 1995; Chapman *et al.*, 1995; Muusze *et al.,* 1998). Fish living in habitats where *chronic* hypoxia occurs naturally (*e.g.* swamps, wetlands), often have special adaptations to these conditions such as a large respiratory surface, a low standard metabolism and high haemoglobin concentrations (Galis and Barel, 1980; Chapman and Liem, 1995; Chapman *et al.*, 2000; Chapman *et al.*, 2002).

In the last century, human-induced perturbations caused chronic hypoxia in water bodies world wide, where it was uncommon previously (Chapman and Chapman, 2002; Wu, 2002; Witte *et al.*, 2005). An example is Lake Victoria in East Africa, where, during the past decades, O_2 concentrations decreased considerably due to eutrophication (Ochumba and Kibaraa, 1989; Kaufman, 1992; Hecky *et al.*, 1994; Wanink *et al.*, 2001; Witte *et al.*, 2005). In 1979-80, at a 14 m-deep sampling station in the Mwanza Gulf, hypoxia (<3 mg L^{-1}) was present near the bottom for several days only during the long rainy season (van Oijen *et al.*, 1981). However, in 1987-88, at the same sampling station severe hypoxia (<1 mg L^{-1}) was present for several months during the rainy season. Moreover, hypoxic water layers reached higher into the water column (Wanink *et al.*, 2001).

Lake Victoria cichlids are rather tolerant to short-term hypoxia (Chapman *et al.*, 1995; 2002a, 2002b; Witte *et al.*, 2005; Chapter 2, 3). However, pilot experiments on some Lake Victoria cichlids show that fish raised at normoxia can only temporarily cope with chronic hypoxia (10% AS, ≈ 0.8 mg L^{-1}). In contrast, 3-4 weeks old animals that were exposed to 10% AS, showed 100% survival, grew well and even reproduced. This indicates that phenotypic plasticity plays a role in survival at lifelong hypoxia. One adaptive response found in hypoxia raised fish, is a considerable enlargement of the respiratory surface area by lengthening of the gill filaments and by size increase of the secondary lamellae (Chapman *et al.*, 2000; Chapter .. this thesis). Similar adaptive changes were found in fish from natural environments, where hypoxia had increased (Wanink and Witte, 2000; Chapman *et al.*, 2000; Witte *et al.*, 2005).

Heads of fish are densely packed with muscles, bones and other structures, that are necessary for respiration, vision, feeding and other functions. As a consequence of limited space, increase of gill size may cause spatial conflicts

with surrounding structures. Such spatial conflicts have been demonstrated in the cichlid *Astatoreochromis alluaudi* (Smits *et al.*, 1996a, b). Animals with a larger pharyngeal jaw apparatus showed a decrease in size of surrounding structures and/or reallocations in the space of the head. Apart from internal reorganisations (Witte *et al.*, 1990; Barel, 1993; Chapman *et al.*, 2000), changing the outer head shape can also create space. It has been suggested that in fish living at low oxygen concentrations, possible enlargement of the gills could have such dramatic effects that the surrounding structures, and even gross morphology of the head, are affected (Smits *et al.*, 1996 b; De Visser and Barel, 2000). Indeed, Chapman *et al.* (2000) found a phenotypically induced increase in head length in the cichlid *Pseudocrenilabrus multicolor victoriae* that was raised at hypoxia, However, it was not determined whether the increase was due to an accelerated growth of the area where the gills are located. Studies done on populations of rock-dwelling cichlids that live at different O_2 concentrations, showed a correlation between volume of the opercular compartment of the head, and environmental oxygen concentrations (Bouton *et. al.*, 2002). In this case however, the size of the gills was not studied. Nor was it clear to what extent enlargement of the head was a phenotypically plastic trait.

In the present paper we investigated the outer head shape of a tilapia hybrid, and three haplochromine species from Lake Victoria. In each of these species, the gill filaments were enlarged due to lifelong hypoxia (Chapter 4 this thesis). We used a three-dimensional model, that has proven to be very sensitive for differences in head shape (De Visser and Barel, 2000) in combination with ANCOVA to test the effects of size, species and hypoxia. The species differ in phylogeny, range of oxygen concentrations in their natural habitat and in morphological parameters relevant to spatial allocation of the gill apparatus, *e.g.* pharyngeal jaw morphology and body depth. Both the pharyngeal jaw size and body depth is supposed to have an impact on the available space for gill increase. We hypothesised that in hypoxia raised fish, as a general phenotypic response, the volume of the head is increased, particularly in the area where the gills are located. In addition we investigated whether transformations in the head are specific for fish with respect to the above mentioned differences.

Materials and methods

Species used

In this study we used three haplochromine species: *Astatoreochromis alluaudi*, *Haplochromis (Labrochromis) ishmaeli* and *Haplochromis (Yssichromis) pyrrhocephalus* and a hybrid between *Oreochromis mossambicus* and *Oreochromis niloticus*. In the following

Table 1: *Summary of species, the habitats, and the morphological parameters relevant for this study. The upper half of each column shows the species specific parameters that possibly influenced the phenotypic responses to hypoxia. Pharyngeal jaw sizes are indicated from -- (very light and slender) to ++ (heavy and robustly developed) following Hoogerhoud (1986). Of each group that was used, the number of fish, average weights (grams) and average standard length (mm) are given. Abbreviations: SL = Standard Length, BD/SL = Body Depth/ Standard length, AS = Air Saturation, LV = Lake Victoria, Nor = Normoxia, Hyp = hypoxia. *, from Van Oijen* et al. *(1981), #, from Welcomme (1964).*

	***A. alluaudi* '92**		***A. alluaudi* '99**		***H. ishmaeli***		***H. pyrrhocephalus***		**Tilapia**	
Species specifics:										
O_2 range in the wild	Hypoxic or normoxic		Hypoxic or normoxic		Stable normoxic		2.5-7.5 mg L^{-1} *		2-5 mg L^{-1} #	
Pharyngeal jaws	+		+		+		0		0	
BD/ SL	34-43%		34-43%		37-45.5%		29-34%		36-49.5%	
Phylogeny	Haplochromine Separate lineage		Haplochromine Separate lineage		Haplochromis LV flock		Haplochromis LV flock		Tilapiine	
Experimental fish:										
Start experiment	1992	1992	1999	1999	1999	1999	1999	1999	1999	1999
AS level	80%	10%	80%	10%	80%	10%	80%	10%	80%	10%
No. of animals	9	6	7	7	6	6	9	6	10	10
Av. Weight (g)	10.5	6.6	22.0	17.5	24.0	23.0	5.6	4.1	27.0	22.3
Av. SL (mm)	82.0	76.6	57.3	67.5	89.4	87.7	67.6	59.6	87.6	83.9

paragraphs we briefly describe the species, habitats and morphological parameters relevant for this study (Table 1).

A.alluaudi is a pharyngeal mollusc crusher that is not endemic to Lake Victoria. It also occurs in Lakes Nabugabo, Edward-George and many small lakes around Lake Victoria (Greenwood, 1959, 1965, 1973; Hoogerhoud, 1986). According to both morphological and molecular studies, *A. alluaudi* is phylogenetically separated from all other Lake Victoria haplochromines (Greenwood, 1959; Meyer *et al.*, 1990; Nagl *et al.*, 2000 Seehausen *et al.*, 2003). The habitat of *A. alluaudi* includes well-oxygenated streams as well as hypoxic swamps and a variety of bottom types (Greenwood, 1974; Witte, 1981). In Lake Victoria, it is rarely found deeper than 20m. The body depth (BD) relative to the standard length (SL) is relatively large (BD/SL = 34-43%) and the maximum SL is 160 mm (Greenwood, 1959). In Lake Victoria molluscs dominate the diet (Greenwood, 1959; Hoogerhoud, 1986), whereas in most of the other lakes the main part of the diet consists of insects. The pharyngeal jaws are hypertrophied in waters where snails are an important part of the diet (Greenwood, 1965; Hoogerhoud, 1986; Smits *et al.*, 1996a).

H. ishmaeli is endemic to Lake Victoria. Though Lake Victoria haplochromines used to be considered as a monophyletic group (Meyer *et al.*, 1990), recent research suggest that there are several lineages in the lake. Regardless of taxonomic debates, the consensus view is that, in contrast to *A. alluaudi, H. ishmaeli* can be considered as a member of the Lake Victoria super-flock (e.g. an assembly of species flocks, see also Nagl *et al.*, 2000; Seehausen *et al.*, 2003; Verheyen *et al.*, 2003). The animals used in the present study were offspring of fish collected in the southern part of the lake, where their distribution was virtually restricted to well oxygenated water of less than 6 meters deep with sand bottoms (Witte, 1981). *H. ishmaeli* is relatively deep-bodied (37.0-45.5% of SL) and reaches a maximum SL of 136 mm (Greenwood, 1960). In spite of the phylogenetic differences between *A. alluaudi* and *H. ishmaeli*, in Lake Victoria both species have a similar pharyngeal jaw apparatus, i.e. hypertrophied and adapted to snail crushing (Greenwood, 1956, 1960, 1974; Hoogerhoud, 1984; Smits *et al.*, 1997).

H. pyrrhocephalus is endemic to Lake Victoria and like *H. ishmaeli*, a member of the Lake Victoria super-flock (Seehausen *et al.*, 2003). This zooplanktivore is more slender bodied than *H. ishmaeli* (BD/SL = 29-34%) and reaches a maximum SL of about 73 mm (Witte and Witte-Maas, 1981). *H. pyrrhocephalus* is found over muddy bottoms between 3 and 21 m deep, where hypoxia (< 3 ppm) occasionally occurred during the rainy season near the bottom in the 1980s, but is now present for longer periods of time and further from the bottom (van Oijen *et al.*, 1981, Wanink *et al.*, 2001). The species is partly pelagic and feeds on zooplankton near the bottom during daytime and on *Chaoborus* larvae near the surface at night (Witte and Witte-Maas 1981; Goldschmidt *et al.*, 1990). The pharyngeal jaws are slender.

As a representative of the tilapiine lineage, we used a hybrid between *Oreochromis mossambicus* and *Oreochromis niloticus* (further referred to as tilapia) that is used in aquaculture. Both parental species are not native to Lake Victoria, though *O. niloticus* has been introduced into the lake. In Lake Victoria it is found at O_2 concentrations between 2 and 5 mg L^{-1} (Welcomme, 1967). *O. niloticus* feeds mainly on phytoplankton when available but it is able to handle a wide range of food types. The diet of *O. mossambicus* consists mainly of detritus, but it also feeds on phyto- and zooplankton, if present (Trewavas, 1983). *O. mossambicus* and *O. niloticus* are relatively deep bodied (BD/SL = 36-49.5%), fast growing species. Maximum weights of more than 2800 g have been reported (Trewavas, 1983).

Raising and sampling of the animals
Experiments were performed in 1992-94 with *A. alluaudi* and in 1999-2002 with all four species. *A. alluaudi* and *H. ishmaeli* were caught in the Mwanza Gulf in 1984 and have been bred in our laboratory since. Also a breeding stock of *H. pyrrhocephalus* was present that originated from the offspring of fish caught by J.H. Wanink in 1987. Of the breeding stocks we obtained broods for our experiments. The tilapia broods that were used were F1 offspring of animals obtained from the University of Nijmegen.

Nests were selected when animals were about 1.5 cm SL (about four weeks after fertilisation). Each nest was split randomly and raised at normoxia (NR) and hypoxia (HR, Table 1). In 1999, all fish were raised in 100-litre aquaria in the same climate room. There were no indications that occasional deaths were related to hypoxia. Before adulthood, survival approximated 100% in both NR and HR groups. Death rate in general was higher in the NR groups where dominant males killed more fish. The water in the aquaria of the NR groups was kept at 80-90% air saturation (AS). The AS-level of the water of the HR groups was lowered stepwise to 10% AS in four weeks. Circulating water was made hypoxic in a vacuum equilibration column in which dissolved air in the water equilibrated at 6-9 kPa. The flow rate through the tanks was about 1-2 L/min. The 100-L aquaria were covered with a metal plate a few centimetres below the water surface to restrict O_2 exchange with the surface and to prevent aquatic surface respiration of the fish. The AS-level was constantly monitored (ADI 1030 biocontroller, Applikon, equipped with polarographic oxygen sensors) and adjusted to 10% AS via a solenoid valve in line with an air stone. The fish were kept at a temperature of 25.5 °C and a day-night cycle of 12-12 hrs. They were given a diverse diet of flake food, frozen midge larvae, frozen zooplankton, and a mixture of pulverized shrimps, mussels and flake food.

After 17-37 months the animals were killed with an overdose of anesthetic (MS222, Finquel) and stored in 3.6% formaldehyde (buffered with Borax) at room temperature. To standardize the morphometric measurements the gill covers and the mouth were closed. After three to 12 months the animals were transferred to 70% ethanol for further storage.

In 1992, the same experiment was conducted on a group of two mixed nests of *A. alluaudi* (Table 1, referred to as *A. alluaudi* '92, the *A. alluaudi* from 1999 are referred to as *A. alluaudi* '99). The methods used were the same, but feeding conditions and aquaria were different and the experiment was done in a different laboratory. The content of the aquaria was about 125 liters. They were fed live chironomid larvae and flake food. Animals were sampled after 15 and 19 months.

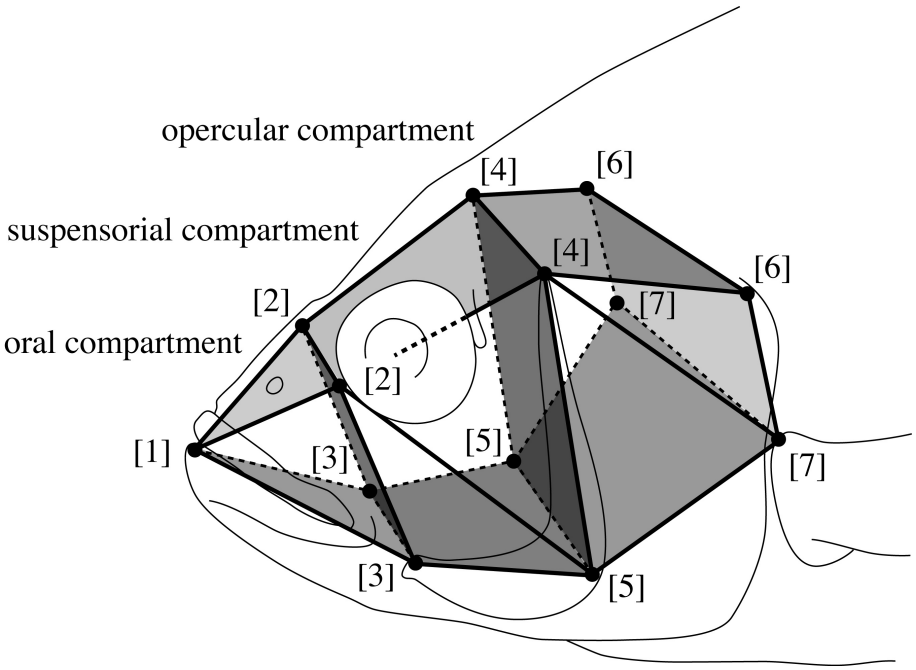

Figure 1: *Schematic drawing of the head of a cichlid and the external framework. The numbers [1]-[7], of which [2]-[7] are found on both sides of the head, indicate the 13 landmarks. The bars that connect the landmarks are indicated as a combination of landmark numbers (e.g. [1-2]), as are the volumes. The bar lengths were averaged between both sides of the fish. These averages were also used for calculating the compartment volumes.*
The landmarks and the 28 bars determine the compartments and sub-compartments in which the head was divided. The combination [1-2-3] corners the oral compartment and [2-3-4-5] the suspensorial compartment, in which [2-3-5] forms the ventral suspensorial and [2-4-5] the dorsal suspensorial compartment. The combination [4-5-6-7] corners the opercular compartment. In which [4-5-7] forms the ventral opercular and [4-6-7] forms the dorsal opercular compartment.

Measuring the external framework

The freshly killed animals were dried with paper tissues and weighed. The SL was measured following Barel *et al.* (1977). All other measures were taken after preservation. Individuals of both sexes were selected randomly in each group. To capture the outer head shape of the animals, the method of De Visser and Barel (2000) for measuring an external framework of cichlid heads was used. This is a 3-dimensional model of 13 landmarks (indicated as numbers) that are connected by 28 bars (indicated as combinations of landmark numbers, Figure 1). The landmarks on both

sides of the head divide the head into an oral [1-2-3], suspensorial [2-3-4-5] and opercular [4-5-6-7] compartment. The suspensorial and opercular compartments are subdivided in a ventral (respectively [2-3-5] and [4-5-7]) and dorsal sub-compartment (respectively [2-4-5] and [4-6-7]). The volumes of all (sub-) compartments were calculated by dividing them into wedges. To measure more precisely, the definitions for two landmarks were modified from those in De Visser and Barel (2000). De Visser and Barel defined point [5] (NPC) as the point halfway SLF_1 (Suspensorial Lateral line Foramen 1) and the neurocranial lateral line crest at NLF_5 (Neurocranial Lateral line Foramen 5). Point [7] (FDO) was measured as: the rostral-dorsal origin of the muscular basis of the pectoral fin at the caudal rim of the gill-cover. We defined point [5] as the point directly under the NLF_5 and point [7] as the most rostral-dorsal cartilaginous part of the pectoral fin base. The latter was visible after removal of the skin. Using these new definitions, the measurements were more accurate, and consequently, resulted in smaller standard deviations. Comparison of the differences in measurements, using the location of the points [5] and [7] used by De Visser and Barel and in this manuscript, resulted in differences less than 3%. In addition to the measurements on the external framework, the HL was measured following Barel *et al*. (1977). The modified definitions were used in the *A. alluaudi* '99 and *H. ishmaeli*.

Before measuring, the circum orbitals and nasal elements (Barel *et al.*, 1976) were removed. Where measuring points were covered by skin, this was removed. After cleaning all the landmarks, they were marked with a black alcohol-proof fineliner (Steadler). The bar lengths were measured three to six times using digital callipers (Sylvac) with needlepoints glued on the ends. Standard deviations were usually between 1% and 2%. If the standard deviation was larger than 5%, more measurements were taken until the standard deviation was below 5%. Both sides of each fish were measured. For calculating the volumes of the compartments the average of the measurements of each bar on both sides was used. Measurements were done without knowing whether animals had been raised at hypoxia or normoxia.

All data were log-transformed and analysed with the software program SPSS V10.0 for Windows. The volumes of all (sub) compartments were analysed with ANCOVA (analysis of covariance). The factors "Species" (4 species) "Environment" (normoxia *vs.* hypoxia) and "Experiment" (*A. alluaudi* '92 *vs.* all four species from 1999, including *A. alluaudi* '99) were investigated. Additional testing for differences between the *A. alluaudi* '92 and *A. alluaudi* '99 separately revealed the same similarities and differences between the two groups. The weight of the animals was used as covariable in the

Table 2: *Morphometric data on bar lengths (See also Figure 1 for notation). Data were collected from 5 different broods (4 species) and raised at normoxic and hypoxic conditions. The mean bar lengths (in mm) per experimental group are estimates based on ANCOVA modelling. Residual variation was normally distributed and hence estimates are reliable approximations of the data. Values are interpolations to a mean standard length (SL) per brood. Since the average SL differs between broods, effects of hypoxia can only be compared within each brood. Bar lengths that differed significantly between normoxia raised and hypoxia raised siblings are given in bold. Where bar lengths did not differ significantly between normoxia raised (NR) and hypoxia raised (HR) fish, identical estimates were given. In Table 3 the percent changes of bar lengths are given. Abbreviations: AS = Air Saturation, HL = Head Length.*

Species SL (mm)	*A. alluaudi* '92 79.1		*A. alluaudi* '99 62.7		*H. ishmaeli* 88.6		*H. pyrrhocephalus* 62.8		Tilapia 85.8	
AS level	80%	10%	80%	10%	80%	10%	80%	10%	80%	10%
HL	**21.5**	20.7	**28.8**	27.8	31.0	**31.9**	19.3	**19.6**	29.4	**29.7**
[1-2]	6.6	**7.9**	**6.3**	6.2	**9.8**	9.7	**5.8**	5.7	**9.8**	9.7
[1-3]	**10.9**	10.2	**8.8**	8.2	12.4	**12.7**	7.4	**8.1**	10.0	**10.1**
[2-3]	5.8		7.4		10.7		7.0		8.4	
[3-5]	**10.4**	9.4	**8.6**	7.8	**11.4**	10.0	7.8	**8.0**	**10.3**	10.2
[2-4]	11.1		10.7		14.9		9.5		13.2	
[5-4]	12.9		10.8		16.3		9.2		15.4	
[2-5]	13.9		12.2		16.5		11.2		14.2	
[5-7]	8.6	**9.3**	6.7	**7.3**	11.8	**12.8**	7.0	**7.5**	10.8	**11.7**
[4-6]	**9.7**	8.7	**8.0**	7.3	12.0	**12.5**	**6.4**	6.1	11.3	
[7-6]	7.1		5.1		16.1	**16.2**	12.0	**14.4**	25.5	**27.9**
[4-7]	12.3	**12.8**	9.8	**10.2**	14.5	**15.2**	7.9	**8.3**	14.3	**14.9**
[5_L-5_R]	8.6	**10.0**	6.9	**7.9**	9.7	**11.1**	6.1	**7.0**	10.8	**12.5**
[4_L-4_R]	11.3	**11.6**	9.1	**9.3**	13.4	**13.8**	8.8	**9.0**	13.1	**13.4**
[6_L-6_R]	12.8		11.1		14.2		8.7		14.8	
[7_L-7_R]	11.5		9.0		12.8		7.8		12.8	
[2_L-2_R]	7.9		8.5		9.4		5.8		10.1	

Table 3: *Percent changes of the bar lengths of hypoxia raised fish compared to normoxia raised siblings for each brood. Only bar lengths that differed significantly between NR and HR siblings (see also Table 2) are given.*
Differences are estimates based on ANCOVA models. Estimates are based only on parameters that contributed significantly to the variation in bar length. Where change in bars due to hypoxia was not significantly different between species, an average was given. Abbreviations: SL = Standard Length, HL = Head Length.

Species SL (mm)	*A. alluaudi* '92 79.1	*A. alluaudi* '99 62.7	*H. ishmaeli* 88.6	*H. pyrrhocephalus* 62.8	Tilapia 85.8
HL	-3.5	-3.5	3.1	1.6	1.0
[1-2]	19.6	-0.9	-0.9	-0.9	-0.9
[1-3]	-6.4	-6.4	1.9	3.5	0.9
[3-5]	-9.0	-9.0	-11.8	2.6	-0.7
[5-7]	8.4	8.4	8.4	8.4	8.4
[4-6]	-9.6	-9.6	4.6	-5.0	-0.6
[7-6]	-0.5	-0.5	0.8	20.7	9.5
[4-7]	4.4	4.4	4.4	4.4	4.4
$[5_L\text{-}5_R]$	15.2	15.2	15.2	15.2	15.2
$[4_L\text{-}4_R]$	2.6	2.6	2.6	2.6	2.6
$[3_L\text{-}3_R]$	0.2	0.2	0.4	30.0	3.7

volume measurements, while the SL was used as covariable in the analyses of the bar lengths.

Results

Head length and bar lengths

The head length and all the bar lengths were analysed with ANCOVA. The head length, which was dependent on both standard length and species, differed significantly between all NR and HR groups (Table 2). Compared to their NR siblings, the head length was 3.5% smaller in HR *A. alluaudi*. In the other three species, the head lenght of the NR animals was 1.0-3.1% larger than that of HR siblings. Seventeen bar lengths were measured of 10 groups. Ten bars were significantly different between NR and HR groups of all broods (Table 2). In bars [1-3], [3-5], [5-7], [4-6], [7-6] and $[3_L\text{-}3_R]$ ($_L$ and $_R$ stand for left- and right side of the head), both hypoxia as well as species effects were found. The bars that represent the ventral part of the head ([3-5], [5-7], $[5_L\text{-}5_R]$, and $[3_L\text{-}3_R]$) changed most in size. From the rostral to the caudal part of the framework the differences in bar lengths were as follows:

Table 4: *Volumes (mm^3) of different head compartments of normoxia raised (NR) and hypoxia raised (HR) cichlids. With ANCOVA the effect was investigated of species, environment, experiment and size on (sub) compartment volumes. Residual variation was normally distributed and hence estimates are reliable approximations of the data. Estimates were interpolations to a mean weight per brood and based only on parameters that significantly contributed to variation. Where change in volume due to hypoxia, was not significantly different between any of the species, estimates are given as being the same for all species. Where the percentage differences between NR and HR groups are 0%, none of the investigated parameters had a significant effect on the volume of (sub) compartments.*

Species	*A. alluaudi* '92		*A. alluaudi* '99		*H. ishmaeli*		*H. pyrrhocephalus*		tilapia	
Weight (g)	8.97		19.43		23.54		4.72		24.66	
AS level	80%	10%	80%	10%	80%	10%	80%	10%	80%	10%
Total volume	1173	1283	2413	2639	3173	3471	787	861	3189	3488
% change	**+9.4%**		**+9.4%**		**+9.4%**		**+9.4%**		**+9.4%**	
Oral	74	79	204	218	246	263	55	59	209	224
% change	**+7.0%**		**+7.0%**		**+7.0%**		**+7.0%**		**+7.0%**	
Ventral susensorial	158	174	426	470	364	402	113	124	345	381
% change	**+10.3%**		**+10.3%**		**+10.3%**		**+10.3%**		**+10.3%**	
Dorsal suspensorial	392		869		1008		293		1044	
% change	**0%**		**0%**		**0%**		**0%**		**0%**	
Ventral opercular	323	377	615	717	991	1156	203	237	932	1088

1. In the <u>oral compartment</u> [1-2-3], the *A. alluaudi* '92 showed significantly different reactions to hypoxia in bar [1-2] (Tables 2, 3). This bar was 19.6% longer in HR than in the NR *A. alluaudi* '92. In all the other HR groups, including the *A. alluaudi* '99, it was 0.9% smaller than in the NR groups. Bar [1-3] was 6.4% smaller in HR than in NR animals for both *A. alluaudi* groups. This bar was slightly larger in HR than in NR groups of the other three species.
2. In the <u>ventral suspensorial sub-compartment</u> [2-3-5] the size of bar [3_L-3_R] was dependent on both species and AS level. In HR *H. pyrrhocephalus* it had increased by 30% compared to NR animals, while hardly any change was found in the other groups. The size of bar [3-5] was dependent on the species and AS level. Bar [3-5] was 9.0% shorter in both HR *A. alluaudi* groups, and 11.8% in HR *H. ishmaeli*, while in *H. pyrrhocephalus* and tilapia little change was found (Tables 2, 3). Bar [5_L-5_R] was 15.2% longer in

all HR groups.

3. The enlarged [5_L-5_R] is also part of the dorsal suspensorial sub-compartment [2-4-5] (Figure 1). In this sub-compartment, bar [4_L-4_R] was slightly enlarged in all HR groups (2.6%).
4. Both the bars [4_L-4_R] and [5_L-5_R] are also a part of the ventral opercular sub-compartment [4-5-7]. Apart from these two, bar [5-7] bar in this sub-compartment was 8.4% longer in all HR groups. Bar [4-7] was slightly enlarged as well (4.4%).
5. Although, the total volume of the dorsal opercular sub-compartment [4-6-7] did not change, four out of 6 measured bars were significantly different between NR and HR animals. As already mentioned, bars [4_L-4_R] and [4-7] were slightly enlarged in all HR groups. In bar [4-6], reactions to hypoxia were different for all species. Bar [4-6] was 4.6% larger in the HR *H. ishmaeli* group but 0.6 to 9.6% smaller in the HR groups of the other species. Bar [7-6] was strongly enlarged in HR *H. pyrrhocephalus* (20.7%) and tilapia (9.5%), while only small differences were found between NR and HR *A. alluaudi* (-0.5% in both *A. alluaudi* ’92 and *A. alluaudi* ’99) and *H. ishmaeli* (0.8%).

Compartment volumes

The head volumes of all HR groups were 9.4% larger than of their NR siblings (Table 4). Relative to body weight, the head volumes (sum of volumes of all compartments) of the tilapias were much smaller than those of the haplochromines (Figure 2).

ANCOVA on the calculated volumes showed that the covariable ‘Weight’ and the factors ‘Species’, ‘Environment’, and ‘Experiment’ all explain a part of the variation found. The volumes of all (sub-) compartments were dependent on the weight of the animals ($P<<0.05$). Test groups that were raised at hypoxia had larger total (Figure 2), oral, ventral suspensorial and ventral opercular (sub-) compartments, but no differently sized dorsal suspensorial and dorsal opercular sub-compartments compared to their NR siblings (Table 4). Differences between NR and HR groups were largest in the ventral opercular (16.6%) and ventral suspensorial (10.3%) compartments.

The proportional change of all the compartments under hypoxia within each nest was the same (Table 4). Analysis of the factor "Experiment" (*A. alluaudi* ’92 *vs.* all four species from 1999, including *A. alluaudi* ’99) showed that the *A. alluaudi* ’92 had relatively smaller oral ($p=0.038$) and suspensorial (sub-) compartments ($p=0.000$), but no significantly different ventral and dorsal opercular sub-compartment. The differences in oral and suspensorial (sub-) compartments between the 1992

and 1999 experiments and between the species cannot be seen in Table 4, since the mean weight was calculated for the NR and HR fish of each brood, and compartment volumes were scaled accordingly. This was done because potential allometric differences between species would make extrapolation of the data unreliable.

Discussion

Replications

Although the experimental set-ups were identical, small differences between tanks were unavoidable. These include variations in social structure within groups, small differences in light conditions, water flow, etc. For such reasons, the use of replications is important to rule out the influence of uncontrollable factors. The only species for which two experiments were performed was *A. alluaudi*. However, these experiments were done seven years apart with a different set-up and feeding regime. As these experiments are rather time consuming no further replicas could be performed at the species level. Consequently, as individuals of each treatment group per species were raised in the same aquarium (pseudo

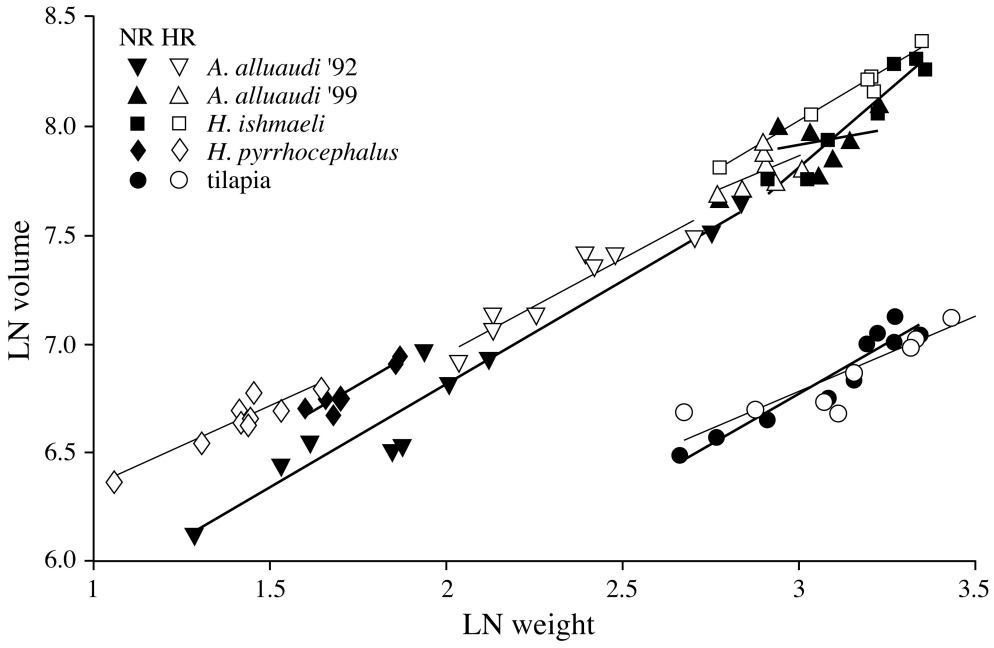

***Figure 2**: Total head volumes, as calculated from the external framework. For estimates on head volumes see Table 4.*

replication), no firm conclusions can be drawn on different responses *among species*. Nevertheless, if we use the data to detect hypoxia responses of *cichlids in general*, the 1999 experiments can be regarded as replications, since the same treatment was given to the four species of cichlids. Similar differences found between the NR and HR groups would then, most likely, be responses to the different AS-levels and not to differences in other factors. In addition, we formulated a prediction about the direction of the differences between NR and HR siblings; namely, an enlarged head volume in HR fishes, specifically in that area where the gills are located. It is unlikely that any random difference between the NR and HR groups of the 5 broods would cause morphological differences exactly according to the prediction.

Bar lengths

Calculation of the head volumes from the bar lengths showed larger volumes in the HR siblings of all 4 species. However, this was only partially achieved in the same manner. Of the ten bars that were different between NR and HR animals, four bars showed the same relative increase in all species: [5_L-5_R], [4_L-4_R], [5-7], and [4-7] (Tables 2, 3). This suggests that to some degree, the underlying mechanism mechanism was the same. By comparing the species according to morphological features, trophic specialisation and habitat we tried to find an explanation for the differences in hypoxia response. When comparing species with hypertrophied (*A. alluaudi* and *H. ishmaeli)* and non-hypertrophied (*H. pyrrhocephalus* and tilapia) pharyngeal jaw apparatuses, two notable differences in reaction to hypoxia were found. Both *A. alluaudi* and *H. ishmaeli* (mollusc crushers) showed a considerable decrease in the length of bar [3-5], while hardly any changes were found in *H. pyrrhocephalus* and tilapia. In contrast, bar [6-7] was hardly different in *A. alluaudi* and *H. ishmaeli*, while in *H. pyrrhocephalus* and tilapia it increased in size. Possibly the functional demands, posed on the enlarged pharyngeal jaw apparatus of the two mollusc crushers, constrains changes in form in the surrounding area (bar [6-7]).

There was no correlation between body depth or oxygen level in the natural habitat of the species (Table 1), and any of the bar lengths.

Volumes

The species used in this study are different with respect to phylogeny, ecology and morphology (see Materials and Methods). Yet, the differences in volumes between NR and HR animals are similar for all 4 species and concentrated mainly in the ventral suspensorial and ventral opercular sub-compartments, strongly suggesting an adaptive response of a general nature. Those regions harbour the major part of the gills, which have increased in

size in the HR groups (Chapter 4). This suggests a causal relationship between enlargement of the gills and volume increase of the compartments. Changes in other regions of the head were smaller or absent. A similar correlation between O_2 concentration and the size of the oral, suspensorial and opercular compartments was recently found in wild haplochromines (Bouton *et al.*, 2002). Respirometry experiments showed that HR fish at 10% AS consume the same amounts of oxygen as NR siblings at 80% AS (Chapters 2, 3 and 4). Since the partial O_2 pressure is eight-fold lower at 10% AS than at 80% AS, HR fish had to ventilate 8 times more water per unit of time to provide the gills with enough O_2 for extraction. In all HR animals, a dramatic increase of both amplitude and frequency of ventilation was observed (unpublished results). Possibly the increase in compartment volumes is related to an increase in the size of the respiratory pump, that comprises the oral, buccal and opercular cavities. As a consequence of the increased effort required for ventilation, the respiratory muscles (Ballintijn, 1969a, b) will likely be increased in size. Increase in the *m. sternohyoideus* and *m. levator hyomandibulae*, requires an increase in the ventral suspensorial and ventral opercular compartment, increase in the *m. adductor operculi, m. levator operculi,* and *m. dilator operculi* requires an increase in size of bar $[4_1\text{-}4_{R]}$.

Possibilities for transformation

If, through a decrease in oxygen availability it is needed to increase the gill size and stroke volume, spatial restrictions may occur. Theoretically, there are several possibilities to accommodate the larger gills: (1) use of the free space within the head; (2) reduction of surrounding structures; (3) increasing the head volume; (4) a combination of the previous possibilities (*e.g.* Witte *et al.,* 1990; Smits *et al.,* 1996 a, b). Apart from the use of free space, solutions 2-4 may have a negative impact on the performance of the animal through transformations of anatomically surrounding structures and decreased streamline (Barel, 1993). In our study, only external features of the head were investigated (possibility 3). We did not investigate whether free space, or a decrease in size of surrounding structures, was involved in allocating larger gills (possibilities 1 and 2). Evidence for combined responses were found in the study of Smits *et al.* (1996) and Chapman *et al.* (2000). In a study on *A. alluaudi* from different locations, two morphs were found (Smits *et al.*, 1996b). Animals that fed on insect larvae and other soft bodied prey had non-hypertrophied pharyngeal jaws, while animals that fed on snails had hypertrophied pharyngeal jaws. In addition to changes in bar lengths and opercular volume, internal changes were also observed, *viz.* the gills showed a change in form, providing extra space

for the pharyngeal jaw apparatus. In a split-brood experiment with NR and HR *Pseudocrenilabrus multicolor victoriae*, an increase in the gills and muscles of the respiratory apparatus was found, together with an increase in head length and a decrease of the *m. sternohyoideus* depth, the *m. retractor dorsalis* depth and the lower pharyngeal jaw depth (Chapman *et al.,* 2000). The studies of Smits *et al.* (1996) and Chapman *et al.* (2000) show that respiratory system and pharyngeal jaw apparatus are phenotypically quite plastic and support the hypothesis that in hypoxia raised cichlids, structures surrounding the enlarged respiratory apparatus may decrease in size or change in shape to provide space.

Barel (1983) recognised two core functions of the oral jaws in food uptake, namely biting and suction, the *m. adductor mandibulae* (*mAM*) being larger in the biters. It has been hypothesised that a thicker *mAM*, which increases biting force (Van Leeuwen and Spoor, 1987), causes a broader head width (Barel, 1983; De Visser and Barel, 1996). In a later study (De Visser and Barel, 2000) it was proven that, in the external framework, the ventral width (bar [5_L-5_R]) was the most important measure that distinguished between biters and suckers. This suggests a correlation between width of the *mAM* and the ventral head width. Indeed, Smits *et al.* (1996 b) found that an increase in head width (bars [5_L-5_R] and [4_L-4_R]), to allocate larger pharyngeal jaws, resulted in an increase of the size of the *mAM*. They suggested that this could be an example of an epiphenomenon (i.e. the *mAM* increased in size, not because of functional demands but because space became available). In our study the ventral width also increased most in size. Thus it is possible that, in HR animals there is space for a larger *mAM*. If a larger *mAM* were indeed realised in HR fish, it would have consequences for the food types that could be utilised.

De Visser and Barel (2000) found that in 73 cichlid species from different East African lakes, with a different phylogenetic background, morphology and ecology, the ventral width (bar [5_l-5_r]) was the most discriminating factor describing inter-specific differences. It seems that, the phenotypic responses to hypoxia that were found in the present study show a large degree of similarity with phylogenetic differences that are found amongst East African cichlids. One could wonder whether phenotypic plasticity in this region was in any way a steerin g factor in the morphological diversification of East African cichlids through evolution.

Ventral width, a hotspot of plasticity in cichlids?

The species flocks of Lake Victoria and of other lakes, e.g. Malawi, Tanganyika are characterised by rapid speciation, and in addition, extreme inter-specific variation in morphology and consequent functional differentiation. Liem

(1980) states that " The morphological novelty characterising the family Cichlidae involves the development of: a synarthrosis between the lower pharyngeal jaws, a strategic shift of insertion of the two fourth *levator externi* muscles, and synovial joints between upper pharyngeal jaws and basicranium. This specialized, highly integrated key innovation enables the cichlids not only to transport (deglutination) but also to prepare food, freeing the premaxillary and mandibular jaws to evolve numerous specializations dealing with the collection of dramatically diverse foods." According to Galis and Metz (1998), this would in turn support the hypothesis of Vermeij (1984) that, an increase in number of independent elements increases the potential for morphological and functional diversification. To our opinion this view should be approached in a more differentiated way. Barel *et al.* (1989) and Witte *et al.* (1990) showed that great levels of dependency exist and can exist between structures within the head of cichlids. Changes in structures within cichlid heads often seem to affect anatomically related structures. In the previous section we have mentioned the close relation between pharyngeal jaw size and gill shape (Smits *et al.*, 1996b) and between gill size and *m. sternohyoideus* depth and lower pharyngeal jaw depth (Chapman *et al.*, 2000). Thus it seems that the fact that many structures are anatomically linked, constrains the possibilities for change. However, when reviewing the studies on which external framework or comparable measurements were used, there is one recurrent phenomenon: variation in the ventral width (bar [5_L-5_R]). These studies concern a wide variation of topics, namely biting force of the oral jaws (De Visser and Barel, 2000), size of the pharyngeal jaw apparatus in relation to food types (Smits *et al.*, 1996 b), phylogenetic differences (Van Velzen *et al.*, 1998; De Visser and Barel 2000), environmentally related differences (Bouton *et al.*, 2002; present study) and phenotypic plasticity (present study). These show that besides all other variation in anatomy, the ventral width is a hot spot for both phenotypic as genotypic plasticity, within East African cichlids. The present study shows that such variation in the ventral width is conceivable by phenotypic plasticity alone. According to our current understanding of the role of phenotypic plasticity, environmentally-induced developmental plasticity may lead to genetic diversity in populations that live in stable different environments (Schlichting and Pigliucci, 1998). In that context, the large phenotypic plasticity of the head shape of East African cichlids, as demonstrated in this study, could be an important factor explaining the vast morphological diversity that is found.

CHAPTER 6:

CLOSELY RELATED FISH SPECIES USE DIFFERENT STRATEGIES TO IMPROVE OXYGEN TRANSPORT AND METABOLISM UNDER CHRONIC HYPOXIA

H.A. Rutjes, M.C. Nieveen, R.E. Weber, F. Witte, G.J.E.E.M. Van den Thillart

Institute of Biology Leiden, Leiden University
P.O. Box 9516, 2300 RA Leiden, The Netherlands

Abstract

We investigated the physiological responses of cichlids exposed to lifelong hypoxia. Broods of *Astatoreochromis alluaudi*, *Haplochromis ishmaeli* and a tilapia hybrid (*Oreochromis niloticus X O. mossambicus*) were split and exposed to normoxia (80-90% air saturation) and hypoxia (10% air saturation). Blood and white muscle tissue samples of adults were taken for analysis of physiological parameters for anaerobic metabolism (glycogen and total creatine levels and lactate dehydrogenase activity) and aerobic metabolism (blood haemoglobin concentration, haematocrit, intra-erythrocytic ATP and GTP levels and citrate synthase activity). Total creatine was not significantly different between normoxia-raised and hypoxia-raised siblings in all three species. Glycogen concentrations were significantly elevated in the white muscle of hypoxia-raised tilapia but not in that of *A. alluaudi* and *H. ishmaeli*. This indicates an increased anaerobic metabolic capacity in tilapia. The activity of the mitochondrial enzyme citrate synthase was not different between normoxia and hypoxia-raised tilapia but was significantly decreased in hypoxia-raised *A. alluaudi* and *H. ishmaeli*, indicating a decreased aerobic capacity. The haemoglobin and haematocrit levels were significantly increased in hypoxia-raised fish of all three species. In hypoxia-raised tilapia, intra-erythrocytic ATP and GTP levels were decreased suggesting an increase in blood oxygen affinity (a decrease in half-saturation oxygen tension, P_{50}) that safeguards sufficient O_2 loading of the haemoglobin under hypoxia. In contrast, no changes were found in *H. ishmaeli*, where different haemoglobin patterns were present in normoxia- and hypoxia-raised siblings. It is likely that these phenotypic differences in hypoxia-raised *H. ishmaeli* increase oxygen affinity of the whole blood. This study clearly shows that within closely related fish species, different strategies exist to cope with similar environmental changes.

Introduction

In general, hypoxia is considered to have detrimental effects on the survival of water breathers (Karlson *et al.*, 2002; Diaz and Rosenberg, 1995). The effects of hypoxia on fish are dependent on the duration of exposure. We distinguish short-term hypoxia from chronic hypoxia, short-term hypoxia lasting from hours up to several days, and chronic hypoxia lasts from a week to permanent hypoxia.

During short-term hypoxia, behavioural and regulatory changes result in a decrease in energy consumption, a maximised O_2 extraction, and an increase of anaerobic metabolism (Van den Thillart and Van Waarde, 1985). Fish that are exposed to short-term hypoxia, normally react with increased ventilation, a reduction in external activity and, if possible, aquatic surface respiration (Van den Thillart and Van Waarde, 1985; Chapman *et al.*, 2002). Upon immediate exposure to hypoxia, fish show stress responses that decrease tolerance. In contrast, when given time to habituate to the new environment, metabolic rates can be decreased (Randall, 1970) and stress responses avoided, resulting in higher hypoxia tolerance (Ultsch *et al.,* 1981). During more gradually induced short-term hypoxia, many fish species are able to reduce their energy consumption to below the standard metabolic rate, which is a determining factor for hypoxia tolerance. If the metabolic rate exceeds the maximum O_2 extraction, suppression of the standard metabolism follows, accompanied by activation of anaerobic metabolism to meet the total energy demand (Van den Thillart *et al*., 1994; Van Ginneken *et al*., 1995). The ability to cope with short-term hypoxia is partly dependent on the coping strategy of the animal. Studies on sole, *Solea solea*, (Van den Thillart *et al.,* 1994) and rainbow trout, *Oncorhynchus mykiss*, (Van Raaij *et al*., 1996) show that fish can either react with tranquil behaviour, or escape responses. The latter is associated with, high levels of catecholamines and cortisol and low survival.

Exposure to chronic hypoxia can, through altered gene expression patterns, lead to the production of new enzymes, proteins etc. (Gracey *et al.*, 2001; Zhou *et al.*, 2001). Hypoxia exposure experiments with immature as well as adult fish, showed that survival of one to two months of hypoxia exposure is mainly based on the reduction of (aerobic) energy expenditure (Lomholt and Johansen, 1979; Johnston and Bernard, 1982a, b; Van den Thillart *et al.*, 1980; Zhou *et al.*, 2000; Wu *et al.*, 2003). After six weeks of chronic hypoxia exposure, the O_2 consumption of tench *Tinca tinca*, and carp was about 50% lower than that of normoxia-acclimated individuals (Lomholt and Johansen, 1979; Johnston & Bernard, 1982a). In the hypoxia-acclimated tench there was a 43-76% decrease in the perimeter of muscle capillaries and a

60% reduction in mitochondrial volume density (Johnston and Bernhard, 1982a).

In the hypoxia tolerant goldfish, *Carassius auratus*, chronic hypoxia exposure led to depression in protein synthesis in the liver, and elevated activity of enzymes that promote conservative use of glycogen stores in the muscles (Van den Thillart and Smit, 1984). In addition, stores of phospho-creatine (PCr) in the white muscles were significantly increased (Van den Thillart *et al.*, 1980). Chronic hypoxia exposure of young carp (35 g) resulted in decreases in serum testosterone, estradiol and triiodothyronine. These hormonal changes were associated with retarded gonadal development and reductions in spawning success, sperm motility, fertilisation success, hatching rate, and larval survival (Wu *et al.*, 2003). Indubitably, the metabolism of the fish in these studies was strongly limited by the ambient oxygen concentration. One might wonder whether this type of response enables lifelong survival. Theoretically, the best adaptation to chronic hypoxia permits uninhibited aerobic energy production and thus, a high oxygen extraction capacity under hypoxic conditions.

Most studies on chronic hypoxia concern exposure for several weeks to two months, performed on (semi)adult fish. Many fish species grow about a thousand-fold larger between the post-larval and adult stage. Conceivable phenotypic responses are larger if fish are exposed from very early stages onwards. Only few studies have addressed the effects of lifelong hypoxia exposure. Rutjes *et al.* (Chapter 2, 3) exposed cichlids to lifelong hypoxia (10% air saturation, 0.8 mg O_2 l^{-1}) starting at an age of about four weeks and a size of 1-1.5 cm standard length. They found routine oxygen consumption of hypoxia-raised (HR) cichlids at 10% air saturation (AS) to be the same or higher than that of normoxia raised (NR) siblings at 80% AS (7.2 mg O_2 l^{-1}). Also growth and routine activity levels were comparable between normoxia (NR) and hypoxia raised (HR) siblings. In contrast, at 10% AS, NR fish showed depression of metabolic rate and died after 12 hours.

Although HR fish at 10% AS showed normal non-depressed O_2 consumption rates it is likely that at peak activity levels, their aerobic metabolism will remain depressed by the low O_2 gradient. An increased anaerobic capacity would enable HR fish to maintain peak metabolic activity longer. In this way, behavioural activity such as fighting or mating is less compromised. Thus, increased levels of glycogen and PCr, which are the main substrates that fuel anaerobic metabolism, are required at chronic hypoxia.

As the P_{50} of the blood is close to the AS level of the inspired water, the gas exchange process has to operate within a small band width between O_2 loading and O_2 unloading. In *Oreochromis niloticus*, the P_{50} of whole blood (without CO_2)

is about 20 mm Hg at 25 ˚C (Verheyen *et al.*, 1985), which corresponds to about 13% AS. Thus, we can assume that the blood will only be partially oxygenated in the gills at 10% AS. (The oxygen carrying capacity stays the same when oxygen saturation decreases). Two common responses to hypoxia exposure in vertebrates are increases in blood Hb concentration (Frey *et al.*, 1998) and blood oxygen affinity (decreased P_{50}). In most fish, the Hb-O_2 affinity can be regulated by altering the intraerythrocytic concentration of organic phosphates, of which ATP and GTP are the most important (Weber, 1989, 1996; Val, 2000). Increases of organic phosphate concentrations reduce the blood-O_2 affinity. An alternative strategy to modulate oxygen affinity is to produce red blood cells with higher proportions of isoHb components with low P_{50}. Such alterations, albeit small, have been demonstrated in temperate fish following thermo- and hypoxia-acclimation (Houston and Cyr, 1974; Houston, 1980; Houston and Tun, 1986; Tun and Houston, 1986; Marinsky *et al.*, 1989). Cases of seasonal variation in the abundance of Hbs in fish erythrocytes are also known in several Amazon fish (Almeida-Val *et al.*, 1999).

Apart from altering fractions of already present Hbs, the P_{50} of the whole blood can also be reduced by production of novel Hbs. The production of new Hbs in fish is almost solely related to transitions in the O_2 availability, for instance during the shift from the water-breathing to the air-breathing stadium in air-breathing fish, or after birth in viviparous fish (Weber, 1990). Phenotypic adaptation by producing novel Hbs is not well explored. Studies adressing this phenomenon concern maximally several weeks of accimation and effects were not very large (Hattingh, 1976; Weber and Jensen, 1988, Weber, 1990).

To investigate the physiological responses of fish to lifelong hypoxia, we tested whether HR cichlids have increased stores of glycogen and PCr in the white muscles and increased enzyme activity that reflect a higher capacity to (an)aerobically produce energy. In addition, we tested whether increases in the Hb concentration, decreases in intraerythrocytic ATP and GTP occur, or changes in the presence of Hbs have occurred in the blood of cichlids that were exposed to lifelong hypoxia. Broods of three species of cichlids were split and raised under normoxia and hypoxia. After 15-19 months, blood and white muscle tissue were tested for specific adaptations to hypoxia.

Materials and methods

Raising and conditioning the animals

In this study we used broods of three species: a commercial strain of tilapia (*Oreochromis mossambicus* hybridised with *Oreochromis niloticus*), *Astatoreochromis alluaudi* and *Haplochromis (Labrochromis) ishmaeli*. The latter two are both haplochromine cichlids. The natural habitat of these species is different. Tilapias (both parental species of the hybrid) live in habitats with varying O_2 concentrations (Welcomme, 1967). The habitat of *A. alluaudi* includes both well-oxygenated streams as hypoxic wetlands (Greenwood, 1974; Witte, 1981). Oxygen concentrations in the habitat of *H. ishmaeli* are at a stable high level and hypoxic events are rare (Witte, 1981). Based on the differences in habitat, it is possible that the responses to chronic hypoxia differ between species.

The *A. alluaudi* and *H. ishmaeli* were obtained from breeding stocks in our laboratory. The breeding stocks are offspring of animals that were caught in the Mwanza Gulf in 1984 and have been bred in our laboratory since then. The tilapias were F1 offspring of animals obtained from the University of Nijmegen. In total, four broods were raised. One brood of each *A. alluaudi* and *H. ishmaeli* was raised in 1999 and a further brood of each *H. ishmaeli* and tilapia in 2002. Due to limitations in time and capacity, no second brood could be raised of *A. alluaudi* and tilapia. Nests of the *A. alluaudi* and *H. ishmaeli* were selected when animals were about 1.5 cm SL (about four weeks after fertilisation). Each nest was split randomly and raised under normoxia (NR) and hypoxia (HR). The tilapia specimens were 0.5-1 cm standard length when the brood was split (three weeks after fertilisation). All fish were raised for 15-19 months in 100-litre aquaria in the same climate room. There were no indications that sporadic deaths were related to hypoxia. Before adulthood, survival approximated 100% in both NR and HR groups. The water in the aquaria of the NR groups was kept at 80-90% air saturation (AS). The AS-level of the water of the HR groups was lowered stepwise to 10% AS in four weeks. The fish were kept at a temperature of 25.5 °C and a light-dark cycle of 12-12 hrs. The *A. alluaudi* and *H. ishmaeli* were given a diverse diet of flake food, frozen midge larvae, frozen zooplankton, and a mixture of pulverized shrimps, mussels and flake food. The tilapias were fed with cyclops, Duplarin (Dupla Aquaristik GmbH) and from about 5 cm SL onwards with commercial 4.5 mm tilapia pellets (Trouw Nutritions BV). The fish were raised in tanks of the same dimensions (45x50x50 cm). The glass tanks contained an extra compartment from where water was pumped into the animal compartment to ensure fast mixing with the inflow of hypoxic filtered water. Hypoxia was reached by a continuous inflow of

degassed water (6-9% AS) at a rate of 1-2 L/min. A stainless steel plate that was placed 3 cm below the water level prevented oxygen uptake from the air by the fish and by the circulating water. The oxygen level of the water was regulated by Applikon biocontrollers (ADI 1030) equipped with polarographic oxygen sensors (Applikon ZZ71202AP10), switching solenoid valves in line with air diffusers. Thus, air bubbling through the water in the extra compartment was initiated automatically when the oxygen level was below the setpoint, whereby the oxygen level in the animal chambers remained constant. Water in the normoxia and hypoxia tanks was continuously refreshed from the same biological filter system. Before flowing into the hypoxia tanks the water was degassed by a vacuum system as described by Van den Thillart and Smit (1984).

Sampling

To minimise possible handling effects that may increase blood catecholamine and cortisol levels and swelling of erythrocytes (Nikinmaa *et al.*, 1987), fish were caught from individual aquaria not more than once in a day. Per aquarium 2 fish were caught each Tuesday and Thursday between 10:00 and 11:00 hours and anaesthetised with MS222 (300 ppm). Animals were completely sedated within 1.5-2 minutes and blood was withdrawn within 3 minutes after sedation. Blood samples were taken from the caudal vein with 1-ml ice cold heparinised syringes (10,000 $IU \cdot ml^{-1}$) and kept on ice. Immediately after blood withdrawal, a sample of white muscle tissue was taken on both sides of the fish from the area between the 5^{th} and 10^{th} dorsal fin ray and above the lateral line. The tissue samples were instantly frozen with freeze clamps that were cooled in liquid nitrogen. The samples were stored in liquid nitrogen until further processing.

Measurements in the white muscle samples

The frozen muscle tissue samples were ground to a fine powder in a mortar that was cooled in liquid nitrogen. The powdered samples were stored in liquid nitrogen for further processing.

Portions of the sample were suspended in nine parts of a 0.1 M KH_2PO_4 buffer pH 7.4, vortexed and centrifuged for ten minutes at 21000 g. The supernatant was stored at -80°C and used for measurement of lactate dehydrogenase, pyruvate kinase and citrate synthase (CS) activities. The suspensions used for CS activity measurements were sonified for 10 seconds before vortexing. The LDH activity (EC 1.1.1.27) was determined spectrophotometrically by measuring the conversion of NADH into NAD^+ at 340 nm (Vassault, 1987). Citrate synthase (EC 4.1.3.7) was determined spectrophotometrically by measuring the conversion of $APAD^+$ into APADH at 340 nm (Stitt, 1984). To correct the enzyme activity for possible

differences in extraction efficiency, total protein content was measured, using a standard protein assay kit (BCA protein assay, #23225, Pierce).

Glycogen was measured from portions of the ground samples by complete hydrolysis into glucose with glucoamylase and spectrophotometrically determining the glucose concentration from the conversion of $NADP^+$ into NADPH at 340 nm (Keppler and Decker, 1988).

The concentration of creatine phosphate in the tissue samples was determined by HPLC, following the protocol of Harmsen *et al.* (1982). In addition the concentration of creatine was determined from the same samples according to Wahlefeld and Siedel (1985). To minimise the bias due to the sampling procedure in the present study, total creatine (creatine + creatine phosphate) was used as a measure.

Measurements in the blood samples

Separate aliquots of whole blood were used for the measurement of hematocrit (HCT), and concentrations of Hb, adenosine triphosphate (ATP) and guanosine triphosphate (GTP). For Hb measurements a standard reagent (Roche) for spectrophotometric Hb-determination was used. For nucleotide measurements, 50 µl of full blood was kept on ice for 10 minutes after admixture of 200 µl PCA solution (8% PCA, 10 mM EDTA, 4 mM NaF, 40% ethanol). The samples were sonified, remixed and resonified for 15 seconds and centrifuged for 10 minutes. The supernatant was separated and 200 µl was treated with 50 µl of a 3M K_3CO_2 solution and centrifuged again for 10 minutes. The supernatant was stored at -80°C, and later used for measurement of GTP and ATP by HPLC following the protocol of Harmsen *et al.* (1982).

The remaining blood (that was not used for measurement of Hb, HCT or ATP and GTP) was centrifuged five minutes at 10,000 g at 4ºC to separate cells and plasma. The erythrocyte pellet was resuspended in saline and centrifuged for 10 minutes after which the saline and red cells were separated. This was repeated twice after which the erythrocytes and plasma were stored at -80°C for further analysis.

Of the stored plasma samples, cortisol levels were measured using an enzyme immunoassay (Oxford Biomedical Research). The washed erythrocyte pellet was thawed and diluted twice with water. For qualitative determination of the isoHbs, isoelectric focussing of the Hb isomorphs was carried out using the PhastSystem^{tm} (Pharmacia biotech) system on polyacrylamide gels in the 5-8 pH range. Marker proteins were applied as a reference. The separated Hbs were fixed and stained with Coomassie blue.

Statistics

Data were statistically analysed wit the program SPSS version 11.0. All data were normally distributed.

Results

Muscle Tissue

There were no significant differences in total creatine stores in *A. alluaudi* and *H. ishmaeli* between species nor between NR and HR siblings (one-sided Independent Samples T-tests, p-values >0.05).

In the white muscle samples, glycogen stores of HR tilapia were significantly greater than of NR siblings (one-sided Independent Samples T-test, p= 0.013). In *A. alluaudi* and *H. ishmaeli*, glycogen stores did not differ significantly between NR and HR siblings (one-sided Independent Samples T-test, p-values >0.05). The activity of LDH was not significantly different between NR and HR siblings of any of the three species (one-sided Independent Samples T-tests, p-values >0.05). When expressed as international units per gram tissue, CS activity differed significantly between NR and HR animals in *A. alluaudi* and *H. ishmaeli* but not in tilapia (one-sided Independent Samples T-tests, p-values respectively 0.002, 0.006 and 0.134).

Blood

The cortisol level was below 100 Ng L^{-1} in all animals and no significant differences were found between NR and HR animals (Independent Samples t-tests p-values >0.05; Table 1).

The Hb and Hct values were significantly higher in HR than in NR siblings of all three species (Table 1). In the *A. alluaudi* and *H. ishmaeli*, the differences in Hb and Hct were larger than in tilapia. No significant differences in MCHC were found between NR and HR siblings in any of the three species. ATP or GTP levels were not measured in the red cells of *A. alluaudi* and *H. ishmaeli* from 1999. In the *H. ishmaeli* from 2002, no differences in ATP and GTP levels were found between NR and HR siblings. (Independent Samples t-test, p=0.847 and 0.181). In tilapia, no significant differences in ATP were found. The GTP concentration however, was significantly lower in HR than in NR fish (Independent Samples t-test, p=0.706 and 0.001).

Thin layer isoelectric focussing was performed on 6-7 individuals of each NR and HR group per species. Of the nests of *H. ishmaeli* from both 1999 and 2002, runs were made of 6 individuals of each NR and HR group. Within each NR or HR group no clear individual variation in band patterns was found in any of the three species. In *A. alluaudi* and in tilapia, no differences were seen in the band patterns between the NR and HR group. All fish showed the same 16 isoHbs (Figure 1). In *H. ishmaeli* the NR animals showed 9 different Hbs while HR animals showed 10 different Hbs. Of the total of 14 mobilities that were found, the mobilities 2, 7, 9, 11 and 13 were absent in NR fish, while mobilities 5, 8, 11 and 14 were lacking in HR fish. Of the fractions that were present in both NR and HR *H. ishmaeli*, mobility 6 was

much more abundant in NR than in HR fish.

Discussion

Behaviour and stress

Both NR and HR fish were active and showed normal social interactions, indicating that the animals in were unstressed in both NR as well as HR groups. Differences in behaviour between both groups did exist. Mortality due to fighting of dominant males was higher in the NR than in HR groups. In addition, the HR fish were less gluttonous than NR siblings. The cortisol level of unstressed fish is generally below 100 $ng \cdot l^{-1}$ (Vianen et al, 2001, 2002). It has been shown that exposure of tilapia to stepwise decreasing AS levels, increased

Table 1: *Body weights and physiological correlates (± SD) for anaerobic and aerobic metabolism in blood and tissue samples from normoxia-raised (NR) and hypoxia-raised (HR) siblings of three species,* A. alluaudi, H. ishmaeli *and tilapia. Units and abbreviations: Hct = haematocrit (%), Hb = haemoglobin concentration (mM), MCHC = mean cellular haemoglobin concentration (Hb/Hct), ATP/Hb and GTP/HB = mM per mM Hb, cortisol (Ng* L^{-1}*), Glycogen = in equivalent of µM glucose* g^{-1} *tissue, Total creatine = µM* g^{-1} *tissue, LDH = lactate dehydrogenase (IU* g^{-1} *tissue), CS = citrate synthase (IU* g^{-1} *tissue). a = significant difference between NR and HR siblings at the 0.05 level b = significantly different from* A. alluaudi *at the 0.05 level c = significantly different from* H. ishmaeli *at the 0.05 level d = significantly different from tilapia at the 0.05 level.*

	NR *A. alluaudi*	HR *A. alluaudi*	NR *H. ishmaeli*	HR *H. ishmaeli*	NR tilapia	HR tilapia
Weight	21.4±2.1	17.8±1.5	19.9±5.7	19.7±4.9	118.3±50	94.0±42
Blood	**Blood**	**Blood**	**Blood**	**Blood**	**Blood**	**Blood**
Hct	31.3±3.5 **a**	44.47±5.1	31.2±4.6 **a**	44.80±8.62	34.4±5.6 **a**	39.4±4.1
Hb	5.0±1.5 **a**	6.8±1.6	4.9±1.0 **a**	5.8±1.8	5.9±0.6 **a**	6.8±0.5
MCHC	0.16±0.04	0.15±0.04	0.15±0.03	0.13±0.03	0.18±0.04	0.17±0.02
No. of Hbs	17	17	8	14	8	8
ATP/Hb	X	X	0.76±0.22	0.77±0.1	0.53±0.3	0.58±0.2
GTP/Hb	X	X	0.32±0.08 **d**	0.28±0.05	1.12±0.57 **a,c**	0.51±0.2
ATP/GTP	X	X	0.44±0.15 **d**	0.36±0.07	2.19±0.44 **a,c**	0.94±0.36
Cortisol	46.8±25.2 **d**	44.4±18.2	45.0±33.9 **d**	33.3±30.1	23.1±22.1 **b,c**	16.4±16.0
Tissue	**Tissue**	**Tissue**	**Tissue**	**Tissue**	**Tissue**	**Tissue**
Glycogen	15.6±5.9	14.0±2.9	16.9±6.9	15.13±8.8	17.7±5.8 **a**	28.3±11.9
Tot. Creatine	30.5±1.1	29.5±4.8	33.5±5.5	33.0±5.5	33.4±6.1	28.3±6.2
LDH	201.7±61.5	204.6±45.2	220.2±70.8	258.2±55.7	304.8±75.3	240.8±97.7
CS	2.64±0.05 **a**	2.00±0.78	2.85±1.01 **a**	2.12±0.44	1.61±0.43	1.46±0.25

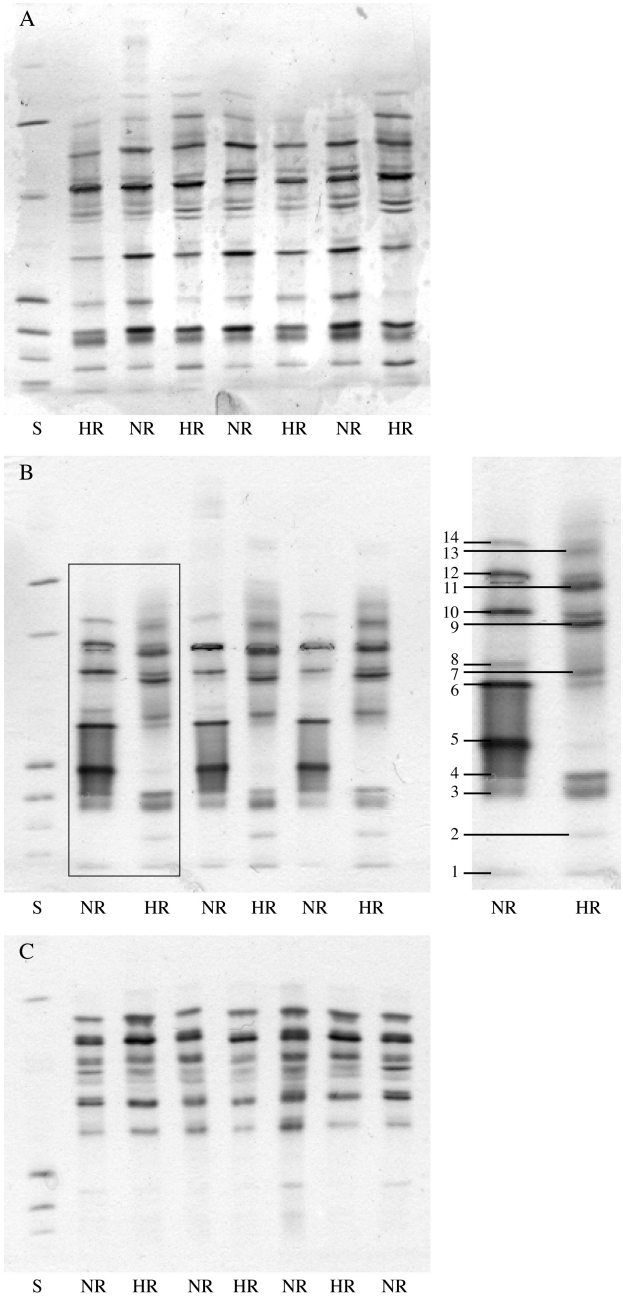

Figure 1: *Gel electrophoresis of blood samples of (A):* A. alluaudi*, (B):* H. ishmaeli *and (C): tilapia. S= standard protein mix, NR = normoxia-raised, HR = hypoxia-raised.* All A. alluaudi *and tilapia specimens showed the same band patterns, indicating that blood of NR and HR fish contained the same isohaemoglobins. Band patterns of NR and HR* H. ishmaeli *were different. In the NR H. ishmaeli bands 2, 7, 9, 11 and 13 were absent while in HR siblings bands 5, 8, 12 and 14 were absent.*

cortisol concentrations at and below 20% AS (Vianen *et al.*, 2002). Cortisol has a suppressing effect on the immune system and is associated with retarded growth (Wendelaar Bonga, 1997). Since growth was similar between NR and HR groups, elevated cortisol levels were not expected in the HR groups. Indeed, no significant differences in cortisol were found in our experiment between NR and HR animals (Table 1). We therefore conclude that10% AS was not a stressful situation for HR cichlids.

Metabolism

In general, glycogen stores in liver and white muscle appear to be higher in fish species that are relatively anoxia/hypoxia-tolerant such as tench, *Tinca tinca*, goldfish, *Carassius auratus*, and Crucian carp, *Carassius carassius*, than in relatively anoxia/hypoxia-intolerant species such as rainbow trout, *Oncorhynchus mykiss*, and cod, *Gadus morhua*, that have very low levels of muscle glycogen (Van den Thillart and Van Raaij, 1995). In *A. alluaudi*, *H. ishmaeli* and tilapia, the white muscle stores of glycogen were about 14-28 μM glucose per gram tissue, which is in the same range as covered by the above-mentioned species.

Glycogen concentrations in the white muscles were significantly elevated in HR tilapia but not in HR *A. alluaudi* and *H. ishmaeli* (Table 1). It supports the idea that HR tilapia compensate limitations in the O_2 consumption by increasing anaerobic capacity of the muscles during peak activity. However, in case of elevated anaerobic metabolism, an elevated activity of the enzyme LDH may also be expected, which was not the case. LDH converts pyruvate into lactate which is an important step in the anaerobic glycolysis. Greany *et al*. (1980) argued that white muscles already have a high glycolytic capacity and would therefore be pre-adapted to hypoxia, obviating the need for a capacity increase during chronic hypoxia.

The CS activity in the white muscles was similar between NR and HR tilapia, indicating an equally high aerobic capacity. However, in HR *A. alluaudi* and *H. ishmaeli*, CS activity was significantly reduced by 25%. Instead of compensations for limited aerobic metabolism at lifelong hypoxia, the unchanged anaerobic capacity and decreased CS activity indicate a reduction in overall maximum metabolic rate. At peak activity levels, for instance during territorial fights or when fleeing for predators, O_2 consumption of HR *A. alluaudi* and *H. ishmaeli* will be impaired and anaerobic energy stores depleted at lower metabolic rates than in NR siblings. We can conclude that acclimation of *A. alluaudi* and *H. ishmaeli* to lifelong hypoxia does not prevent impairment of maximum behavioural activity. This may have serious consequences for the ecology and survival of these species in the wild. However the decreased CS activity, did not affect routine activity

levels since O_2 consumption levels of HR cichlids was the same or higher than those of NR siblings (Chapter 3). CS is part of the TCA cycle and located within the mitochondria. It is known that in man, exercise at high altitude causes a reduction in the mitochondria content of the muscles (Hoppeler *et al*., 2003). Similarly, Johnston and Bernhard (1982a) showed that tench acclimated to 8.5% AS for six weeks have a decreased mitochondrial density in the white muscles. Thus, it is plausible that the decreased CS activity was a result of a reduction in the mean density of mitochondria in the white muscles.

Although, the creatine and glycogen stores in the white muscles did not differ between NR and HR siblings of *A. alluaudi* and *H. ishmaeli*, HR individuals of both species exhibited a marked increase in anoxia tolerance (Chapter 3). Possibly increased deposition of glycogen in the liver, an important storage organ of glycogen, could explain the increased anoxia tolerance in *A. alluaudi* and *H. ishmaeli*. Depletion of glycogen and PCr stores during hypoxia exposure in liver was demonstrated in goldfish Van den Thillart *et al.* (1980). Instead of attributing it to increased energy stores, the difference in anoxia tolerance between NR and HR fish could also be based on an increased ability regulate metabolic rate. For *A. alluaudi* this seems a likely possibility since particularly the HR individuals of this species, which were most hypoxia tolerant, reacted to anoxia exposure by lying on the bottom of the tank. Keeping this posture instead of an upright posture probably allows a greater depression of metabolic rate. Additionally, from visual observations it was very clear that those animals which showed the least external activity, were able to tolerate anoxia the longest (Chapter 3).

Responses of the haemoglobin system

Common responses in vertebrates to hypoxia are increases in Hb concentration occur as well as shifts in the P_{50} of the blood. However, hypoxia exposure did not change Hb and Hct levels in the hypoxia-tolerant carp and tench (Weber and Jensen, 1988). In HR fish from all three species used in this study, both total Hb and Hct levels increased but the mean cellular Hb concentration remained unchanged (Table 1), showing that the increase in Hb concentration was due to a higher amount of erythrocytes.

In vertebrates, organic phosphates are the most important allosteric effectors of Hb and provide a rapid means of adapting Hb function to tissue O_2 demand (Weber and Jensen, 1988; Weber, 1996; Val, 2000). In most fish, hypoxia exposure causes a decrease in organic phosphate concentration, mostly ATP and GTP, and is associated with an increase in Hb-O_2 affinity (Val, 1995). In most fish that have significant amounts of GTP in the red blood cells, the effect of GTP on Hb-O_2 affinity is stronger than that of ATP (Weber 1996). In tilapia Hb, the

effects of ATP and GTP are very similar (Babiker, 1985). As the P_{50} of the whole blood of tilapia is about 13% AS (20 mm Hg; Verheyen et al, 1995), a decrease in organic phosphate concentration would seem advantageous under hypoxia. In the red blood cells of NR tilapia used in this study, about twice as much GTP as ATP was found (Table 1). HR fish showed a 55% reduction in GTP levels resulting in equimolar ATP and GTP concentrations.

In contrast to tilapia, red blood cells of both NR as well as HR *H. ishmaeli* contained approximately equimolar concentrations of ATP and GTP. Apparently *H. ishmaeli* does not use organic phosphates to increase its Hb-O_2 affinity, which contrasts with the hypoxic response generally seen in fish. However, routine O_2 consumption rates in this species were similar between NR and HR siblings (Chapter 3). Apparently, a decrease in the P_{50} is regulated differently in this species.

Isoelectric focussing of the Hbs of *A. alluaudi* and tilapia showed no differences in Hb multiplicity between NR and HR siblings. However, in both batches of *H. ishmaeli* that were used, clear differences were observed between NR and HR siblings (Figure 1). HR fish lacked four isoHbs that were present in NR siblings. However, five new Hbs were seen that were lacking in NR *H. ishmaeli*. This pattern was consistent for all NR and HR fish examined. This clear cut difference has to our knowledge not earlier been observed in fish. The presence of such a distinct Hb pattern in hypoxia-raised *H. ishmaeli*, indicates that a regulatory mechanism is involved. These changes may well have resulted in a decreased P_{50} of the whole blood, thereby increasing the Hb-O_2 loading in the gills at 10% AS.

Production of different Hbs in different environments was up till now only known from animals that undergo drastic changes in ontogenetic development, for instance birth in humans, viviparity in fish, or water to air transitions in amphibians (Weber, 1990; 1994) Thus, the ability of *H. ishmaeli* to produce different Hbs under different environmental conditions is quite unique in the respect that it seems to be a real phenotypically plastic trait that is not bound to ontogenetic shifts.

The hypoxia response of tilapia *viz.* decreasing the concentration of GTP that increases Hb-O_2 affinity fits very well for life in environments where O_2 concentrations fluctuate (hours-weeks). The concentration of organic phosphates in the red blood cells can be altered in a matter of minutes to a few hours (Val, 2000). This fits well with the wide dispersion and variety of O_2 concentrations that is found in the habitats *O. niloticus* and *O. mossambicus* (Welcomme, 1967, Trewavas, 1983). The hypoxia responses of *H. ishmaeli viz.* a switch between phenotypes with different Hb composition of the blood, can only be used in environments with

stable O_2 concentrations (months-years) since a large fraction of the erythrocyte pool needs to be replaced for the response to be effective. Considering the life span of red blood cells, this can take several months. The habitat in which *H. ishmaeli* was found before the introduction of lake-wide chronic hypoxia, was thought to be a stable normoxic environment (Van Oijen *et al*., 1981). Apparently, in the evolutionary past of *H. ishmaeli*, selective forces (occurrence of hypoxia) must have occurred, favouring animals with the capacity to develop distinct alternative phenotypes at different stable AS levels. Possibly this capacity evolved during the desiccation of Lake Victoria 14,000 years ago (^{14}C 12,400 years; Johnson et al., 1996). In the present, *H. ishmaeli* could benefit from the increased occurrence of hypoxia in Lake Victoria and settle in hypoxic areas that have become less suitable habitats for other species. Although each of the three species of cichlids here studied cope with hypoxia very well, this study clearly shows that very closely related fish species deploy widely different strategies to cope with the same environmental challenges.

CHAPTER 7:

SYNTHESIS

H. A. Rutjes, F. Witte, G.J.E.E.M. Van den Thillart

Institute of Biology Leiden, Leiden University
P.O. Box 9516, 2300 RA Leiden, The Netherlands

Introduction

When exposed to chronically hypoxic conditions, fish have few options to balance metabolic rate and O_2 extraction ability: the metabolic rate can be decreased to match the O_2 extraction ability, the maximum O_2 extraction can be increased to match the metabolic rate or a combination of the two. The literature that is available on chronic hypoxia exposure of fish, shows that fish commonly respond by decreasing the metabolic rate. This is done by a reduced growth (Chabot and Dutil, 1999; Thetmeyer *et al.*, 1999), a lower reproduction capacity (Wu *et al.*, 2003), a large reduction of the O_2 consumption rate (Lomholt and Johansen, 1979; Johnston and Bernard, 1982a, b) and a reduction of the aerobic capacity of the muscles (Lomholt and Johansen, 1979; Johnston and Bernard, 1982a, b). In the present study, fish that were exposed to hypoxia from their youth up, remained active, grew well and even reproduced, indicating that their routine metabolic rate was unaffected. Therefore, we expected that hypoxia-raised fish were able to maintain high O_2 consumption rates under 10% AS and that, concomitantly, the aerobic capacity was not reduced. To realise this, an enlarged gill surface and a decreased P_{50} of the haemoglobin (Hb) would be necessary. Additionally, we expected that increased gill size would affect structures surrounding the gills.

Maintenance of metabolic rate

In Chapters 2, 3 and 4, respirometry was performed on split broods of tilapia (crossbreed between *Oreochromis niloticus* and *O. mossambicus*), *Astatoreochromis alluaudi* *Haplochromis* (*labrochromis*) *ishmaeli* and *Haplochromis (Yssichromis) pyrrhocephalus* that were normoxia-raised (NR) or hypoxia-raised (HR). Although slight differences in behaviour were observed, no differences in growth rate could be distinguished between NR and HR fish. In addition, no hypoxia-related mortality was found. NR fish showed depressed O_2 consumption levels below 10% air saturation (AS) and were not able to survive 10% AS for more than 12 hours. In addition, NR fish showed an increase in O_2 consumption during recovery (under 80% AS) from hypoxia exposure, indicating an oxygen debt due to an increased anaerobic metabolism during the foregoing hypoxia exposure. In contrast, O_2 consumption levels of HR fish under 10% AS was the same or higher than that that of NR siblings under 80% AS. This indicates that phenotypic responses occurred that enabled them to follow a strategy of optimising the O_2 extraction instead of decreasing the metabolic rate.

In our experiments, fish could thrive under 10% AS but not in studies performed by other authors (*e.g.* Lomholt and Johansen, 1979; Johnston and Bernard, 1982a, b; Zhou *et al.*, 2000; Wu, 2003). There are two likely explanations for this difference.

One explanation is that the duration of hypoxia exposure was different between our experiments and that of other authors. The duration of the experiments in this study (up to two years) was much longer than in comparable studies (up to

two months). Possibly, if the duration of hypoxia was extended in those studies, similar increases in the O_2 extraction capacity as in this thesis could be found. Literature reports some increase in the O_2 consumption (Lomholt and Johansen, 1979; Johnston and Bernard, 1982) and normalisation of enzyme activity (Greany *et al.*, 1980) after several weeks of hypoxia.

A second explanation is the difference in growth of fish during our experiments and those of other authors. The fish used in other studies were (almost) adult and hardly grew. In contrast, the fish in our study were exposed to hypoxia from a very young post-larval stage up to adulthood (Chapter 2 and 3) and grew several hundred times larger. Thus, only a small change in growth rate will result in large phenotypic differences in the adult stage. Therefore, it is likely that adaptive responses to hypoxia are much larger during growth.

Limitations in metabolism

Several observations indicate that HR cichlids from this study were limited by lifelong exposure to 10% AS. First, during feeding, the NR fish were very eager to eat and food was usually eaten directly whereas HR fish did not eat the food directly and feeding took longer. This indicates that HR fish spent less energy on feeding and competing for food with group mates than NR siblings did (Chapter 2). Second, pilot studies were performed on a split brood of *H. piceatus* in the aquarium where the animals were raised. Of the males in each group, frequencies of aggressive and mating behaviour were scored. The results showed that all behavioural parameters that were measured occurred less often in males of the HR group but the standard deviation was large. Only the parameters 'butting' and 'quivering' towards females were scored significantly less often in HR than in NR *H. piceatus*. 'Butting' was defined as accelerating towards and bumping into the glass wall behind which a stimulus male was present. 'Quivering' was defined as a shiver of the body with anal fin erect and dorsal fin folded (Seehausen, 1996). This suggests that the behavioural activity was depressed in HR fish, thus, reducing energy expenditure (Rutjes, Senadheera, Van den Thillart and Witte, unpublished). Third, the activity of the mitochondrial enzyme citrate synthase (CS) in the white muscles was decreased by 25% in HR *A. alluaudi* and *H. ishmaeli*. In tilapia, the difference between NR and HR siblings was smaller and not significant (Chapter 6). The CS activity is a limiting step in the citric acid cycle. Thus, it can be said that the maximum aerobic metabolic rate of the white muscles was decreased in HR *A. alluaudi* and *H. ishmaeli*. In contrast, the stores of glycogen in HR tilapia were increased, suggesting that they have an increased anaerobic capacity. In *A. alluaudi* and *H. ishmaeli* the differences between NR and HR siblings were small and not significant. Differences in muscle glycogen stores were not found by others in tench or carp (Johnston and Bernard, 1982a; Zhou *et al.*, 2000). HR *A. alluaudi* and *H. ishmaeli* were able to tolerate anoxia longer than NR siblings

were (Chapter 3). Possibly, this tolerance was based on behavioural differences or increased glycogen stores in the liver (Chapter 6).

The observations discussed above, indicate reductions in energy expenditure. This should result in decreased O_2 consumption rates but respirometry experiments clearly showed that O_2 consumption was maintained under normal levels. However, these results do not contradict each other. The total O_2 consumption of a fish is the result of the O_2 cost of all physiological processes together. While the amount of energy used for some processes was decreased in HR fish, others for instance respiration, became more energy demanding. Under the assumption that NR fish under 80% AS and HR fish under 10% AS had the same O_2 extraction efficiency, the HR fish had to ventilate eight times more water through the gills per unit of time, thereby drastically increasing the cost of respiration. The energy that was allocated to the different physiological processes that were needed to sustain normal metabolic rates and development, must have been different between NR and HR siblings.

Increased Oxygen extraction by transformations in the gills

Under hypoxic conditions, normoxia-acclimated fish are generally unable to maintain normal O_2 consumption levels, since it dramatically decreases their O_2 extraction efficiency (Randall, 1970; Fernandes and Rantin, 1994). According to Fick's law, the O_2 flux is directly proportional to the difference in partial O_2 pressure between water and blood. Furthermore, the O_2 flux is dependent on the respiratory surface and inversely proportional to the diffusion distance. While during short-term hypoxia exposure fish have a limited capacity to alter the respiratory surface and diffusion distance, we expected that lifelong hypoxia exposure of young post-larval fish would result in an increased gill surface and smaller diffusion distance. Measurements on the third gill arch showed that the respiratory surface had increased by 80%, indicating that gills of fish are highly plastic organs (Chapter 4). This increase was realised by 26.9% longer primary filaments, and 9.2% higher and 37.7% longer secondary lamellae.

The differences in gill size and shape that were observed between NR and HR *H. pyrrhocephalus* showed large similarities compared to differences found between fish living in normoxic and hypoxic habitats (Galis and Barel, 1980; Hoogerhoud *et al.*, 1983; Chapman *et al.*, 2002). As the present study shows that gill size and shape are to a large extent phenotypically plastic we should be careful when interpreting the cause of differences in gill morphology between species of different habitats and life style.

For HR fish to be able of the same O_2 consumption under 10% AS as NR siblings under 80% AS, an eight-fold increase in the water flow through the gills was necessary to supply the gills with the same amount of O_2. Since gas exchange is slower at a lower difference in partial O_2 pressure, the retention time of water

in the gills should increase to achieve the same extraction efficiency. This conflicts with the increased water flow through the gills. An increase of the total cross-sectional area of the respiratory channels would reduce water flow speed. In addition, longer respiratory channels would further increase the retention time of water in the gills. Measurements on the third gill arch of *H. pyrrhocephalus* showed that the cross-sectional area of individual respiratory channels was increased in HR fish as well as their number and secondary lamellae were longer. However, a ~26.9% increase of the number of respiratory channels, a 9.2% increase of the height of the channels and a 37.7% increase of the length of the channels, increases the retention time of water in the gills by less than a factor 2. Thus, at an eight-fold increased water flow, the retention time of water in the gills was more than 4 times shorter, resulting in lower efficiency. Additionally, an 80% increase in gill surface does not compensate for the eight-fold decrease in O_2 flux caused by the lower partial gas pressure under 10% AS. Since total O_2 consumption was not affected in HR fish, we expect that other responses, such as a decrease of the thickness of the water-blood barrier, occurred that enabled a higher O_2 flux over the gills and increased O_2 extraction efficiency.

Anatomical effects of gill increase

As there is little space available in the head of fish, it is likely that the large increase in gill size that was observed in HR *H. pyrrhocephalus*, resulted in transformations of surrounding structures. This in turn could affect the functionality of these structures, for instance the ability for biting or sucking. Evidence that structures surrounding the filaments are flexible is given by Smits *et al.* (1996b). They found two different morphs of *A. alluaudi* at different locations. Animals from one location that fed on insect larvae and other soft bodied prey had non-hypertrophied pharyngeal jaws, while animals from the other location that fed on snails and had hypertrophied pharyngeal jaws. In addition to changes in external structures and opercular volume, internal changes were also observed, *viz.* the gills showed a change in form, providing extra space for the pharyngeal jaw apparatus. In the study of Smits *et al.* (1996b) it was not determined whether the observed responses were a phenotypically plastic trait. In a split-brood experiment with NR and HR *Pseudocrenilabrus multicolor victoriae* carried out by Chapman *et al.* (2000), a phenotypic increase of the gills by 22% as well as an increase of several muscles of the respiratory apparatus was found. This was accompanied by an increase in head length and a decrease of the *m. sternohyoideus* depth, the *m. retractor dorsalis* depth and the lower pharyngeal jaw depth.

In Chapter 5, split brood experiments on tilapia, *A. alluaudi*, *H. ishmaeli* and *H. pyrrhocephalus* were performed. A modified three-dimensional model was used to measure the outer head shape and the volume of the oral, suspensorial and opercular compartments (De Visser and Barel, 2000). In spite of the fact that

the species are different in phylogeny, morphology and have different life styles, volume enlargements were realised in a similar way and magnitude. Volume increases were most prominent in the ventral suspensorial and ventral opercular sub-compartments. In that area also the enlarged gills were situated. It is likely that other structures within the head of HR cichlids had also changed shape and size as a result of spatial conflicts with the enlarged gills. When reviewing studies on which the external framework or comparable measurements were used, there is one recurrent phenomenon: variation in the ventral width (bar [5_L-5_R]; see Chapter 4). These studies concern a wide variation of topics, namely biting force of the oral jaws (De Visser and Barel, 2000), size of the pharyngeal jaw apparatus in relation to food types (Smits *et al.*, 1996 b), phylogenetic differences (Van Velzen *et al.*, 1998; De Visser and Barel 2000) and environmentally related differences (Bouton *et al.*, 2002; present study). These studies show that the ventral width is a hot spot for both phenotypic and genotypic plasticity. The present study shows that such variation in the ventral width is possible by phenotypic plasticity alone. According to our current understanding of the role of phenotypic plasticity, environmentally-induced developmental plasticity may lead to genetic diversity in populations that live in stable different environments (Schlichting and Pigliucci, 1998). In that context, the large phenotypic plasticity of the head shape of East African cichlids, as demonstrated in this study, could be an important factor explaining the vast morphological diversity that is found.

Increased oxygen extraction by transformations in the blood

In addition to the gas exchange process in the gills, O_2 loading of the blood and transport to the tissues is an essential process that is influenced by hypoxia exposure. As the P_{50} of whole blood of tilapia is about 13% AS (20 mm Hg; Verheyen et al., 1985), it is obvious that in normoxia acclimated cichlids, O_2 uptake is impaired during exposure to 10% AS. In the HR fish from all three species used in this study, the total Hb concentration was increased, which increased the capacity for O_2 transport. Additionally, in erythrocytes of HR tilapia, a 55% reduction of the GTP level was observed. In fish, the nucleotides ATP and GTP are the most important allosteric effectors of Hb. A reduction of the intraerythrocytic nucleotide concentration results in an increase of the Hb-O_2 affinity *viz.* a reduction of the P_{50} (Val, 2000). In contrast, in *H. ishmaeli* no difference in ATP and GTP concentrations were found between NR and HR siblings, suggesting that the Hbs of *H. ishmaeli* are not sensitive to these molecules. However, routine O_2 consumption rates in this species were similar between NR and HR siblings (Chapter 3). It is likely that a decrease in the P_{50} was regulated differently in this species. While no differences in Hb multiplicity were found between NR and HR siblings of tilapia and *A. alluaudi*, very clear differences were observed between NR and HR siblings of *H.*

ishmaeli. HR *H. ishmaeli* lacked four isoHbs that were present in NR siblings while five new Hbs were found that were lacking in NR *H. ishmaeli*. This pattern was consistent for both split broods of *H. ishmaeli* examined. It is likely that the P_{50} of the Hbs that appeared in HR *H. ishmaeli* is lower than the ones that disappeared. This would result in a decreased P_{50} of the whole blood, thereby increasing the Hb-O_2 loading in the gills under 10% AS. This clear-cut difference in the presence of isoHbs has to our knowledge not earlier been observed in fish. Such a phenotypic response is even more unique considering the fact that production of different Hbs in different environments was up till now only known from animals that undergo drastic changes in ontogenetic development, for instance birth in humans, viviparity in fish, or water to air transitions in amphibians (Weber, 1994; 1990).

Conclusions

Four different cichlid species that were exposed to gradually decreasing AS levels within the first two months after fertilisation survived lifelong exposure to 10% AS (0.8 mg O_2 L^{-1}). In these HR fish, routine aerobic metabolic rate was the same as in NR siblings. Hypoxia-raised cichlids, grew well and could even reproduce under these conditions. Although a greater anaerobic capacity was found, survival was based on an increase of the O_2 extraction capacity under hypoxia, rather than a decrease of metabolic rates. The increase in O_2 extraction capacity was realised by an 80% increase in the gill surface area. Concomitantly, an increase of the volume of the head was found particularly in the region where the gills were located. The phenotypic transformations in the size and shape of the gills and head that were demonstrated in this thesis were of the same magnitude as inter-specific differences between species from different habitats and life styles. Transport of O_2 to the tissues was improved in two ways. First, an increase of the Hb concentration was found in all species investigated. Second, the Hb-O_2 affinity was increased. Closely related cichlids showed different strategies to achieve this. While HR tilapia increased the Hb-O_2 affinity by a reduction in intraerythrocytic GTP levels *H. ishmaeli* probably did this by producing different types of Hb that have higher O_2-affinity.

The results from this thesis show that cichlids have remarkable phenotypic plasticity that enables them to survive and thrive under low oxygen conditions. Hence, the general belief that chronic hypoxia have detrimental effects on fish should be more differentiated. This helps us to a better understanding of the effects of (human-induced) O_2 fluctuations on aquatic organisms. While acute hypoxia results in direct limitations for O_2 uptake in cichlids, chronic hypoxia exposure does not harm young fish of the studied species and allows new generations to adapt to life under such conditions.

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NEDERLANDSE SAMENVATTING

In het stuk hieronder zijn alleen de belangrijkste referenties gegeven. De rest van de referenties kan worden gevonden in de hoofdstukken 1 en 7.

Het Victoriameer

In oppervlakte is het Victoriameer het grootste tropische meer ter wereld. Met een maximum diepte van 70 meter is het een ondiep meer in vergelijking met de andere grote meren in Afrika (Tanganyika en Malawi). Tot in de jaren 80 van de twintigste eeuw werd de visfauna van het meer gedomineerd door een groep van meer dan 500 soorten nauwverwante haplochromine cichliden (familie Cichlidae). Deze vormen samen een soortenzwerm die, op evolutionaire schaal, pas zeer recentelijk uit een of enkele vooroudersoorten zijn ontstaan. De grote diversiteit en nauwe verwantschap van deze vissen maakt hen tot een populair studieobject. Voor bijna elke beschikbare voedselbron in het meer bestaan er wel soorten haplochromine cichliden die gespecialiseerd zijn in het eten ervan. Het Victoriameer zelf is minder dan een miljoen jaar oud en de laatste gegevens wijzen erop dat aan het eind van het Pleistoceen het meer droog is komen te staan. Ongeveer 14.000 jaar geleden (12.400 ^{14}C jaren) vulde het meer zich weer met water. Men is het er nog niet over eens of het Victoriameer tijdens deze uitdrogingsperiode geheel droog stond, of dat er nog kleine meertjes zijn blijven bestaan in het Victoriameerbassin. Dit vormt een belangrijk discussiepunt rondom de evolutie van de Victoriacichliden. Er zijn sterke aanwijzingen dat de genetische diversiteit van deze vissen al 250.000 tot 750.000 jaar geleden is ontstaan. Toen vooroudersoorten het meer bevolkte nadat het weer met water gevuld was, stelde hun genetische diversiteit hen in staat om zeer snel in de verschillende soorten te divergeren die de latere soortenzwerm zou vormen. Een alternatieve theorie stelt dat de meeste soorten al vóór de uitdroging van het Victoriameer zijn ontstaan en dus als gescheiden soorten de uitdrogingsperiode moeten hebben overleefd. Feit blijft dat de vissen die het Victoriameer meer bevolkten nadat het 14.000 jaar geleden weer gevuld was met water, tijdens de uitdrogingsperiode ergens moeten hebben overleefd. Het ene kamp beweert dat deze hebben overleefd in kleine moerasachtige meertjes die in het Victoriameerbassin zouden zijn achtergebleven. Er zijn gegevens die erop wijzen dat soorten die daar oorspronkelijk niet voorkomen zich er toch in stand kunnen houden. Het andere kamp denkt dat juist de stroompjes en rivieren die naar het Victoriameer bassin toe liepen, een toevluchtsoord vormden voor de toekomstige meerbewoners. Ondersteuning hiervoor is gevonden toen zeer recentelijk een grote groep cichlidensoorten is gevonden in een rivier in de Kalahariwoestijn (Joyce *et al.*, 2005). De verscheidenheid aan specialisaties aan verschillende voedselbronnen binnen deze groep doet niet onder voor de verscheidenheid die in het Vicoriameer werd aangetroffen. De rivier waar deze nieuw gevonden soorten in leven, voedde een meer dat 2000 jaar geleden uitdroogde tot een zoutwoestijn. Men concludeerde dat de soorten uit de

rivier dus een overblijfsel van de soorten die ooit in het meer leefden.

Recente veranderingen

In de afgelopen eeuw hebben menselijke invloeden voor dramatische veranderingen in de ecologie van het Victoriameer gezorgd. Sinds het begin van de twintigste eeuw is de visserijdruk continu toegenomen, wat leidde tot steeds lagere vangsten van de inheemse tilapiasoorten in het meer. In de jaren ‘50 en ’60 zijn exotische tilapiasoorten, in het bijzonder de Nijltilapia, en de roofzuchtige Nijlbaars (*Lates niloticus*) in het meer uitgezet maar aanvankelijk namen populaties ervan niet in aantal toe. Nijlbaars en Nijltilapia worden veel groter dan de vinger- tot handgrote cichliden en zijn daardoor van veel grotere economische waarde. In de jaren ’60 begon men met lichtvisserij specifiek op de talrijk voorkomende *Rastrineobola argentea*, een kleine karperachtige, te bevissen. Bij deze vorm van visserij lokt men de visjes ‘s nachts met lampen waarna zij met netten worden gevangen. In 1976 is men begonnen om met trailschepen de haplochromine cichliden te bevissen. Hierdoor werden populaties cichliden locaal overbevist. In de jaren ’80 namen nijlbaarspopulaties explosief in aantal toe. Dit ging gepaard met een dramatische afname van de cichlidenpopulaties die tot prooi dienden aan de nijlbaars. Vele soorten zijn sindsdien nooit meer waargenomen en we kunnen aannemen dat de meeste van hen zijn uitgestorven. Tegelijkertijd nam *R. argentea* op veel plaatsen sterk in aantal toe. Een van de getroffen soorten, waar in dit proefschrift veel experimenten mee zijn gedaan, is *Haplochromis* (*Yssichromis*) *pyrrhocephalus*. Vangsten hiervan liepen terug van ongeveer 20.000 per trailvangst van een half uur naar minder dan een. Behalve verschuivingen in de visfauna nam men in de jaren ’80 ook belangrijke veranderingen van omgevingsfactoren waar. De troebelheid van het water nam toe, deels veroorzaakt door algenbloei. De hoeveelheid cyanobacteriën (blauwalgen) in het meer nam flink in aantal toe ten koste van diatomeën die eerst de dominante groep vormden. Uit boorproeven in de sedimentlaag van het Victoriameer is gebleken dat eutrofiëring[1] van het meer, als waarschijnlijke oorzaak van deze verschuiving, al rond de jaren ‘20 of ‘30 van de vorige eeuw in gang is gezet. De eutrofiëring vertoont een sterke correlatie met de toename van het aantal mensen dat rond het Victoriameer woont. Bronnen van eutrofiëring zijn intensieve landbouw, ontbossing en industrieel en huishoudelijk afvalwater. Door de opkomst van de Nijlbaars nam het aantal vissen dat algen at af en nam de ontbossing verder toe omdat hout nodig was voor het roken en vakken van de gevangen Nijlbaars.

Door menselijke activiteiten veroorzaakte eutrofiëring is een wereldwijd probleem. Algenbloei en de daaropvolgende sterfte heeft in veel gevallen hypoxie tot gevolg. Onder hypoxie verstaan we het voorkomen van te lage zuurstofconcentraties. Het voorkomen van hypoxie in wateren waar dit ongewoon is heeft een negatief effect op de soortenrijkdom en dichtheden van

[1] Verrijking van het water met nutriënten.

waterademhalende dieren (zoöplankton, macroëvertebraten, schaaldieren vissen etc.).

Seizoensgebonden terugkeer van hypoxie veroorzaakt grootschalige ontvolking van getroffen regio's door sterfte en migratie. In het Victoriameer zorgt acute opwelling van hypoxische waterlagen voor grootschalige vissterfte (Wanink *et al.*, 2001). Dit fenomeen is in de laatste decennia sterk in frequentie toegenomen. In het Victoriameer komt chronische hypoxie tegenwoordig in veel grotere gebieden en gedurende langere periodes voor dan vroeger. In 1960-61 kwamen zeer lage zuurstofconcentraties (< 1 mg l^{-1}) alleen voor op plaatsen dieper dan 60 meter. Echter, in 1990-91 werd dit al op dieptes van 45 tot 54 meter (35% van de bodemoppervlakte) waargenomen in een aaneengesloten periode van oktober 1990 tot maart 1991. In de ondiepere Mwanza golf (<20m), in het zuiden van het Victoriameer, werden tussen 1979 en 1988 de periodes van hypoxie langer en hypoxische waterlagen werden steeds verder van de bodem waargenomen. Hypoxie maakt de waterlaag vlak boven de bodem een minder geschikt habitat voor vissen die ervan afhankelijk zijn. Het is dan ook niet verassend dat een aantal wetenschappers met de hypothese kwam dat, naast predatie door nijlbaars, hypoxie een belangrijke oorzaak was voor het afnemen van cichlidenpopulaties in de jaren '80 van de vorige eeuw.

Hoe gaan vissen om met hypoxie?

De duur van blootstelling aan hypoxie heeft een belangrijke invloed op de respons van vissen hierop. Deze kunnen gedragsmatig, fysiologisch, biochemisch en anatomisch van aard zijn. Echter, de relatie tussen de blootstellingduur aan hypoxie en het type respons is bijna nooit gespecificeerd. In dit proefschrift is onderscheid gemaakt tussen korte-termijn hypoxie en chronische hypoxie. Korte-termijn hypoxie kan enkele uren tot dagen duren terwijl chronische hypoxie vanaf een week tot permanente blootstelling kan inhouden.

Korte-termijn hypoxie

Tijdens korte-termijn hypoxie kunnen veranderingen van het gedrag en de regulering van de stofwisseling de energieconsumptie verlagen, zuurstofextractie verhogen en het anaërobe metabolisme[2] activeren. Vissen die aan korte-termijn hypoxie worden blootgesteld zullen doorgaans reageren met een verhoogde ademhalingsactiviteit, een afname van de bewegingsactiviteit en oppervlakterespiratie. Dit laatste houdt in dat vissen het laagje water direct aan de oppervlakte opzuigen daar dit meer zuurstof bevat dan de rest van de waterkolom. Een plotselinge blootstelling aan hypoxie leidt tot stress-responsen die resulteren in een lage tolerantie voor hypoxie. Als vissen de tijd krijgen om zich aan een nieuwe omgeving aan te passen, het metabolisme te verlagen en andere stressfactoren minimaal blijven, is de tolerantie voor hypoxie veel hoger. De mogelijkheid om korte-termijn hypoxie te overleven is deels afhankelijk van de "coping strategie"[3] van het individu. Onderzoek aan tong (*Solea solea*) en regenboogforel

[2] Stofwisseling in het lichaam.

[3] Het type reactie op een verandering in de omgeving, deze zijn vaak een combinatie van gedrag en fysiologische veranderingen

(*Oncorhynchus mykiss*) heeft aangetoond dat zij óf met een onderdrukking van de gedrags- en metabole activiteit reageren, óf proberen weg te komen. Dit laatste gaat gepaard met hoge concentraties van catecholamines en cortisol[4] en snelle sterfte van de dieren als zij niet kunnen ontsnappen.

Veel vissen reageren op geleidelijke blootstelling aan korte-termijn hypoxie met een reductie van het metabolisme die tot onder het standaardmetabolisme[5] kan uitkomen. Dit stelt hen in staat om hun stofwisseling aëroob te houden, hetgeen een bepalende factor is voor de tolerantie. Bij steeds lager wordende zuurstofconcentraties zal op een gegeven moment de zuurstofbehoefte groter zijn dan de opname en is activering van het anaërobe metabolisme nodig om aan de energiebehoefte te voldoen. In reactie op steeds lagere zuurstofconcentraties, zal de perfusie van de kieuwen met bloed verhoogd worden. Door de hogere doorbloeding zal het maximale kieuwoppervlak worden gebruikt voor zuurstofopname. Onder normale omstandigheden en routine activiteit is het gebruikte kieuwoppervlak lager omdat niet alle secundaire lamellen doorbloed zijn.

In de nijltilapia (*Oreochromis niloticus*) ligt de P_{50} [6] van het volbloed bij ongeveer 20 mm Hg. Dus bij blootstelling aan zulke lage zuurstofconcentraties kunnen we aannemen dat het bloed maar gedeeltelijk met zuurstof beladen wordt als het de kieuwen passeert. Veel vertebraten kunnen de zuurstofaffiniteit van het hemoglobine (Hb) veranderen door allostere[7] interacties met verschillende verbindingen in de rode bloedcellen. In vissen zijn de organische fosfaten ATP (adenosine trifosfaat) en GTP (guanosine trifosfaat) de belangrijkste verbindingen om de Hb-O_2 [8] affiniteit te reguleren. Tijdens hypoxie kan binnen een a twee dagen de concentratie ATP en GTP verlaagd worden waardoor de Hb-O_2 affiniteit stijgt (Weber, 1996, 2000; Val, 2000).

Fysiologie tijdens chronische hypoxie

Tijdens blootstelling aan chronische hypoxie resulteert de activering en deactivering van genen in de productie van andere proteïnen en weefsels. Dit kan leiden tot verhoging van de zuurst ofextractiecapaciteit, aërobe capaciteit en veranderingen op weefselniveau zoals erythropoiese[9] en angiogenese[10]. Er zijn totnogtoe maar enkele studies gepubliceerd over blootstelling van vissen aan chronische hypoxie. Experimenten met zowel juveniele als volwassen vissen lieten zien dat het overleven ervan voornamelijk gebaseerd was op het reduceren van energieverbruik. In zeelten (*Tinca tinca*) die 6 weken geacclimatiseerd werden aan 8% luchtverzadiging werd een afname van de routine zuurstofconsumptie van

[4] Hormonen die vrijkomen bij acute en chronische stress
[5] Minimum aan stofwisselingsactiviteit om in leven te blijven onder normale omstandigheden
[6] De zuurstofconcentratie waarbij 50% van de maximale verzadiging van het bloed bereikt is
[7] Een interactie door binding van een stof op een andere plaats. In dit geval: door binding van een stof op een andere plaats dan waar zuurstof bindt op het Hb molecuul, verandert de bindingsaffiniteit met zuurstof.
[8] O_2 is de chemische notatie voor zuurstof.
[9] Aanmaak van rode bloedcellen.
[10] Aanmaak en onderhoud van bloedvaten.

gemiddeld 48% waargenomen (Johnston en Bernard, 1982a). Bovendien werd gevonden dat de totale oppervlakte van de bloedcapillairen in doorsnedes van spieren met 43-76% was afgenomen en de volumedichtheid van de mitochondriën[11] met 60%. Glycogeenvoorraden in de witte spieren en de activiteit van het enzym lactaatdehydrogenase[12], beide indicatoren voor de anaërobe capaciteit, waren verhoogd maar niet significant. Van karpers (*Cyprinus carpio*) die 6 weken aan hypoxie werden geacclimatiseerd (20% luchtverzadiging ofwel 30 mm Hg), was de gemiddelde zuurstofconsumptie 50% lager dan in karpers die aan normale zuurstofconcentraties waren geacclimatiseerd(>80% luchtverzadiging ofwel 120 mm Hg; Lomholt en Johansen, 1979). In de goudvis (*Carassius auratus*) die erg tolerant is voor hypoxie, leidde chronische hypoxie tot afname van de proteïnesynthese in de lever en een toename in activiteit van enzymen die een conservatief verbruik van glycogeenvoorraden in de spieren bevorderen. Glycogeen is een van de belangrijkste substraten die onder anaërobe omstandigheden gebruikt kan worden voor de energieproductie. In een andere studie leidde chronische hypoxie bij juveniele karpers (35 g) tot lagere in het bloed van testosteron, oestradiol en triiodothyronine. Deze hormonale veranderingen werden geassocieerd met een vertraagde gonadengroei, een afname van het paaisucces, spermamotiliteit, bevruchting van de eieren, aantal uitgekomen eieren en overleving van de larven (Wu, 2003). In de bovengenoemde studies aan zeelt, karper en goudvis, duurde de blootstelling aan hypoxie 6-8 weken en is het vrij duidelijk dat de dieren hiervan negatieve gevolgen ondervonden in hun fysiologie. Men kan zich afvragen of deze vissen erg veel langer of zelfs levenslang onder hypoxie zouden kunnen overleven. In het wild zouden zij dan ook nog in staat moeten zijn om voldoende te foerageren, territoria te verdedigen tegen indringers, vluchten voor predatoren etc. Theoretisch zou de beste hypoxieadaptatie vissen in staat moeten stellen om hun energieverbruik te kunnen handhaven. Dat impliceert dat aanpassingen nodig zijn om onder hypoxie niet beperkt te zijn in zuurstof opname.

Functionaliteit van de kieuwen

Een van de belangrijkste functies van de kieuwen is het realiseren van optimale gasuitwisseling tussen het water en bloed dat door de kieuwen stroomt. Bij maximale efficiëntie is al het zuurstof uit het water in het bloed opgenomen als het de kieuwen verlaat. De wet van Fick kan gebruikt worden voor berekeningen aan gasuitwisselingsprocessen:

$$J_{net} = D\ A\ \Delta PO_2/\Delta\chi$$

Hierin is J_{net} de netto gasuitwisseling tussen water en bloed, A de oppervlakte, ΔPO_2 het verschil in partiële zuurstofdruk en $\Delta\chi$ de diffusieafstand.

Volgens deze wet is de gasuitwisseling per tijdseenheid onder andere afhankelijk van het kieuwoppervlak. Dus zijn vissen die leven onder lage zuurstofconcentraties gebaat bij een groter kieuwoppervlak om

[11] Energie producerende onderdelen in de cel. Hier vinden de verbrandingsprocessen plaats.

[12] Een enzym dat een belangrijke stap in de citroenzuurcyclus vormt. De citroenzuurcyclus is een belangrijk proces in de aerobe omzetting van voedsel.

een hoge zuurstofopname te kunnen handhaven. Bij een geringe verlaging van de zuurstofconcentratie is een vergroting van het aantal doorbloede kieuwlamellen en een verhoging van de ventilatiefrequentie voldoende om dezelfde zuurstofopname te realiseren. Bij een steeds lager zuurstofniveau zal op een gegeven moment de snelheid van de waterstroom langs de kieuwlamellen zo hoog zijn dat de gasuitwisseling efficiëntie sterk daalt. In de Nijltilapia gaat de zuurstofopname efficiëntie al achteruit als de luchtverzadiging van het water 50% is.

Om de gasuitwisseling van een complex ademhalingsorgaan als de kieuwen beter te kunnen begrijpen kunnen ze vereenvoudigd worden tot een serie van rechthoekige kanaaltjes waardoor het water stroomt en terwijl in de schotten het bloed stroomt; vergelijkbaar met een radiator. Gebaseerd hierop kan men beredeneren wat het effect zal zijn van een groter kieuwoppervlak op de waterstroom en de weerstand van de kieuwen op het doorstromende water. Een groter kieuwoppervlak heeft onder hypoxie niet alleen maar voordelen. Als bijvoorbeeld de oppervlakte wordt vergroot door de dichtheid van de secundaire lamellen te vergroten, zullen de kanaaltjes kleiner worden hetgeen een verhoging van de weerstand oplevert. Door de secundaire lamellen langer te maken, zal de weglengte voor het doorstromende water toenemen wat ook voor meer weerstand zorgt. Om de stroomsnelheid van het water bij zulke veranderingen toch even hoog te houden zal meer energie voor de ademhaling gebruikt moeten worden. Deze energie gaat dan verloren voor andere investeringen als voedsel zoeken of voor nageslacht zorgen. Een mogelijke vergroting van de totale oppervlakte van de secundaire lamellen zónder de weerstand per kanaaltje te verhogen wordt gerealiseerd door langere filamenten (dus meer kanaaltjes) en door hogere secundaire lamellen (dus grotere kanaaltjes).

Er is veel gelijkenis tussen de vorm en grootte van kieuwen van vissen die in een vergelijkbaar habitat leven en tussen vissen die een vergelijkbare levensstijl en zuurstofbehoefte hebben. In een studie waarin kieuwdimensies van cichliden uit verschillende Afrikaanse meren werden vergeleken, zag men dat de soorten die gespecialiseerd zijn in het kraken van slakken met hun keelkaken, een hogere dichtheid van secundaire lamellen hadden (dus een grotere oppervlakte maar kleinere kanaaltjes voor waterdoorstroming) naarmate zij in dieper water leefden. Dit werd gerelateerd aan de lagere zuurstofconcentratie op grotere diepte. In een andere studie werden *Haplochromis hiatus* en *H. iris* vergeleken. Morfologisch en ecologisch lijken deze twee Victoriacichliden sterk op elkaar maar er zijn belangrijke verschillen. *H. hiatus* komt voor op dieptes tussen 3 en 9 meter terwijl *H. iris* tussen de 8 en 15 meter voorkomt. Tijdens het regenseizoen komt stratificatie van het water voor en komen zuurstofconcentraties van 2-3 mg L^{-1} voor in het habitat van *H. iris*. Het totale kieuwoppervlak van deze soort is 1.6 maal groter dan dat van *H. hiatus*

(Hoogerhoud, 1983). Contrasterend met de vele studies die de verschillen in kieuwen tussen soorten beschrijven in relatie tot de habitat en levensstijl, is de fenotypische plasticiteit van de kieuwen in relatie tot omgevingsfactoren nauwelijks onderzocht. Of er een plastische respons bestaat van de kieuwen in reactie op veranderingen in de zuurstofconcentratie, is dus niet bekend.

Anatomische veranderingentijdens korte-termijn hypoxie

Sommige gespecialiseerde vissoorten kunnen hun anatomie veranderen in reactie op korte-termijn hypoxie. De kroeskarper (*Carassius carassius*) bijvoorbeeld, is in staat om zijn kieuwoppervlak in enkele dagen tijd te vergroten. Onder normale omstandigheden zijn op de kieuwfilamenten slechts rudimentaire secundaire lamellen te zien. Deze zijn ingebed in een sponsachtig weefsel dat, na enkele dagen van blootstelling aan hypoxie, door apoptose[13], verdwijnt (Sollid *et al.*, 2003). Hierdoor neemt het functionele kieuwoppervlak sterk in grootte toe. In een latere studie bleek ook dat dit verschijnsel bij zowel de kroeskarper als de goudvis (*Carassius auratus*) optreedt als zij aan warm water worden blootgesteld (Sollid *et al.*, 2005). Dit kan verklaard worden door het feit dat de maximale hoeveelheid opgeloste zuurstof in het water afneemt bij hogere temperaturen. Een totaal andere soort fenotypische respons wordt gevonden bij de Zuid-Amerikaanse Tambaqui (*Colossoma macroponum*). Onder hypoxie groeit bij deze vis binnen een uur de onderlip sterk uit waardoor deze geschikt wordt om de altijd goed beluchte bovenste millimeters van de waterkolom te benutten voor de respiratie. De hypoxieresponsen in de kroeskarper, goudvis en tambaqui zijn volledig reversibel en bij uitstek geschikt om onder wisselende zuurstofconcentraties te leven.

Anatomische veranderingen bij chronische hypoxie

Relaties tussen kieuwgrootte en chronische hypoxie zijn enkele malen eerder gevonden. In het begin van de jaren ’80, de periode dat chronische hypoxie zich manifesteerde in het Victoriameer, groeiden de populaties van *R. argentea*. Het totale aantal kieuwfilamenten op de eerste kieuwboog van *R. argentea* die in 1988 gevangen werden was 3.6% groter dan van vissen die in 1983 gevangen werden. Het is niet bekend of deze verandering een resultaat was van fenotypische plasticiteit of dat ook natuurlijke selectie een rol hierin heeft gespeeld. Er is een studie bekend waarin juist de fenotypisch geïnduceerde veranderingen van de kieuwen zijn onderzocht (Chapman *et al.*, 2000). In deze studie zijn gesplitste nesten van de niet endemische[14] *Pseudocrenilabrus multicolor victoriae* (in Nederland bekend als de kleine muilbroeder, een aquariumvis) bij normoxie (<7.5 mg L^{-1}) en hypoxie (1 mg L^{-1}) opgegroeid. Toen zij 6 maanden oud waren had de hypoxiegroep een 22% groter kieuwoppervlak, voornamelijk veroorzaakt door een

[13] Gereguleerde celdood.

[14] Soorten zijn endemisch als zij maar op een locatie voorkomen, bijvoorbeeld een meer of rivier.

groter filamentaantal en –lengte. Wilde exemplaren uit een normoxisch habitat hadden een 41% groter kieuwoppervlak dan vissen uit een hypoxisch habitat. De vissen uit het hypoxische habitat vertoonden naast een verandering in de filamenten ook grotere secundaire lamellen.

Consequenties en beperkingen van grotere kieuwen

In de kop van een vis liggen spieren, botten en andere weefsels, die betrokken zijn bij respiratie, visie, eten etc. tegen elkaar aan. Als door blootstelling aan chronische hypoxie de kieuwen en het ademvolume groter zouden worden, zou er extra ruimte ingenomen worden door de kieuwen en spieren van het ademhalingsapparaat. In andere studies is al gesuggereerd dat een dergelijke vergroting zulke grote effecten heeft op omliggende structuren dat deze zelfs de vorm van de kop zullen veranderen om genoeg ruimte te creëren. Theoretisch zou extra ruimte in de kop op verschillende manieren verkregen kunnen worden (Witte *et al.*, 1990): (1) het gebruik van de vrije beschikbare ruimte; (2) verkleining van omliggende structuren; (3) toename van het kopvolume; (4) een combinatie van de voorgaande mogelijkheden. Oplossingen 2-4 zouden een negatieve invloed kunnen hebben op de functies van omliggende structuren en de stroomlijn van de vis nadelig kunnen beïnvloeden.

Bewijs dat structuren die de kieuwfilamenten omringen plastisch zijn is al eerder geleverd (Smits *et al.*, 1996a,b). Van twee populaties van *A. alluaudi* is bekend dat de een vooral van slakken leeft en vergrote keelkaken heeft. De andere populatie leeft van zachtere prooien zoals insecten en heeft kleinere, minder robuuste keelkaken. De kieuwen, die tegen de keelkaken aan liggen, hebben bij de slakkenetende *A. alluaudi* een andere vorm, waardoor er meer ruimte beschikbaar is voor de vergrote keelkaken. Bij de *A. alluaudi* met grote keelkaken was ook de kop breder op de plaats waar de keelkaken en kieuwen zitten. In die studie kon niet vastgesteld worden of de gevonden verschillen een resultaat waren van fenotypische plasticiteit of dat genetische verschillen ook een rol speelden.

Centraal in dit proefschrift

Gegeven de wereldwijde trend van een toenemend aantal locaties waar chronische hypoxie voorkomt, en het feit dat tot nu toe geen voorbeeld bekend is van herstel, is er behoefte aan meer begrip van de effecten van hypoxie op vissen. De huidige kennis hiervan is voornamelijk gebaseerd op korte-termijn hypoxie. In de weinige studies die bekend zijn over chronische hypoxie, was de duur van de blootstelling maar enkele weken en de vissen die gebruikt werden groeiden niet veel. Deze studies, waarin parameters voor bewegingsactiviteit, zuurstofconsumptie, enzymactiviteit, reproductie, groei en voedselopname zijn meegenomen, laten zonder uitzondering zien dat vissen beperkt worden door blootstelling aan hypoxie en overleven doorhun energieverbruik zo veel mogelijk te beperken. Ook uit eerdere studies met Victoriacichliden in

ons laboratorium, bleek dat exemplaren die bij normale zuurstofconcentraties (≥ 80% luchtverzadiging) zijn opgegroeid en geleidelijk in enkele uren tijd aan 10% luchtverzadiging (LVZ) worden blootgesteld, niet langer dan een dag kunnen overleven. Echter, als twee weken oude visjes van dezelfde soorten aan in een periode van vier weken geleidelijk aan 10% LVZ werden blootgesteld, overleefden zij dit en trad er geen sterfte op. De vissen groeiden net zo snel als nestgenoten die bij normale zuurstofconcentraties (80% LVZ) opgroeiden en volgroeide individuen produceerden regelmatig nesten met levensvatbare jongen. Dit zijn sterke aanwijzingen dat cichliden die bij 10% LVZ opgroeien hun energieverbruik (lees zuurstofconsumptie) kunnen handhaven en dus weinig fysieke hinder ondervinden van de lage zuurstofconcentratie. Dit in tegenstelling met de studies die hierboven zijn beschreven.

Hiermee komen we bij de centrale vraag in dit proefschrift:

Welke fenotypische responsen zijn verantwoordelijk voor het feit dat cichliden die vanaf de vrijzwemmende fase aan chronische hypoxie worden blootgesteld zich veel beter kunnen handhaven onder hypoxie dan nestgenoten die niet onder hypoxie opgroeien? Experimenten waarin nesten worden gesplitst zijn erg nuttig voor het beantwoorden van deze vraag. In het onderzoek dat voor dit proefschrift werd uitgevoerd zijn nesten cichliden gesplitst waarna een helft is opgegroeid onder hypoxie (10% LVZ) en de andere helft onder normoxie (80% LVZ). Van de volgroeide individuen zijn parameters van het gedrag, de fysiologie en de anatomie gemeten. Hiermee is geprobeerd te achterhalen of onder hypoxie opgegroeide (OHO) vissen juist hun energieconsumptie beperken of zuurstofextractie vergroten om onder hypoxie te overleven.

Limitaties in zuurstofopname onder hypoxie

Uit respirometrie-experimenten is gebleken dat de zuurstofconsumptie van OHO cichliden bij 10% LVZ en onder normoxie opgegroeide (ONO) nestgenoten bij 80% LVZ niet wezenlijk van elkaar verschilt. Als de ONO cichliden onder hypoxie gezet worden dan is hun zuurstofconsumptie lager dan die van OHO nestgenoten (Hoofdstuk 2,3 en 4). Echter, er waren verschillende aanwijzingen dat cichliden die onder hypoxie opgroeiden toch beperkt waren in hun zuurstofopnamecapaciteit. Ten eerste, tijdens het voeren was het gedrag van ONO en OHO vissen duidelijk verschillend (Hoofdstuk 2 en 3). De ONO vissen waren altijd erg actief tijdens het eten en aten al het voer zo snel mogelijk op. OHO nestgenoten wachtten juist tot de verzorger ze met rust liet en begonnen daarna rustig met eten, wat dan ook langer duurde. Dit impliceert dat OHO vissen minder energie spenderen aan eten en concurrentie om voer. Ten tweede, aan een gesplitst nest van *H. piceatus* is een voorstudie uitgevoerd waarin verschillende gedragsparameters zijn gemeten die representatief zijn voor paaigedrag en agressief gedrag. Al deze gedragingen werden minder

vaak waargenomen in de OHO groep dan in de ONO groep. Paaigedrag en agressief gedrag kosten erg veel energie en het gevonden verschil duidt erop dat OHO vissen minder energie spenderen aan deze gedragspatronen en dat het totale energieverbruik dus lager ligt. Ten derde, de activiteit van het mitochondriale enzym citraatsynthase in de witte spieren van OHO *A. alluaudi* en *H. ishmaeli* was 25% lager dan in dat van ONO nestgenoten (Hoofdstuk 6). In tilapia was het verschil kleiner en niet significant. De citraatsynthas eactiviteit is een beperkende stap in de citroenzuurcyclus. Dus kan gezegd worden dat de maximale aerobe activiteit van de witte spieren lager was in OHO dan in ONO exemplaren van *A. alluaudi* en *H. ishmaeli*. Verder werd in de witte spieren van OHO tilapia een verhoogde concentratie glycogeen aangetroffen ten opzichte van ONO nestgenoten (Hoofdsuk 6). Dus kan er meer energie door anaërobe energie omzetting worden geproduceerd. In *A. alluaudi* en *H. ishmaeli* waren de verschillen in glycogeen voorraad kleiner en niet significant. Andere onderzoeken lieten zien dat ook in de karper en zeelt geen verhoging van glycogeenvoorraden optraden na langdurige blootstelling aan hypoxie. Echter, OHO *A. alluaudi* en *H. ishmaeli* konden zuurstofloze condities veel langer tolereren dan ONO nestgenoten (Hoofdstuk 3). Wellicht wordt de hogere tolerantie van *H. ishmaeli* en *A. alluaudi* verklaard door gedragsverschillen of verhoogde glycogeenvoorraden in de lever. De lever is naast de spieren de belangrijkste opslagplaats voor glycogeen.

De observaties hierboven zijn aanwijzingen voor een verlaagd energieverbruik in OHO vissen. Dit zou moeten resulteren in een verlaagd zuurstofverbruik maar experimenten met respirometers hebben juist aangetoond dat de zuurstofconsumptie van ONO vissen bij 80% LVZ niet verschilde van de zuurstofconsumptie van OHO nestgenoten bij 10% LVZ (Hoofdstuk 2, 3). Echter, zuurstofconsumptie is een weergave van het totale energie budget van een vis terwijl gedragsactiviteit, glycogeenvoorraden en citraatsynthase activiteit iets zeggen over de verdeling van het energie budget. Onder hypoxie is dus de verdeling van het energie budget anders dan onder normoxie. Behalve afname van energiebudget in sommige fysiologische processen onder hypoxie, zal er ook een toename van het budget zijn in andere. Bijvoorbeeld in de kosten voor ademhaling; OHO vissen moeten, om bij 10% LVZ dezelfde hoeveelheid zuurstof te kunnen opnemen als ONO nestgenoten bij 80% LVZ, ongeveer acht keer meer water over hun kieuwen pompen. Logischerwijs kost dit veel extra energie.

Verhoogde zuurstofextractie door veranderingen in de kieuwen

Normaliter kunnen vissen die onder normoxie opgroeien hun normale zuurst ofconsumptiepatroon niet handhaven bij hypoxie omdat dan hun zuurstofextractie efficiëntie verlaagd is. Uitgaande van de wet van Fick, is de zuurstofflux direct

afhankelijk van het verschil in partiële druk tussen water en bloed. Verder is de zuurstofopname per tijdseenheid direct afhankelijk van het totale oppervlak en omgekeerd evenredig met de diffusieafstand. Terwijl de meeste vissen tijdens korte-termijn hypoxie maar beperkte middelen hebben om hun zuurstofopname capaciteit te verhogen, was de verwachting dat blootstelling van jonge vissen aan chronische hypoxie zou leiden tot een vergroot kieuwoppervlak en een kleinere diffusieafstand (Hoofdstuk 4). Metingen aan de derde kieuwboog van *H. pyrrhocephalus* wezen uit dat het kieuwoppervlak van OHO vissen maarliefst 80% groter was dan dat van ONO nestgenoten. Kieuwen zijn dus erg plastische organen. Deze vergroting van het kieuwoppervlak werd veroorzaakt door 26.9% grotere primaire filamenten, 9.2% hogere en 37.7% langere secundaire lamellen.

De verschillen in kieuwgrootte die we hebben waargenomen tussen ONO en OHO *H. pyrrhocephalus* vertoonden grote overeenkomsten met de verschillen tussen vissen die leven in habitats met veel of weinig zuurstof. Omdat uit onze studie duidelijk blijkt dat kieuwen erg plastische organen zijn, kan men zich afvragen in hoeverre de verschillen in de kieuwen van deze vissen een fenotypische respons zijn op het leven in een verschillende omgeving. In ieder geval moeten we voorzichtig zijn met de interpretatie van morfologische verschillen tussen vissen die in verschillende habitats leven. Behalve de acht maal lagere zuurstofconcentratie bij 10% LVZ, zorgt de relatief grote toename in de hoeveelheid water die over de kieuwen gepompt moet worden voor voldoende zuurstofaanvoer in OHO vissen, voor sterk verschillende condities waaronder gasuitwisseling moet plaatsvinden. Bij een verlaging van het verschil in zuurstofconcentratie tussen water en bloed gaat de gasuitwisseling in theorie evenredig veel trager. Dus zou het water juist langer in de kieuwen moeten blijven om nagenoeg al het zuurstof uit het water op te kunnen nemen. Dit lijkt op het eerste gezicht niet te kunnen vanwege de grote toename in hoeveelheid water die door de kieuwen stroomt. Echter, als het totaal van de oppervlaktes van de dwarsdoorsneden van de respiratiekanalen groter wordt, verlaagt dit de stroomsnelheid van het water. Ook langere secundaire lamellen zorgen voor een langer verblijf van water in de kieuwen. Metingen toonden aan dat, bij OHO vissen zowel de dwarsdoorsnede per kanaal als het aantal kanalen groter was dan bij ONO nestgenoten (Hoofdstuk 4). Bovendien waren de secundaire lamellen en dus de respiratiekanalen langer. Echter, een ~26.9% toename van het aantal kanalen, een 9.2% toename van de hoogte en een 37.7% toename van de lengte van de kanalen, verhoogt de verblijftijd van het water in de kieuwen met minder dan een factor twee. Dus bij een acht maal lagere zuurstofconcentratie, lees acht maal langzamere gasuitwisseling snelheid, is de efficiëntie nog steeds erg verlaagd. Samen met de waargenomen vergroting van het kieuwoppervlak van 80%, kan dus niet verklaard worden dat OHO vissen toch in staat zijn tot even

grote zuurstofopname als ONO vissen. Het vermoeden bestaat dan ook dat er nog meer veranderingen in de kieuwen hebben plaatsgevonden. Bijvoorbeeld een afname van de dikte van de water-bloedbarrière. De hieruit volgende verlaging van de diffusieafstand zorgt voor een rechtevenredige vergroting van de diffusiesnelheid.

Anatomische effecten van grotere kieuwen

Omdat er maar weinig vrije ruimte beschikbaar is in de kop van een vis, was de hypothese dat een mogelijke vergroting van de kieuwen zulke veranderingen met zich meebrengt dat dit de kopvorm beïnvloedt. In hoofdstuk 5 zijn experimenten met gesplitste nesten van tilapia, *A. alluaudi*, *H. ishmaeli* en *H. pyrrhocephalus* beschreven. Met een behulp van driedimensionaal model is de vorm van de kop en de volumes van de orale, suspensoriale en operculaire[15] compartimenten gemeten. Ondanks het feit dat de gebruikte soorten een verschillende fylogenetische achtergrond hebben en verschillen in hun leefwijze en morfologie, waren de gevonden toenames van dezelfde aard en omvang. De sterkste vergroting van het kopvolume vond plaats in het suspensoriale compartiment, waarin het grootste gedeelte van de kieuwen zich bevindt. Gebaseerd op de studies die hierboven zijn beschreven is het waarschijnlijk dat andere structuren die tegen de kieuwen aanliggen, zoals de keelkaken en spieren die de keelkaken bedienen, ook van vorm zijn veranderd. Een dergelijke correlatie tussen fenotypische vergroting van de kieuwen en verandering van omliggende structuren is recentelijk gevonden (Chapman *et al*., 2000). In een experiment met een gesplitst nest van *P. multicolor*, vond men dat de OHO groep een langere kop had dan de ONO groep en een afgenomen diepte van de *m. sternohyoideus*, de *m. retractor dorsalis* en onderste keelkaak. Deze structuren liggen dicht tegen de kieuwen.

Als we alle andere publicaties raadplegen waar een zelfde of een vergelijkbare meetmethode is gebruikt, zien we steeds een terugkerend fenomeen: een grote variatie in de ventrale[16] breedte van de kop (stang [5_L-5_R], zie Hoofdstuk 4). Deze studies gaan over bijtkracht van de orale kaken, grootte van de keelkaken in relatie tot voedseltypen, fylogenetische verschillen en verschillen in leefomgeving tussen soorten. Dit suggereert dat de ventrale breedte in zowel genotypisch als fenotypisch opzicht veel plasticiteit vertoont. De huidige studie toont aan dat een dergelijke variatie ook door fenotypische plasticiteit alleen gerealiseerd kan worden.

Toegenomen zuurstofextractie door veranderingen in het bloed

Naast de gasuitwisseling in de kieuwen is de zuurstofbelading van het bloed en het transport naar de weefsels een essentieel proces dat beïnvloed wordt door hypoxie. De P_{50} van het bloed van tilapia is 20 mmHg (vergelijkbaar met 13% LVZ in onze experimenten), dus kan aangenomen worden dat bij 10% LVZ de relatieve zuurstofbelading van het bloed in de kieuwen erg laag

[15] Holtes ter hoogte van opeenvolgend: de bek, het oog en de kieuwdeksels, zie hfst. 5 fig 1.
[16] De kant waar de buik zit.

is. Bij alle drie soorten die onderzocht zijn, was de haemoglobineconcentratie (Hb-concentratie) in het bloed van de OHO groep significant hoger dan van de ONO groep. Daarnaast was de GTP-concentratie in de rode bloedcellen van de OHO tilapia 55% verlaagd (Hoofdstuk 6). Een verlaging van de GTP-concentratie in de rode bloedcellen verlaagt in vissen de P_{50} van het Hb. Hierdoor wordt dus meer zuurstof in het bloed opgenomen onder hypoxie. In rode bloedcellen van *H. ishmaeli* werd geen verschil in GTP-concentratie gevonden. Dit suggereerd dat rode bloedcellen van *H. ishmaeli* niet gevoelig zijn voor veranderingen in de GTP-concentratie. Echter, ook in *H. ishmaeli* werd geen verschil in zuurstofconsumptie gevonden tussen OHO vissen bij 10% LVZ en ONO vissen bij 80% LVZ. Het is dus waarschijnlijk dat in *H. ishmaeli* op een andere manier voor voldoende Hb-O_2 binding wordt gezorgd. Terwijl in tilapia en *A. alluaudi* geen verschillen gevonden werden in de types Hb van ONO en OHO dieren, hadden alle ONO *H. ishmaeli* duidelijk andere Hbs in hun bloed dan OHO nestgenoten. OHO *H. ishmaeli* misten vier isoHbs[17] die wel aanwezig waren in ONO vissen terwijl in rode bloedcellen van OHO vissen vijf isoHbs aanwezig waren die niet gevonden werden in die van ONO nestgenoten. Het is waarschijnlijk dat het verdwijnen van vier isoHbs en het verschijnen van vijf nieuwe isoHbs een verlaging van de P_{50} van het bloed met zich meebrengt. Hierdoor neemt de zuurstofopnamecapaciteit in de kieuwen dus toe bij 10% LVZ. Zo een eenduidig verschil in hemoglobines is totnogtoe nooit gevonden in vissen. Een dergelijke fenotypische respons is extra uniek als men bedenkt dat de productie van verschillende Hbs onder verschillende omstandigheden alleen bekend was bij dieren die drastische ontogenetische veranderingen doormaken zoals de na de geboorte bij mensen, bij levendbarende vissen of de overgang van water naar lucht bij amfibieën.

Conclusies

Van vier verschillende soorten cichliden zijn broedsels enkele weken na de bevruchting, in een postlarvaal stadium, gesplitst en permanent bij 10% en 80% luchtverzadiging gehouden. In de groepen bij 10% LVZ trad geen mortaliteit op en de vissen groeiden net zo snel als OHO nestgenoten. Hoewel er duidelijke aanwijzingen waren voor een enigszins verlaagde maximum aërobe capaciteit, resulteerde hun fenotypische respons voornamelijk in een toename van de zuurstofopnamecapaciteit onder hypoxie. Deze toename werd deels gerealiseerd door een 80% vergroting van het kieuwoppervlak. Hiermee samenhangend nam ook het kopvolume toe. De sterkste volumevergroting vond plaats op de locatie waar de kieuwen zitten. De fenotpische veranderingen in de kieuwen en kop waren van vergelijkbare aard en omvang als verschillen tussen soorten uit verschillende habitats of met verschillende levensstijlen (lees zuurstofbehoefte). In OHO vissen verschilde het transport van zuurstof naar de weefsels op twee manieren van dat van ONO dieren: ten eerste was de

[17] Haemoglobines die verschillen in structuur en daardoor vaak ook in functie.

Hb-concentratie verhoogd en ten tweede was de Hb-O_2 affiniteit verhoogd. Dit laatste werd op verschillende manieren gerealiseerd. Terwijl in tilapia lagere GTP-concentraties in de rode bloedcellen een hogere Hb-O_2 affiniteit tot gevolg hadden, had in *H. ishmaeli* het produceren van andere Hbs dan onder normoxie waarschijnlijk hetzelfde resultaat.

Het effect van hypoxie op belangrijke aspecten als het gedrag, voortplanting en embryoontwikkeling zijn in dit proefschrift amper belicht maar de resultaten van dit proefschrift laten zien dat cichliden over buitengewoon plastische eigenschappen beschikken die hen in staat stellen om onder zeer zuurstofarme omstandigheden te leven. Daarom moet de algemene gedachte dat chronische hypoxie desastreuze gevolgen heeft op vissen, meer worden genuanceerd. Dit proefschrift helpt ons om de effecten van zuurstoffluctuaties, een belangrijke menselijke invloed op waterademhalende organismen, beter te begrijpen. Terwijl acute en korte-termijn hypoxie resulteert in beperking van de zuurstofopname in cichliden, stelt chronische hypoxie jonge cichliden in staat om zich aan te passen aan het leven onder deze omstandigheden.

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Curriculum Vitae

Hendrikus Antonius Rutjes (roepnaam Carlo) wordt op 9 april 1977 geboren te Velp. In zijn woonplaats Silvolde doorloopt hij de basisschool en het Atheneum waar hij in 1995 eindexamen doet. Later dat jaar begint hij aan zijn studie Biologie aan de Universiteit Wageningen waar hij in 2000 zijn diploma haalt. Hij doet daar drie stages: De eerste gaat over competitie tussen 0+ roofblei (*Aspius aspius*) en snoekbaars (*Stizostedion lucioperca*) in de Rijn en wordt begeleid door promovendus Ir. Rob Grift (IMARES, IJmuiden). Carlo zijn tweede stage gaat over de rol van kleine barbelensoorten in het Tanameer (Ethiopië). Hiervoor verblijft hij vijf maanden in Ethiopië. De supervisie wordt gedaan door Dr. Nand Sibbing (Wageningen Universiteit). Na Ethiopië vertrekt hij opnieuw vier maanden naar het buitenland. Dit maal betreft het een stage over de inventarisatie en verspreiding van slakken in het oerwoud van Maleisisch Borneo. Dit ter ondersteuning van het onderzoek van Dr. Menno Schilthuizen die aan de Universiteit van Sabah op Borneo werkte. Kort na het behalen van zijn doctoraal diploma in 2000 begint Carlo aan zijn promotie onderzoek aan de Universiteit Leiden bij de sectie Integratieve Zoölogie. De dagelijkse begeleiding wordt gedaan door Dr. F. Witte en Dr. G.J.E.E.M. van den Thillart. Uit dit onderzoek vloeit het huidige proefschrift voort. Na het beëindigen van zijn dienstverband bij de Universiteit Leiden is Carlo achtereenvolgens werkzaam bij Velopa BV en bij INC Research. Sinds augustus 2006 werkt hij als adviseur aquatische ecologie bij Grontmij Aquasense BV.