Phenotypic responses to lifelong hypoxia in cichlids

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CHAPTER 2:

UNAFFECTED OXYGEN CONSUMPTION UNDER LIFELONG HYPOXIA IN TILAPIA

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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%)

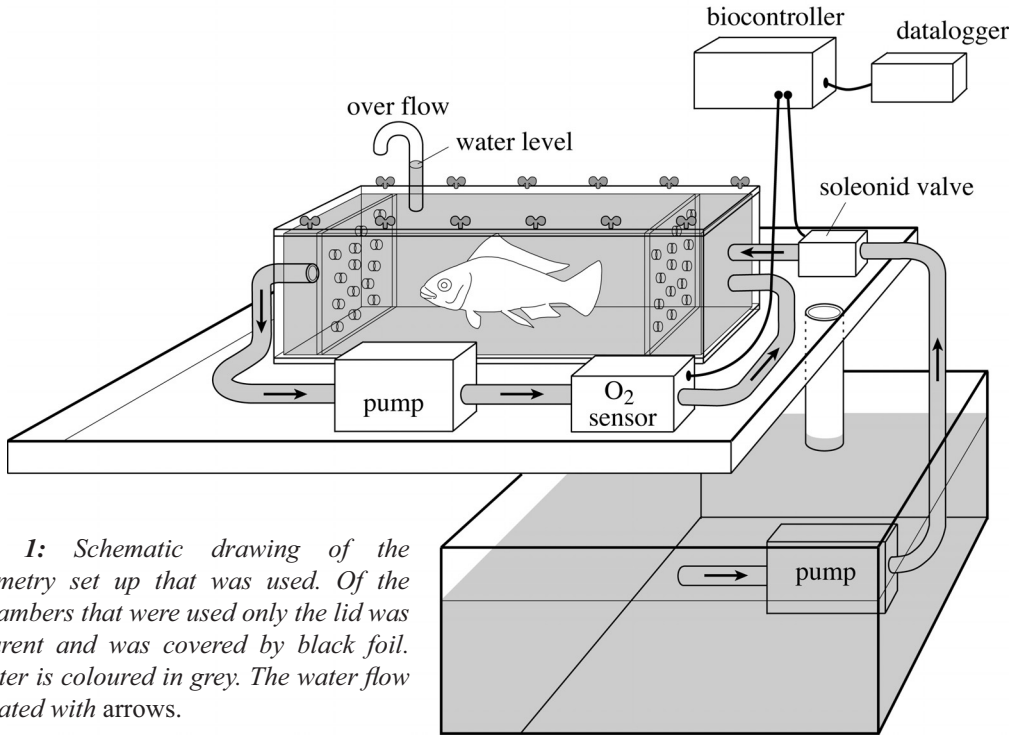

Figure 1: *Schematic drawing of the respirometry set up that was used. Of the flow chambers that were used only the lid was transparent and was covered by black foil. The water is coloured in grey. The water flow is indicated with* arrows.

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.

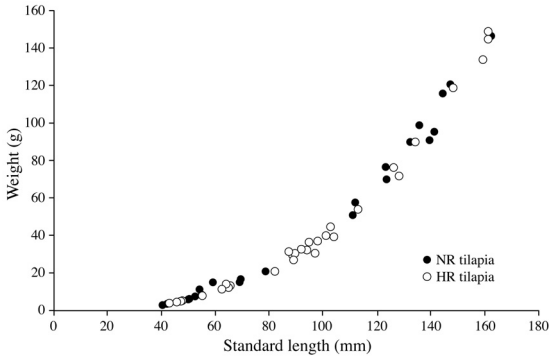

Figure 2: *Standard length against weight of harvested tilapia. Data from NR and HR siblings in neighbouring aquaria were included.*

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

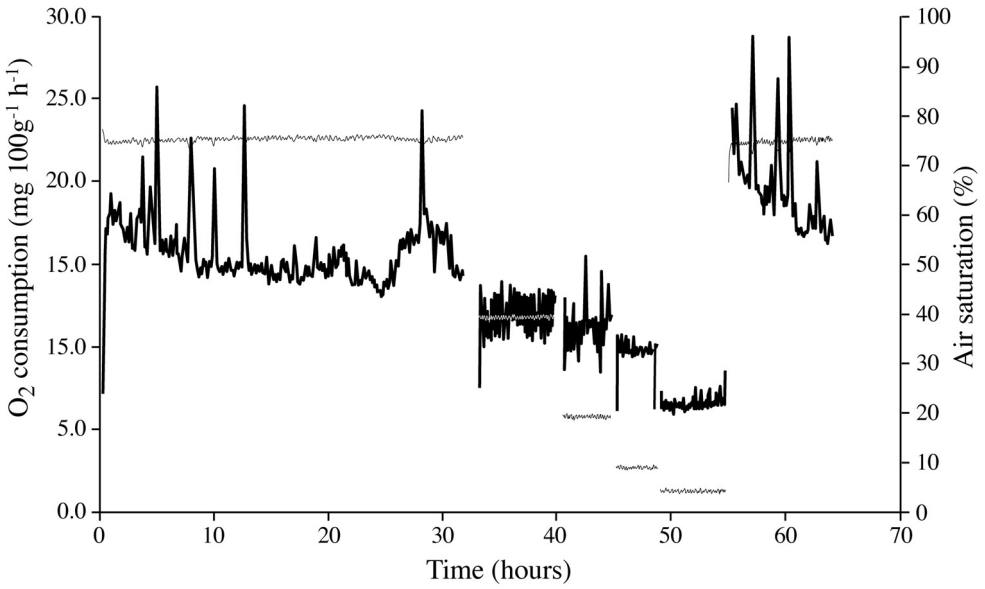

Figure 3: *Oxygen consumption of a tilapia in a typical respirometry experiment. Oxygen (fat line) consumption was measured of individual tilapias at the conditions they were raised at and during a series of stepwise decreasing AS levels (thin line, for protocol see Table 2). The protocol used is given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

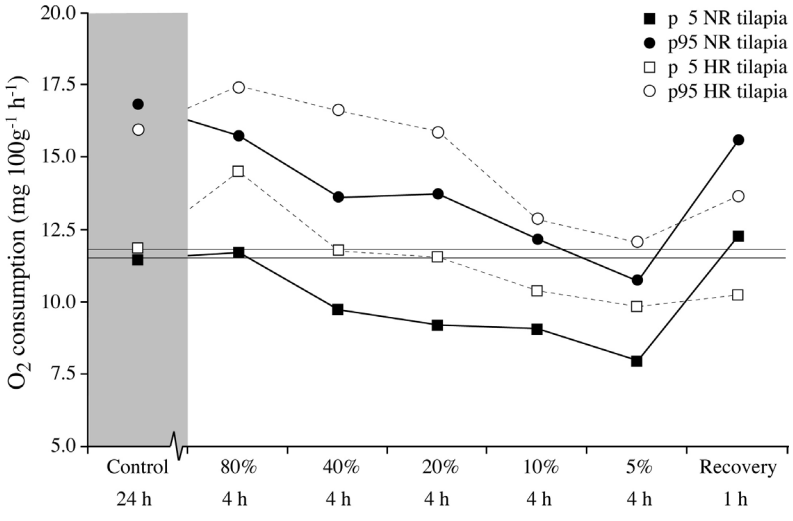

Figure 4:
The averaged O_2 consumption patterns of normoxia and hypoxia-raised tilapia measured at the acclimation level (Control, respectively 80% and 10% air saturation for normoxia and hypoxia-raised fish) and a series of decreasing AS levels. For the protocol and animals used, see table 2. Of the 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

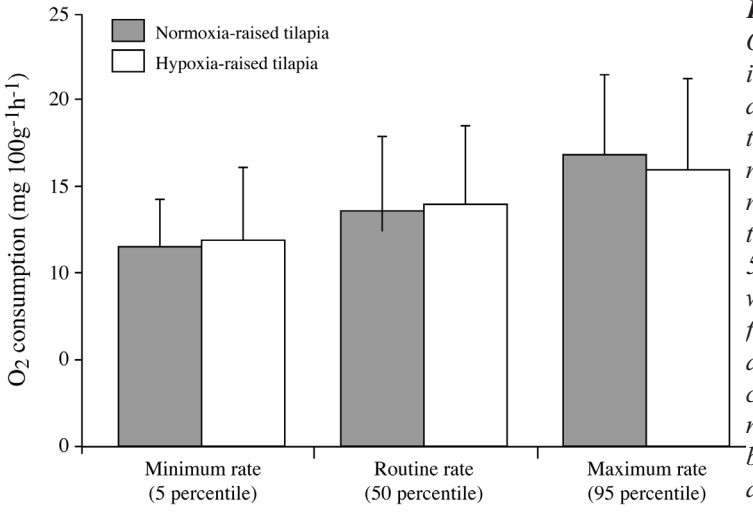

Figure 5:
Oxygen consumption of individual NR and HR tilapia during 24 hours of exposure to the AS level that they were raised under. NR tilapias were raised under 80% AS and HR tilapia under 10% AS. The 5-, 50-, and 95-percentile were calculated from the frequency distribution and averaged. Oxygen consumption 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.