Phenotypic responses to lifelong hypoxia in cichlids

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CHAPTER 3:

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

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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

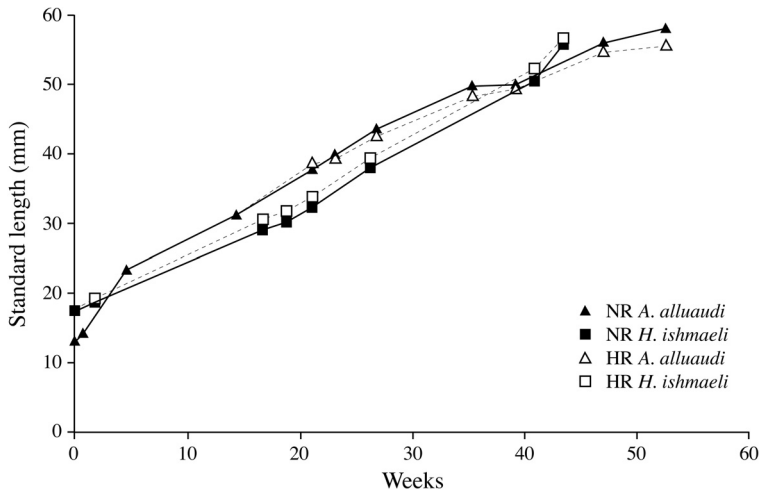

Figure 1: *Growth of normoxia-raised (NR) and hypoxia-raised (HR)* A. alluaudi *and* H. ishmaeli*, which were used in the respirometry experiments. The x-axis represents the time that animals were in the experiment. NR animals were raised at 80-90% air saturation (AS) and HR animals at 10% AS. After 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

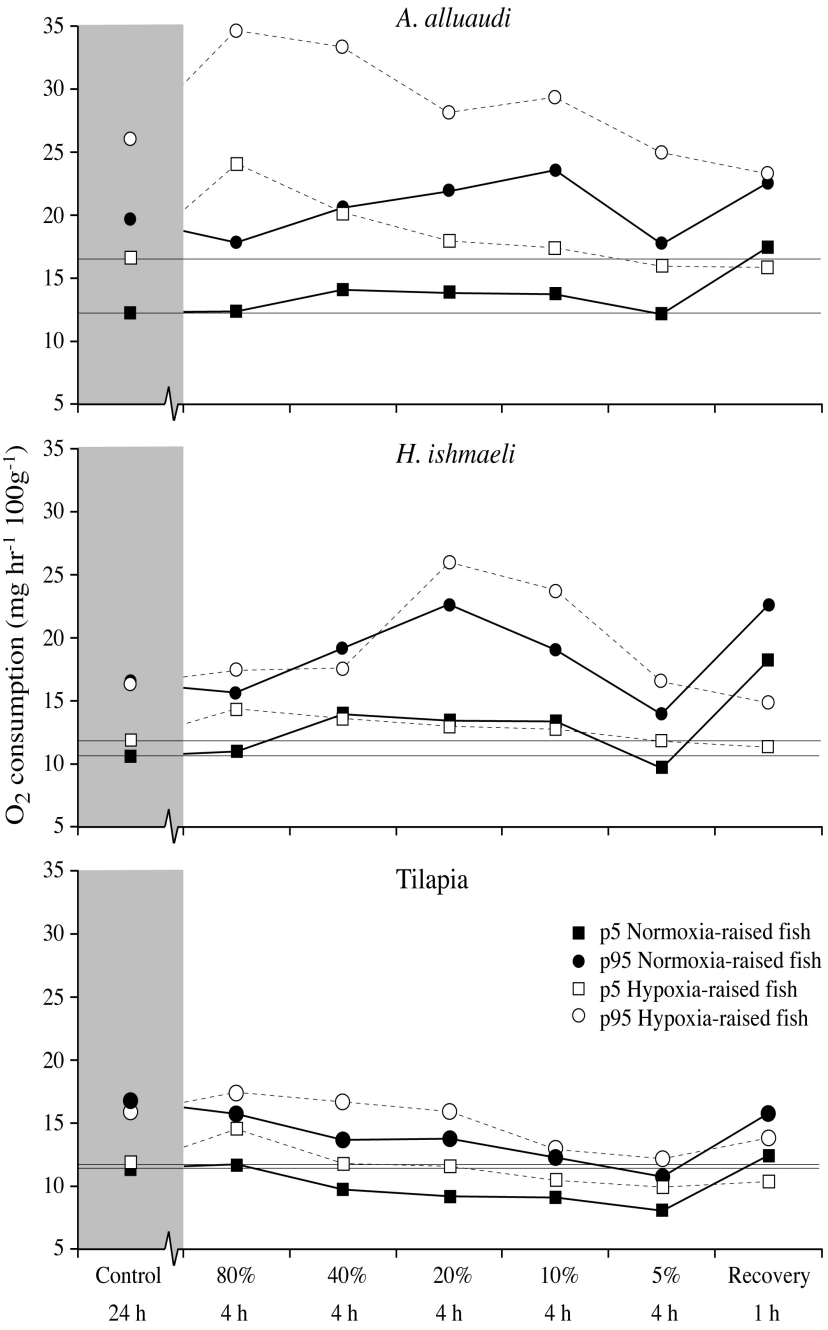

Figure 2: *The averaged O_2 consumption patterns of NR and HR* A. alluaudi, H. ishmaeli *and tilapia under control and experimental conditions. Of the frequency distribution of the data per condition, the 5-percentile and 95-percentiles are given. Horizontal lines represent the standard metabolic rate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

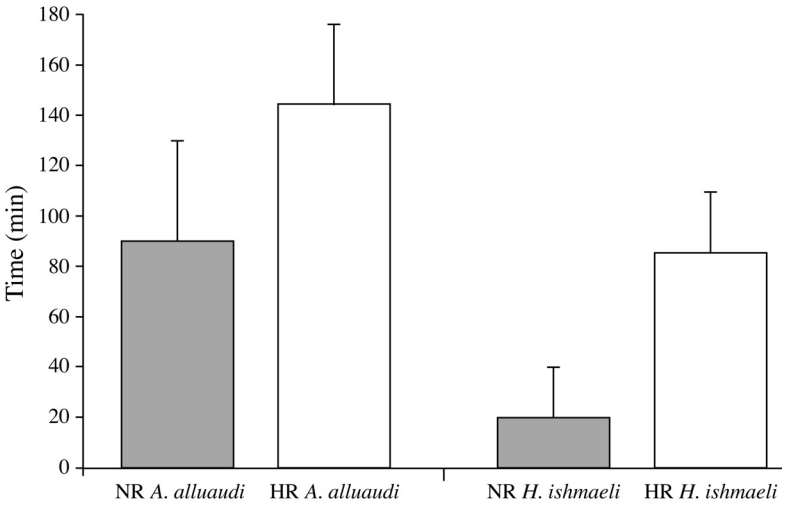

Figure 3: *Anoxia tolerance of NR and HR fish. ANCOVA showed no effects of weight*. A. alluaudi *could spend significantly more time under anoxia than* H. ishmaeli *(MANOVA, P=0.000). In addition HR animals could spend more time under anoxia than NR animals (MANOVA, p=0.000).*

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.

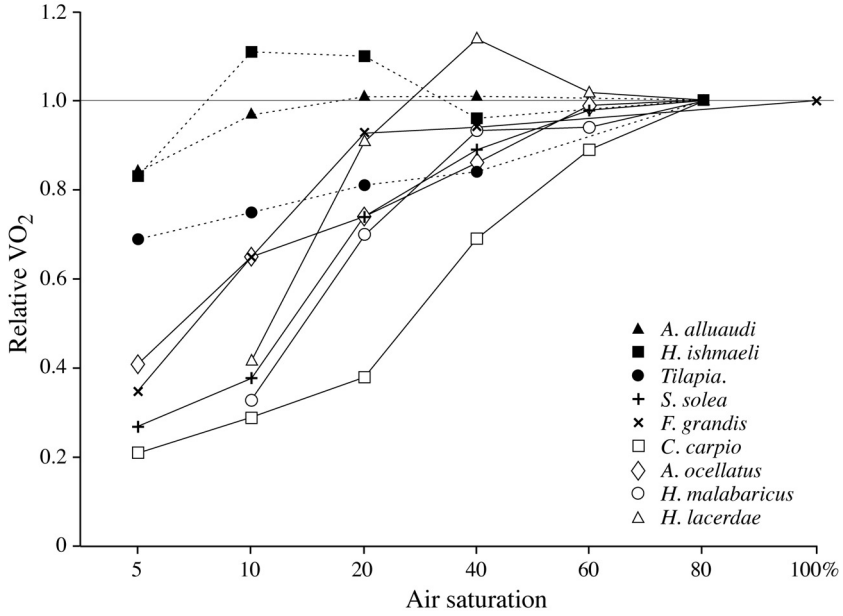

Figure 4: *Relative O_2 consumption at decreasing AS-levels for different species.* A. alluaudi *and* H. ishmaeli *and tilapia were able to maintain high O_2 consumption rates in comparison to other species. References for the data used: Astatoreochromis alluaudi, this study;* Haplochromis ishmaeli*, 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.