Phenotypic responses to lifelong hypoxia in cichlids

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CHAPTER 4:

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

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Abstract

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

Introduction

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

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

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

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

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

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

Materials and Methods

Raising and sampling of the animals

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

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

Respirometry Experiments

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

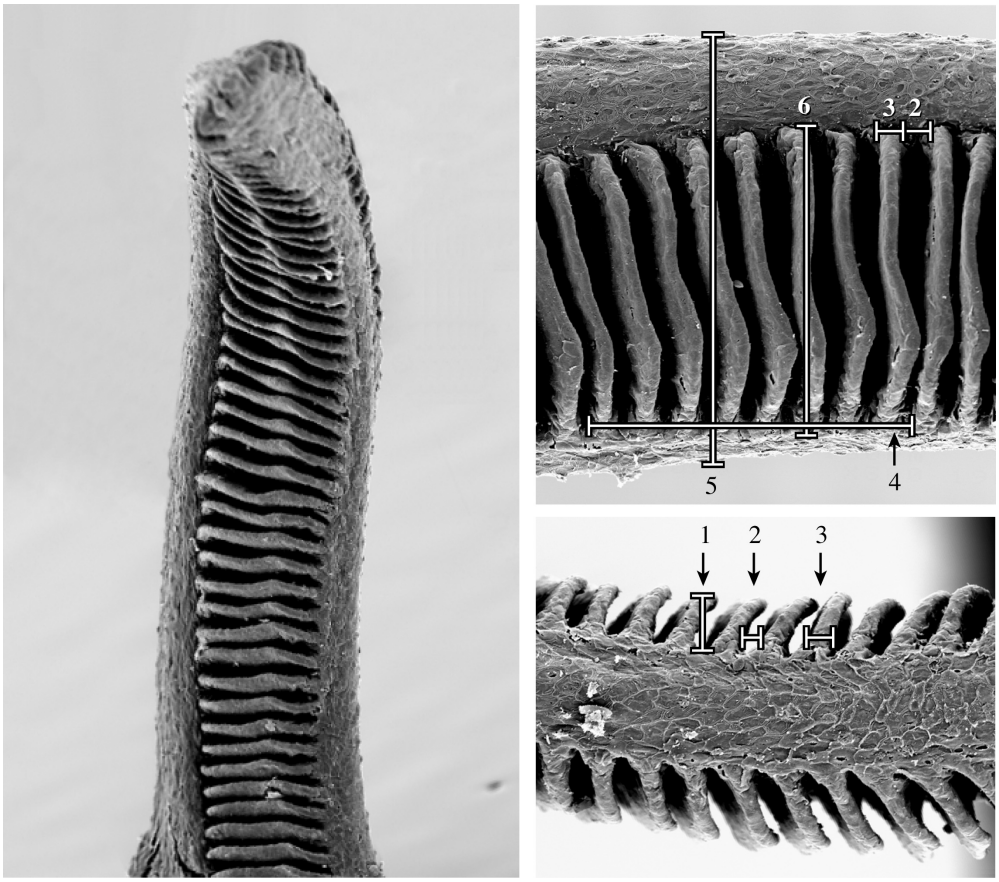

Figure 1: *Measures that were taken with light microscopy and scanning electron microscopy (SEM) of filaments on the lateral hemibranch of the third gill arch of H. pyrrhocephalus. The photograph left above shows the four positions where filaments were selected for measurement of the densities of the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

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

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

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

Measurements

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

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

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

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

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

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

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

Surface area = 2F L 2A

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

Statistics

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

Results

Respirometry

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

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

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

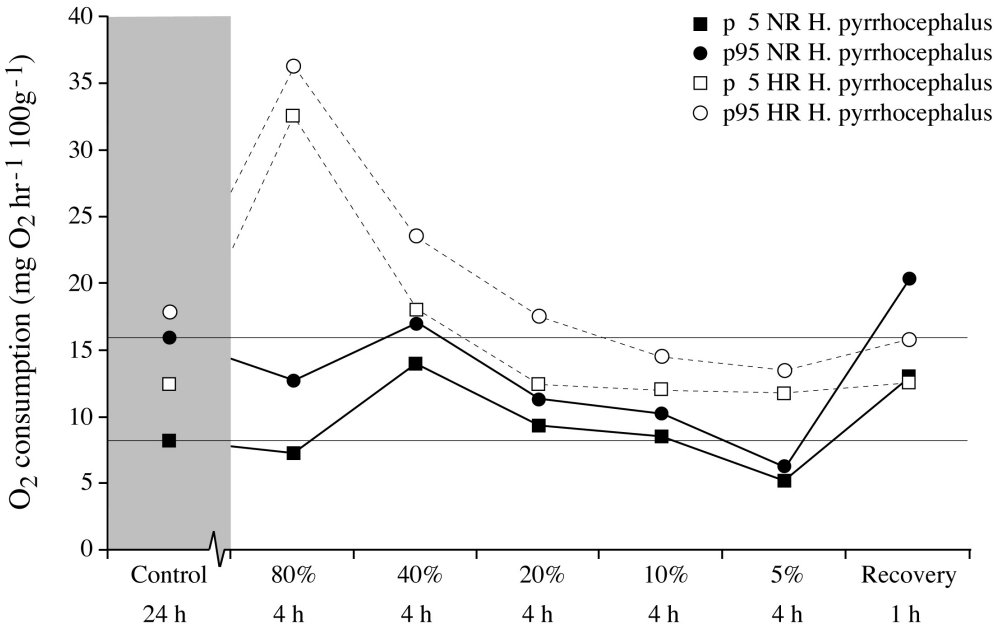

Figure 2: *The averaged O_2 consumption patterns of the normoxia- (n=7) and hypoxia-raised (n=9)* H. pyrrhocephalus *group, measured at the acclimation level (Control, respectively 80% and 10% air saturation for normoxia and hypoxia-raised fish) and a series of decreasing AS levels, followed by recovery.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

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

Filaments

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

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

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

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

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

Secondary lamellae

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

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

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

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

Gill surface

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

Discussion

Experimental design

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

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

Respirometry

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

Effects of changed gill dimensions

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

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

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

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

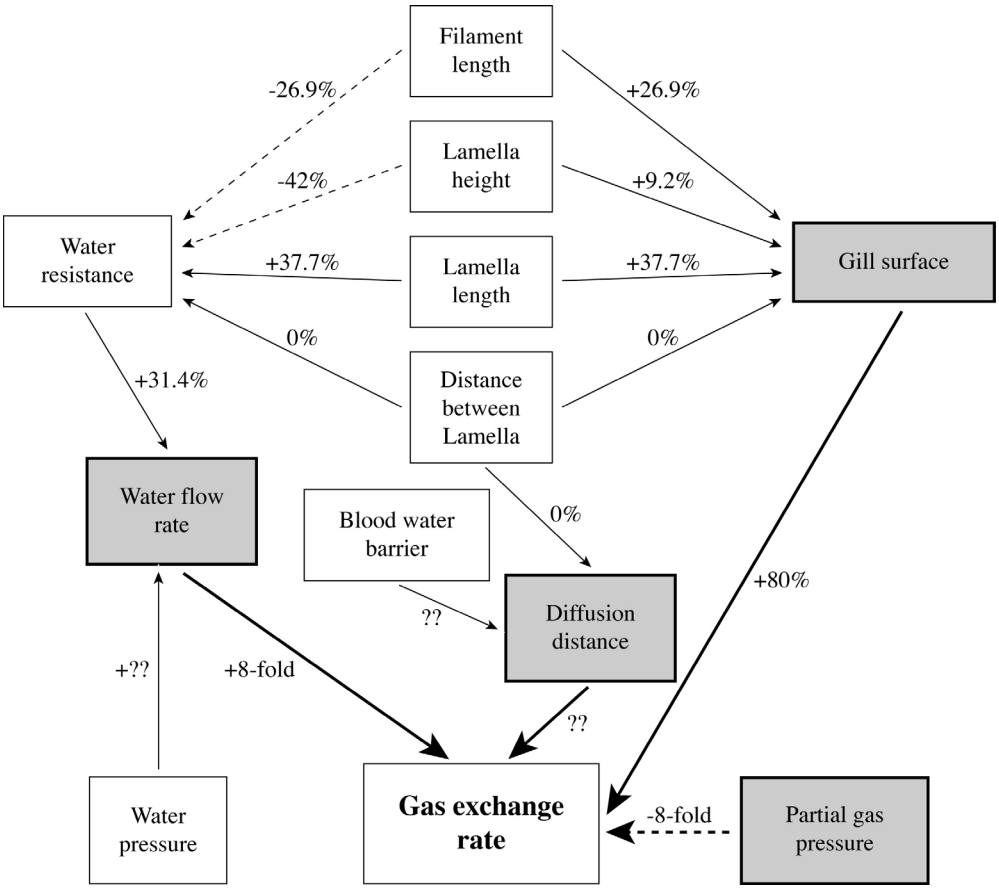

Figure 3: *The most important parameters that affect gas exchange rate in the gills. Gas exchange principally is dependent on sufficient supply of oxygen. As the partial gas pressure at 10% air saturation was eight-fold decreased compared to 80% AS, the same increase in water flow rate is necessary to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

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

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

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

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

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

Gill resistance and water flow

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

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

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

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

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

Oxygen diffusion

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

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

Efficiency and respiratory cost

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

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

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

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

Comparison with other species

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

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

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

Plasticity and its ecological consequences

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

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