Phenotypic responses to lifelong hypoxia in cichlids

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CHAPTER 7:

SYNTHESIS

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INTRODUCTION

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

MAINTENANCE OF METABOLIC RATE

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

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

One explanation is that the duration of hypoxia exposure was different between our experiments and that of other authors. The duration of the experiments in this study (up to two years) was much longer than in comparable studies (up to two months). Possibly, if the duration of hypoxia was extended in those studies, similar increases in the O_2 extraction capacity as in this thesis could be found. Literature reports some increase in the O_2 consumption (Lomholt and Johansen, 1979; Johnston and Bernard, 1982) and normalisation of enzyme activity (Greany *et al.*, 1980) after several weeks of hypoxia.

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

Limitations in metabolism

Several observations indicate that HR cichlids from this study were limited by lifelong exposure to 10% AS. First, during feeding, the NR fish were very eager to eat and food was usually eaten directly whereas HR fish did not eat the food directly and feeding took longer. This indicates that HR fish spent less energy on feeding and competing for food with group mates than NR siblings did (Chapter 2). Second, pilot studies were performed on a split brood of *H. piceatus* in the aquarium where the animals were raised. Of the males in each group, frequencies of aggressive and mating behaviour were scored. The results showed that all behavioural parameters that were measured occurred less often in males of the HR group but the standard deviation was large. Only the parameters ‘butting’ and ‘quivering’ towards females were scored significantly less often in HR than in NR *H. piceatus*. ‘Butting’ was defined as accelerating towards and bumping into the glass wall behind which a stimulus male was present. ‘Quivering’ was defined as a shiver of the body with anal fin erect and dorsal fin folded (Seehausen, 1996). This suggests that the behavioural activity was depressed in HR fish, thus, reducing energy expenditure (Rutjes, Senadheera, Van den Thillart and Witte, unpublished). Third, the activity of the mitochondrial enzyme citrate synthase (CS) in the white muscles was decreased by 25% in HR *A. alluaudi* and *H. ishmaeli*. In tilapia, the difference between NR and HR siblings was smaller and not significant (Chapter 6). The CS activity is a limiting step in the citric acid cycle. Thus, it can be said that the maximum aerobic metabolic rate of the white muscles was decreased in HR *A. alluaudi* and *H. ishmaeli*. In contrast, the stores of glycogen in HR tilapia were increased, suggesting that they have an increased anaerobic capacity. In *A. alluaudi* and *H. ishmaeli* the differences between NR and HR siblings were small and not significant. Differences in muscle glycogen stores were not found by others in tench or carp (Johnston and Bernard, 1982a; Zhou *et al.*, 2000). HR *A. alluaudi* and *H. ishmaeli* were able to tolerate anoxia longer than NR siblings were (Chapter 3). Possibly, this tolerance was based on behavioural differences or increased glycogen stores in the liver (Chapter 6).

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

INCREASED OXYGEN EXTRACTION BY TRANSFORMATIONS IN THE GILLS

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

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

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

Anatomical effects of gill increase

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

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

INCREASED OXYGEN EXTRACTION BY TRANSFORMATIONS IN THE BLOOD

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

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

Conclusions

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

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