Phenotypic responses to lifelong hypoxia in cichlids

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CHAPTER 1:

GENERAL INTRODUCTION

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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.