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Phenotypic responses to lifelong hypoxia in cichlids

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CHAPTER 5:

A DISCRIMINATING SHAPE FACTOR AMONG AFRICAN CICHLIDS CAN BE INDUCED PHENOTYPICALLY

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ABSTRACT

A massive enlargement of the gill surface proved to be an important factor in the hypoxia survival of young cichlids. As heads of cichlids are densely packed with structures related to both feeding and breathing, we hypothesised that gill enlargement requires such large internal reorganisations that outer head shape is affected. We used a three-dimensional model to describe changes in the outer head shape of cichlids. The model estimates the dimensions of the oral, suspensorial and opercular compartments in the head. Broods of cichlids of different phylogenetic lineages, habitats and trophic specialisation, were split and raised at either 10% or 80-90% air saturation until adulthood. They comprised two endemic Lake Victoria haplochromine species, a non-endemic haplochromine and a tilapia species. In spite of the above-mentioned differences between the species that were used, all hypoxia raised groups showed similar volume enlargements. Volume increases were most prominent in the ventral suspensorial and ventral opercular sub-compartments. A relation with the enlarged gills of hypoxia raised fish is likely, as the gills are mainly located in these compartments. Differences in ventral width are found in other studies comprising a wide variety of genotypic and phenotypic variations. The present study shows that such variation in the ventral width is conceivable by phenotypic plasticity alone. That head shape is to a large extent phenotypically plastic could be an important factor explaining the vast morphological diversity that is found in East African cichlids.

INTRODUCTION

Many fish may encounter abnormally low ambient oxygen concentrations (hypoxia) at some stage during their life. There are several mechanisms to cope with *short* periods of hypoxia, *e.g.* aquatic surface respiration, metabolic depression, and migration to areas with higher O₂ concentrations (Lomholt and Johansen, 1978; Van den Thillart and Van Waarde, 1985; Verheyen *et al.*, 1994; Almeida-Val *et al.*, 1995; Chapman *et al.*, 1995; Muusze *et al.*, 1998). Fish living in habitats where *chronic* hypoxia occurs naturally (*e.g.* swamps, wetlands), often have special adaptations to these conditions such as a large respiratory surface, a low standard metabolism and high haemoglobin concentrations (Galis and Barel, 1980; Chapman and Liem, 1995; Chapman *et al.*, 2000; Chapman *et al.*, 2002).

In the last century, human-induced perturbations caused chronic hypoxia in water bodies world wide, where it was uncommon previously (Chapman and Chapman, 2002; Wu, 2002; Witte *et al.*, 2005). An example is Lake Victoria in East Africa, where, during the past decades, O₂ 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, hypoxia (<3 mg L⁻¹) was present near the bottom for several days only during the long rainy season (van

Oijen *et al.*, 1981). However, in 1987-88, at the same sampling station severe hypoxia (<1 mg L⁻¹) was present for several months during the rainy season. Moreover, hypoxic water layers reached higher into the water column (Wanink *et al.*, 2001).

Lake Victoria cichlids are rather tolerant to short-term hypoxia (Chapman *et al.*, 1995; 2002a, 2002b; Witte *et al.*, 2005; Chapter 2, 3). However, pilot experiments on some Lake Victoria cichlids show that fish raised at normoxia can only temporarily cope with chronic hypoxia (10% AS, ≈ 0.8 mg L⁻¹). In contrast, 3-4 weeks old animals that were exposed to 10% AS, showed 100% survival, grew well and even reproduced. This indicates that phenotypic plasticity plays a role in survival at lifelong hypoxia. One adaptive response found in hypoxia raised fish, is a considerable enlargement of the respiratory surface area by lengthening of the gill filaments and by size increase of the secondary lamellae (Chapman *et al.*, 2000; Chapter .. this thesis). Similar adaptive changes were found in fish from natural environments, where hypoxia had increased (Wanink and Witte, 2000; Chapman *et al.*, 2000; Witte *et al.*, 2005).

Heads of fish are densely packed with muscles, bones and other structures, that are necessary for respiration, vision, feeding and other functions. As a consequence of limited space, increase of gill size may cause spatial conflicts

with surrounding structures. Such spatial conflicts have been demonstrated in the cichlid *Astatoreochromis alluaudi* (Smits *et al.*, 1996a, b). Animals with a larger pharyngeal jaw apparatus showed a decrease in size of surrounding structures and/or reallocations in the space of the head. Apart from internal reorganisations (Witte *et al.*, 1990; Barel, 1993; Chapman *et al.*, 2000), changing the outer head shape can also create space. It has been suggested that in fish living at low oxygen concentrations, possible enlargement of the gills could have such dramatic effects that the surrounding structures, and even gross morphology of the head, are affected (Smits *et al.*, 1996 b; De Visser and Barel, 2000). Indeed, Chapman *et al.* (2000) found a phenotypically induced increase in head length in the cichlid *Pseudocrenilabrus multicolor victoriae* that was raised at hypoxia. However, it was not determined whether the increase was due to an accelerated growth of the area where the gills are located. Studies done on populations of rock-dwelling cichlids that live at different O₂ concentrations, showed a correlation between volume of the opercular compartment of the head, and environmental oxygen concentrations (Bouton *et al.*, 2002). In this case however, the size of the gills was not studied. Nor was it clear to what extent enlargement of the head was a phenotypically plastic trait.

In the present paper we investigated the outer head shape of a tilapia hybrid,

and three haplochromine species from Lake Victoria. In each of these species, the gill filaments were enlarged due to lifelong hypoxia (Chapter 4 this thesis). We used a three-dimensional model, that has proven to be very sensitive for differences in head shape (De Visser and Barel, 2000) in combination with ANCOVA to test the effects of size, species and hypoxia. The species differ in phylogeny, range of oxygen concentrations in their natural habitat and in morphological parameters relevant to spatial allocation of the gill apparatus, *e.g.* pharyngeal jaw morphology and body depth. Both the pharyngeal jaw size and body depth is supposed to have an impact on the available space for gill increase. We hypothesised that in hypoxia raised fish, as a general phenotypic response, the volume of the head is increased, particularly in the area where the gills are located. In addition we investigated whether transformations in the head are specific for fish with respect to the above mentioned differences.

MATERIALS AND METHODS

Species used

In this study we used three haplochromine species: *Astatoreochromis alluaudi*, *Haplochromis (Labrochromis) ishmaeli* and *Haplochromis (Yssichromis) pyrrhocephalus* and a hybrid between *Oreochromis mossambicus* and *Oreochromis niloticus*. In the following

Table 1: Summary of species, the habitats, and the morphological parameters relevant for this study. The upper half of each column shows the species specific parameters that possibly influenced the phenotypic responses to hypoxia. Pharyngeal jaw sizes are indicated from -- (very light and slender) to ++ (heavy and robustly developed) following Hoogerhoud (1986). Of each group that was used, the number of fish, average weights (grams) and average standard length (mm) are given. Abbreviations: SL = Standard Length, BD/SL = Body Depth/Standard length, AS = Air Saturation, LV = Lake Victoria, Nor = Normoxia, Hyp = hypoxia. *, from Van Oijen et al. (1981), #, from Welcomme (1964).

	<i>A. alluaudi</i> '92		<i>A. alluaudi</i> '99		<i>H. ishmaeli</i>		<i>H. pyrrhocephalus</i>		Tilapia	
Species specifics:										
O ₂ range in the wild	Hypoxic or normoxic		Hypoxic or normoxic		Stable normoxic		2.5-7.5 mg L ⁻¹ *		2-5 mg L ⁻¹ #	
Pharyngeal jaws	+		+		+		0		0	
BD/ SL	34-43%		34-43%		37-45.5%		29-34%		36-49.5%	
Phylogeny	Haplochromine Separate lineage		Haplochromine Separate lineage		Haplochromis LV flock		Haplochromis LV flock		Tilapiine	
Experimental fish:										
Start experiment	1992	1992	1999	1999	1999	1999	1999	1999	1999	1999
AS level	80%	10%	80%	10%	80%	10%	80%	10%	80%	10%
No. of animals	9	6	7	7	6	6	9	6	10	10
Av. Weight (g)	10.5	6.6	22.0	17.5	24.0	23.0	5.6	4.1	27.0	22.3
Av. SL (mm)	82.0	76.6	57.3	67.5	89.4	87.7	67.6	59.6	87.6	83.9

paragraphs we briefly describe the species, habitats and morphological parameters relevant for this study (Table 1).

A. alluaudi is a pharyngeal mollusc crusher that is not endemic to Lake Victoria. It also occurs in Lakes Nabugabo, Edward-George and many small lakes around Lake Victoria (Greenwood, 1959, 1965, 1973; Hoogerhoud, 1986). According to both morphological and molecular studies, *A. alluaudi* is phylogenetically separated from all other Lake Victoria haplochromines (Greenwood, 1959; Meyer *et al.*, 1990; Nagl *et al.*, 2000; Seehausen *et al.*, 2003). The habitat of

A. alluaudi includes well-oxygenated streams as well as hypoxic swamps and a variety of bottom types (Greenwood, 1974; Witte, 1981). In Lake Victoria, it is rarely found deeper than 20m. The body depth (BD) relative to the standard length (SL) is relatively large (BD/SL = 34-43%) and the maximum SL is 160 mm (Greenwood, 1959). In Lake Victoria molluscs dominate the diet (Greenwood, 1959; Hoogerhoud, 1986), whereas in most of the other lakes the main part of the diet consists of insects. The pharyngeal jaws are hypertrophied in waters where snails are an important part of the diet (Greenwood, 1965; Hoogerhoud, 1986; Smits *et al.*, 1996a).

H. ishmaeli is endemic to Lake Victoria. Though Lake Victoria haplochromines used to be considered as a monophyletic group (Meyer *et al.*, 1990), recent research suggest that there are several lineages in the lake. Regardless of taxonomic debates, the consensus view is that, in contrast to *A. alluaudi*, *H. ishmaeli* can be considered as a member of the Lake Victoria super-flock (e.g. an assembly of species flocks, see also Nagl *et al.*, 2000; Seehausen *et al.*, 2003; Verheyen *et al.*, 2003). The animals used in the present study were offspring of fish collected in the southern part of the lake, where their distribution was virtually restricted to well oxygenated water of less than 6 meters deep with sand bottoms (Witte, 1981). *H. ishmaeli* is relatively deep-bodied (37.0-45.5% of SL) and reaches a maximum SL of 136 mm (Greenwood, 1960). In spite of the phylogenetic differences between *A. alluaudi* and *H. ishmaeli*, in Lake Victoria both species have a similar pharyngeal jaw apparatus, i.e. hypertrophied and adapted to snail crushing (Greenwood, 1956, 1960, 1974; Hoogerhoud, 1984; Smits *et al.*, 1997).

H. pyrrhocephalus is endemic to Lake Victoria and like *H. ishmaeli*, a member of the Lake Victoria super-flock (Seehausen *et al.*, 2003). This zooplanktivore is more slender bodied than *H. ishmaeli* (BD/SL = 29-34%) and reaches a maximum SL of about 73 mm (Witte and Witte-Maas, 1981). *H. pyrrhocephalus* is found over muddy

bottoms between 3 and 21 m deep, where hypoxia (< 3 ppm) occasionally occurred during the rainy season near the bottom in the 1980s, but is now present for longer periods of time and further from the bottom (van Oijen *et al.*, 1981, Wanink *et al.*, 2001). The species is partly pelagic and feeds on zooplankton near the bottom during daytime and on *Chaoborus* larvae near the surface at night (Witte and Witte-Maas 1981; Goldschmidt *et al.*, 1990). The pharyngeal jaws are slender.

As a representative of the tilapiine lineage, we used a hybrid between *Oreochromis mossambicus* and *Oreochromis niloticus* (further referred to as tilapia) that is used in aquaculture. Both parental species are not native to Lake Victoria, though *O. niloticus* has been introduced into the lake. In Lake Victoria it is found at O₂ concentrations between 2 and 5 mg L⁻¹ (Welcomme, 1967). *O. niloticus* feeds mainly on phytoplankton when available but it is able to handle a wide range of food types. The diet of *O. mossambicus* consists mainly of detritus, but it also feeds on phyto- and zooplankton, if present (Trewavas, 1983). *O. mossambicus* and *O. niloticus* are relatively deep bodied (BD/SL = 36-49.5%), fast growing species. Maximum weights of more than 2800 g have been reported (Trewavas, 1983).

Raising and sampling of the animals

Experiments were performed in 1992-94 with *A. alluaudi* and in 1999-2002 with all four species. *A. alluaudi* and *H. ishmaeli* were caught in the Mwanza Gulf in 1984 and have been bred in our laboratory since. Also a breeding stock of *H. pyrrhocephalus* was present that originated from the offspring of fish caught by J.H. Wanink in 1987. Of the breeding stocks we obtained broods for our experiments. The tilapia broods that were used were F1 offspring of animals obtained from the University of Nijmegen.

Nests were selected when animals were about 1.5 cm SL (about four weeks after fertilisation). Each nest was split randomly and raised at normoxia (NR) and hypoxia (HR, Table 1). In 1999, all fish were raised in 100-litre aquaria in the same climate room. There were no indications that occasional deaths were related to hypoxia. Before adulthood, survival approximated 100% in both NR and HR groups. Death rate in general was higher in the NR groups where dominant males killed more fish. The water in the aquaria of the NR groups was kept at 80-90% air saturation (AS). The AS-level of the water of the HR groups was lowered stepwise to 10% AS in four weeks. Circulating water was made hypoxic in a vacuum equilibration column in which dissolved air in the water equilibrated at 6-9 kPa. The flow rate through the tanks was about 1-2 L/min. The 100-L aquaria were covered with a metal

plate a few centimetres below the water surface to restrict O₂ 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 day-night cycle 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.

After 17-37 months the animals were killed with an overdose of anesthetic (MS222, Finquel) and stored in 3.6% formaldehyde (buffered with Borax) at room temperature. To standardize the morphometric measurements the gill covers and the mouth were closed. After three to 12 months the animals were transferred to 70% ethanol for further storage.

In 1992, the same experiment was conducted on a group of two mixed nests of *A. alluaudi* (Table 1, referred to as *A. alluaudi* '92, the *A. alluaudi* from 1999 are referred to as *A. alluaudi* '99). The methods used were the same, but feeding conditions and aquaria were different and the experiment was done in a different laboratory. The content of the aquaria was about 125 liters. They were fed live chironomid larvae and flake food. Animals were sampled after 15 and 19 months.

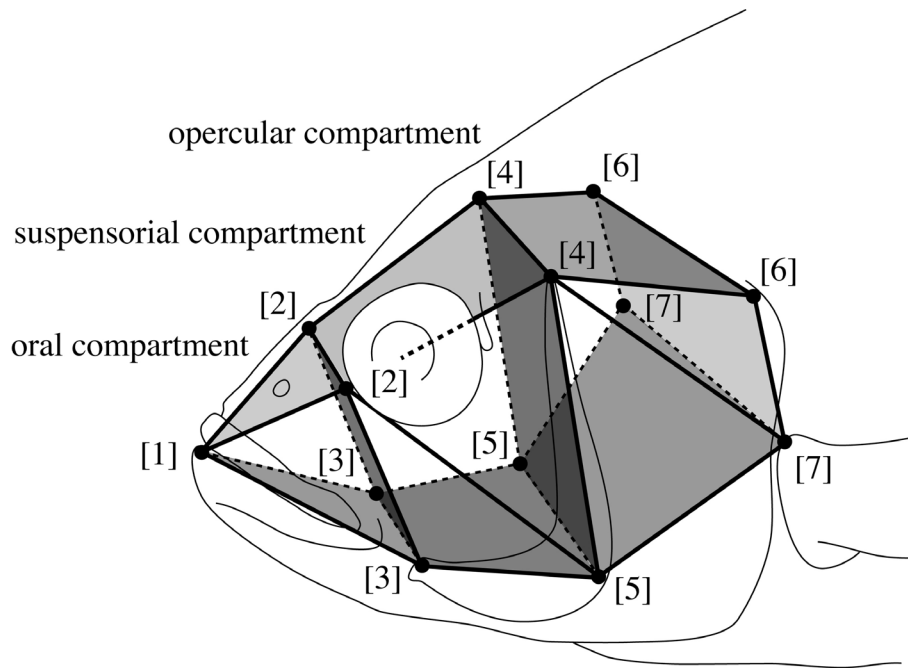


Figure 1: Schematic drawing of the head of a cichlid and the external framework. The numbers [1]-[7], of which [2]-[7] are found on both sides of the head, indicate the 13 landmarks. The bars that connect the landmarks are indicated as a combination of landmark numbers (e.g. [1-2]), as are the volumes. The bar lengths were averaged between both sides of the fish. These averages were also used for calculating the compartment volumes.

The landmarks and the 28 bars determine the compartments and sub-compartments in which the head was divided. The combination [1-2-3] corners the oral compartment and [2-3-4-5] the suspensorial compartment, in which [2-3-5] forms the ventral suspensorial and [2-4-5] the dorsal suspensorial compartment. The combination [4-5-6-7] corners the opercular compartment. In which [4-5-7] forms the ventral opercular and [4-6-7] forms the dorsal opercular compartment.

Measuring the external framework

The freshly killed animals were dried with paper tissues and weighed. The SL was measured following Barel *et al.* (1977). All other measures were taken after preservation. Individuals of both sexes were selected randomly in each group. To capture the outer head

shape of the animals, the method of De Visser and Barel (2000) for measuring an external framework of cichlid heads was used. This is a 3-dimensional model of 13 landmarks (indicated as numbers) that are connected by 28 bars (indicated as combinations of landmark numbers, Figure 1). The landmarks on both

sides of the head divide the head into an oral [1-2-3], suspensorial [2-3-4-5] and opercular [4-5-6-7] compartment. The suspensorial and opercular compartments are subdivided in a ventral (respectively [2-3-5] and [4-5-7]) and dorsal sub-compartment (respectively [2-4-5] and [4-6-7]). The volumes of all (sub-) compartments were calculated by dividing them into wedges. To measure more precisely, the definitions for two landmarks were modified from those in De Visser and Barel (2000). De Visser and Barel defined point [5] (NPC) as the point halfway SLF₁ (Suspensorial Lateral line Foramen 1) and the neurocranial lateral line crest at NLF₅ (Neurocranial Lateral line Foramen 5). Point [7] (FDO) was measured as: the rostral-dorsal origin of the muscular basis of the pectoral fin at the caudal rim of the gill-cover. We defined point [5] as the point directly under the NLF₅ and point [7] as the most rostral-dorsal cartilaginous part of the pectoral fin base. The latter was visible after removal of the skin. Using these new definitions, the measurements were more accurate, and consequently, resulted in smaller standard deviations. Comparison of the differences in measurements, using the location of the points [5] and [7] used by De Visser and Barel and in this manuscript, resulted in differences less than 3%. In addition to the measurements on the external framework, the HL was measured following Barel *et al.* (1977). The modified definitions were used in

the *A. alluaudi* '99 and *H. ishmaeli*.

Before measuring, the circum orbitals and nasal elements (Barel *et al.*, 1976) were removed. Where measuring points were covered by skin, this was removed. After cleaning all the landmarks, they were marked with a black alcohol-proof fineliner (Steadler). The bar lengths were measured three to six times using digital callipers (Sylvac) with needlepoints glued on the ends. Standard deviations were usually between 1% and 2%. If the standard deviation was larger than 5%, more measurements were taken until the standard deviation was below 5%. Both sides of each fish were measured. For calculating the volumes of the compartments the average of the measurements of each bar on both sides was used. Measurements were done without knowing whether animals had been raised at hypoxia or normoxia.

All data were log-transformed and analysed with the software program SPSS V10.0 for Windows. The volumes of all (sub) compartments were analysed with ANCOVA (analysis of covariance). The factors "Species" (4 species) "Environment" (normoxia vs. hypoxia) and "Experiment" (*A. alluaudi* '92 vs. all four species from 1999, including *A. alluaudi* '99) were investigated. Additional testing for differences between the *A. alluaudi* '92 and *A. alluaudi* '99 separately revealed the same similarities and differences between the two groups. The weight of the animals was used as covariable in the

Table 2: Morphometric data on bar lengths (See also Figure 1 for notation). Data were collected from 5 different broods (4 species) and raised at normoxic and hypoxic conditions. The mean bar lengths (in mm) per experimental group are estimates based on ANCOVA modelling. Residual variation was normally distributed and hence estimates are reliable approximations of the data. Values are interpolations to a mean standard length (SL) per brood. Since the average SL differs between broods, effects of hypoxia can only be compared within each brood. Bar lengths that differed significantly between normoxia raised and hypoxia raised siblings are given in bold. Where bar lengths did not differ significantly between normoxia raised (NR) and hypoxia raised (HR) fish, identical estimates were given. In Table 3 the percent changes of bar lengths are given. Abbreviations: AS = Air Saturation, HL = Head Length.

Species SL (mm)	<i>A. alluaudi</i> '92 79.1		<i>A. alluaudi</i> '99 62.7		<i>H. ishmaeli</i> 88.6		<i>H. pyrrhocephalus</i> 62.8		Tilapia 85.8	
AS level	80%	10%	80%	10%	80%	10%	80%	10%	80%	10%
HL	21.5	20.7	28.8	27.8	31.0	31.9	19.3	19.6	29.4	29.7
[1-2]	6.6	7.9	6.3	6.2	9.8	9.7	5.8	5.7	9.8	9.7
[1-3]	10.9	10.2	8.8	8.2	12.4	12.7	7.4	8.1	10.0	10.1
[2-3]	5.8		7.4		10.7		7.0		8.4	
[3-5]	10.4	9.4	8.6	7.8	11.4	10.0	7.8	8.0	10.3	10.2
[2-4]	11.1		10.7		14.9		9.5		13.2	
[5-4]	12.9		10.8		16.3		9.2		15.4	
[2-5]	13.9		12.2		16.5		11.2		14.2	
[5-7]	8.6	9.3	6.7	7.3	11.8	12.8	7.0	7.5	10.8	11.7
[4-6]	9.7	8.7	8.0	7.3	12.0	12.5	6.4	6.1	11.3	
[7-6]	7.1		5.1		16.1	16.2	12.0	14.4	25.5	27.9
[4-7]	12.3	12.8	9.8	10.2	14.5	15.2	7.9	8.3	14.3	14.9
[5 _r -5 _r]	8.6	10.0	6.9	7.9	9.7	11.1	6.1	7.0	10.8	12.5
[4 _r -4 _r]	11.3	11.6	9.1	9.3	13.4	13.8	8.8	9.0	13.1	13.4
[6 _r -6 _r]	12.8		11.1		14.2		8.7		14.8	
[7 _r -7 _r]	11.5		9.0		12.8		7.8		12.8	
[2 _r -2 _r]	7.9		8.5		9.4		5.8		10.1	

Table 3: Percent changes of the bar lengths of hypoxia raised fish compared to normoxia raised siblings for each brood. Only bar lengths that differed significantly between NR and HR siblings (see also Table 2) are given.

Differences are estimates based on ANCOVA models. Estimates are based only on parameters that contributed significantly to the variation in bar length. Where change in bars due to hypoxia was not significantly different between species, an average was given. Abbreviations: SL = Standard Length, HL = Head Length.

Species SL (mm)	<i>A. alluaudi</i> '92 79.1	<i>A. alluaudi</i> '99 62.7	<i>H. ishmaeli</i> 88.6	<i>H. pyrrhocephalus</i> 62.8	Tilapia 85.8
HL	-3.5	-3.5	3.1	1.6	1.0
[1-2]	19.6	-0.9	-0.9	-0.9	-0.9
[1-3]	-6.4	-6.4	1.9	3.5	0.9
[3-5]	-9.0	-9.0	-11.8	2.6	-0.7
[5-7]	8.4	8.4	8.4	8.4	8.4
[4-6]	-9.6	-9.6	4.6	-5.0	-0.6
[7-6]	-0.5	-0.5	0.8	20.7	9.5
[4-7]	4.4	4.4	4.4	4.4	4.4
[5 _L -5 _R]	15.2	15.2	15.2	15.2	15.2
[4 _L -4 _R]	2.6	2.6	2.6	2.6	2.6
[3 _L -3 _R]	0.2	0.2	0.4	30.0	3.7

volume measurements, while the SL was used as covariable in the analyses of the bar lengths.

RESULTS

Head length and bar lengths

The head length and all the bar lengths were analysed with ANCOVA. The head length, which was dependent on both standard length and species, differed significantly between all NR and HR groups (Table 2). Compared to their NR siblings, the head length was 3.5% smaller in HR *A. alluaudi*. In the other three species, the head length of the NR

animals was 1.0-3.1% larger than that of HR siblings. Seventeen bar lengths were measured of 10 groups. Ten bars were significantly different between NR and HR groups of all broods (Table 2). In bars [1-3], [3-5], [5-7], [4-6], [7-6] and [3_L-3_R] (_L and _R stand for left- and right side of the head), both hypoxia as well as species effects were found. The bars that represent the ventral part of the head ([3-5], [5-7], [5_L-5_R], and [3_L-3_R]) changed most in size. From the rostral to the caudal part of the framework the differences in bar lengths were as follows:

Table 4: Volumes (mm³) of different head compartments of normoxia raised (NR) and hypoxia raised (HR) cichlids. With ANCOVA the effect was investigated of species, environment, experiment and size on (sub) compartment volumes. Residual variation was normally distributed and hence estimates are reliable approximations of the data. Estimates were interpolations to a mean weight per brood and based only on parameters that significantly contributed to variation. Where change in volume due to hypoxia, was not significantly different between any of the species, estimates are given as being the same for all species. Where the percentage differences between NR and HR groups are 0%, none of the investigated parameters had a significant effect on the volume of (sub) compartments.

Species	<i>A. alluaudi</i> '92		<i>A. alluaudi</i> '99		<i>H. ishmaeli</i>		<i>H. pyrrhocephalus</i>		tilapia	
Weight (g)	8.97		19.43		23.54		4.72		24.66	
AS level	80%	10%	80%	10%	80%	10%	80%	10%	80%	10%
Total volume	1173	1283	2413	2639	3173	3471	787	861	3189	3488
% change	+9.4%		+9.4%		+9.4%		+9.4%		+9.4%	
Oral	74	79	204	218	246	263	55	59	209	224
% change	+7.0%		+7.0%		+7.0%		+7.0%		+7.0%	
Ventral susensorial	158	174	426	470	364	402	113	124	345	381
% change	+10.3%		+10.3%		+10.3%		+10.3%		+10.3%	
Dorsal suspensorial	392		869		1008		293		1044	
% change	0%		0%		0%		0%		0%	
Ventral opercular	323	377	615	717	991	1156	203	237	932	1088

1. In the oral compartment [1-2-3], the *A. alluaudi* '92 showed significantly different reactions to hypoxia in bar [1-2] (Tables 2, 3). This bar was 19.6% longer in HR than in the NR *A. alluaudi* '92. In all the other HR groups, including the *A. alluaudi* '99, it was 0.9% smaller than in the NR groups. Bar [1-3] was 6.4% smaller in HR than in NR animals for both *A. alluaudi* groups. This bar was slightly larger in HR than in NR groups of the other three species.
2. In the ventral suspensorial sub-

compartment [2-3-5] the size of bar [3_L-3_R] was dependent on both species and AS level. In HR *H. pyrrhocephalus* it had increased by 30% compared to NR animals, while hardly any change was found in the other groups. The size of bar [3-5] was dependent on the species and AS level. Bar [3-5] was 9.0% shorter in both HR *A. alluaudi* groups, and 11.8% in HR *H. ishmaeli*, while in *H. pyrrhocephalus* and tilapia little change was found (Tables 2, 3). Bar [5_L-5_R] was 15.2% longer in

all HR groups.

3. The enlarged $[5_L-5_R]$ is also part of the dorsal suspensorial sub-compartment $[2-4-5]$ (Figure 1). In this sub-compartment, bar $[4_L-4_R]$ was slightly enlarged in all HR groups (2.6%).
4. Both the bars $[4_L-4_R]$ and $[5_L-5_R]$ are also a part of the ventral opercular sub-compartment $[4-5-7]$. Apart from these two, bar $[5-7]$ bar in this sub-compartment was 8.4% longer in all HR groups. Bar $[4-7]$ was slightly enlarged as well (4.4%).
5. Although, the total volume of the dorsal opercular sub-compartment $[4-6-7]$ did not change, four out of 6 measured bars were significantly different between NR and HR animals. As already mentioned, bars $[4_L-4_R]$ and $[4-7]$ were slightly enlarged in all HR groups. In bar $[4-6]$, reactions to hypoxia were different for all species. Bar $[4-6]$ was 4.6% larger in the HR *H. ishmaeli* group but 0.6 to 9.6% smaller in the HR groups of the other species. Bar $[7-6]$ was strongly enlarged in HR *H. pyrrhocephalus* (20.7%) and tilapia (9.5%), while only small differences were found between NR and HR *A. alluaudi* (-0.5% in both *A. alluaudi* '92 and *A. alluaudi* '99) and *H. ishmaeli* (0.8%).

Compartment volumes

The head volumes of all HR groups were 9.4% larger than of their NR siblings (Table 4). Relative to body weight, the head volumes (sum of volumes of all compartments) of the tilapias were much smaller than those of the haplochromines (Figure 2).

ANCOVA on the calculated volumes showed that the covariable 'Weight' and the factors 'Species', 'Environment', and 'Experiment' all explain a part of the variation found. The volumes of all (sub-) compartments were dependent on the weight of the animals ($P < 0.05$). Test groups that were raised at hypoxia had larger total (Figure 2), oral, ventral suspensorial and ventral opercular (sub-) compartments, but no differently sized dorsal suspensorial and dorsal opercular sub-compartments compared to their NR siblings (Table 4). Differences between NR and HR groups were largest in the ventral opercular (16.6%) and ventral suspensorial (10.3%) compartments.

The proportional change of all the compartments under hypoxia within each nest was the same (Table 4). Analysis of the factor "Experiment" (*A. alluaudi* '92 vs. all four species from 1999, including *A. alluaudi* '99) showed that the *A. alluaudi* '92 had relatively smaller oral ($p=0.038$) and suspensorial (sub-) compartments ($p=0.000$), but no significantly different ventral and dorsal opercular sub-compartment. The differences in oral and suspensorial (sub-) compartments between the 1992

and 1999 experiments and between the species cannot be seen in Table 4, since the mean weight was calculated for the NR and HR fish of each brood, and compartment volumes were scaled accordingly. This was done because potential allometric differences between species would make extrapolation of the data unreliable.

DISCUSSION

Replications

Although the experimental set-ups were identical, small differences between tanks were unavoidable. These

include variations in social structure within groups, small differences in light conditions, water flow, etc. For such reasons, the use of replications is important to rule out the influence of uncontrollable factors. The only species for which two experiments were performed was *A. alluaudi*. However, these experiments were done seven years apart with a different set-up and feeding regime. As these experiments are rather time consuming no further replicas could be performed at the species level. Consequently, as individuals of each treatment group per species were raised in the same aquarium (pseudo

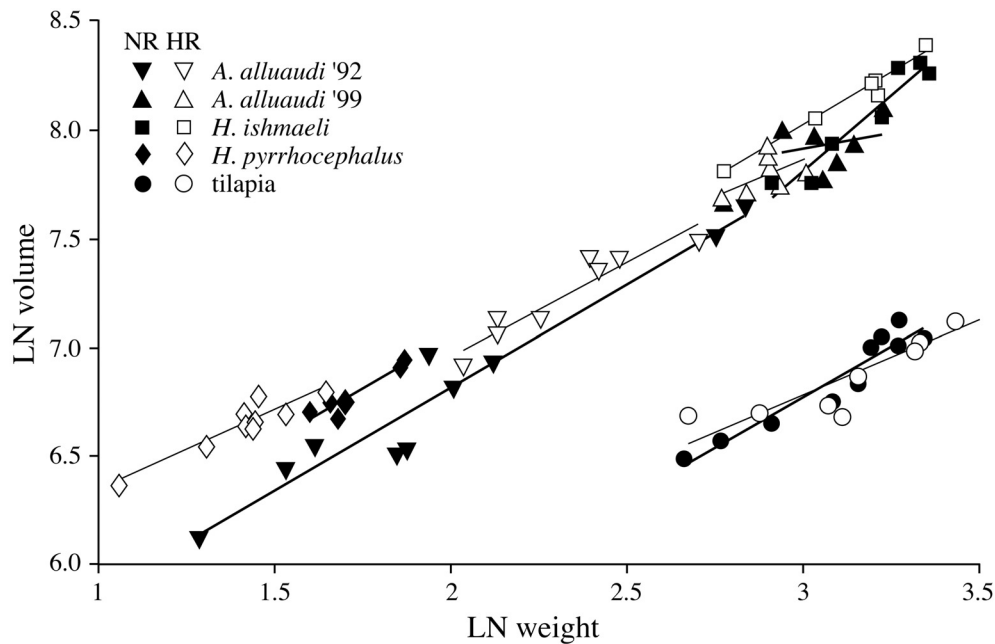


Figure 2: Total head volumes, as calculated from the external framework. For estimates on head volumes see Table 4.

replication), no firm conclusions can be drawn on different responses *among species*. Nevertheless, if we use the data to detect hypoxia responses of *cichlids in general*, the 1999 experiments can be regarded as replications, since the same treatment was given to the four species of cichlids. Similar differences found between the NR and HR groups would then, most likely, be responses to the different AS-levels and not to differences in other factors. In addition, we formulated a prediction about the direction of the differences between NR and HR siblings; namely, an enlarged head volume in HR fishes, specifically in that area where the gills are located. It is unlikely that any random difference between the NR and HR groups of the 5 broods would cause morphological differences exactly according to the prediction.

Bar lengths

Calculation of the head volumes from the bar lengths showed larger volumes in the HR siblings of all 4 species. However, this was only partially achieved in the same manner. Of the ten bars that were different between NR and HR animals, four bars showed the same relative increase in all species: $[5_L-5_R]$, $[4_L-4_R]$, $[5-7]$, and $[4-7]$ (Tables 2, 3). This suggests that to some degree, the underlying mechanism mechanism was the same. By comparing the species according to morphological features, trophic specialisation and habitat we

tried to find an explanation for the differences in hypoxia response. When comparing species with hypertrophied (*A. alluaudi* and *H. ishmaeli*) and non-hypertrophied (*H. pyrrhocephalus* and tilapia) pharyngeal jaw apparatuses, two notable differences in reaction to hypoxia were found. Both *A. alluaudi* and *H. ishmaeli* (mollusc crushers) showed a considerable decrease in the length of bar [3-5], while hardly any changes were found in *H. pyrrhocephalus* and tilapia. In contrast, bar [6-7] was hardly different in *A. alluaudi* and *H. ishmaeli*, while in *H. pyrrhocephalus* and tilapia it increased in size. Possibly the functional demands, posed on the enlarged pharyngeal jaw apparatus of the two mollusc crushers, constrains changes in form in the surrounding area (bar [6-7]).

There was no correlation between body depth or oxygen level in the natural habitat of the species (Table 1), and any of the bar lengths.

Volumes

The species used in this study are different with respect to phylogeny, ecology and morphology (see Materials and Methods). Yet, the differences in volumes between NR and HR animals are similar for all 4 species and concentrated mainly in the ventral suspensorial and ventral opercular sub-compartments, strongly suggesting an adaptive response of a general nature. Those regions harbour the major part of the gills, which have increased in

size in the HR groups (Chapter 4). This suggests a causal relationship between enlargement of the gills and volume increase of the compartments. Changes in other regions of the head were smaller or absent. A similar correlation between O_2 concentration and the size of the oral, suspensorial and opercular compartments was recently found in wild haplochromines (Bouton *et al.*, 2002). Respirometry experiments showed that HR fish at 10% AS consume the same amounts of oxygen as NR siblings at 80% AS (Chapters 2, 3 and 4). Since the partial O_2 pressure is eight-fold lower at 10% AS than at 80% AS, HR fish had to ventilate 8 times more water per unit of time to provide the gills with enough O_2 for extraction. In all HR animals, a dramatic increase of both amplitude and frequency of ventilation was observed (unpublished results). Possibly the increase in compartment volumes is related to an increase in the size of the respiratory pump, that comprises the oral, buccal and opercular cavities. As a consequence of the increased effort required for ventilation, the respiratory muscles (Ballintijn, 1969a, b) will likely be increased in size. Increase in the *m. sternohyoideus* and *m. levator hyomandibulae*, requires an increase in the ventral suspensorial and ventral opercular compartment, increase in the *m. adductor operculi*, *m. levator operculi*, and *m. dilator operculi* requires an increase in size of bar [4_l-4_R].

Possibilities for transformation

If, through a decrease in oxygen availability it is needed to increase the gill size and stroke volume, spatial restrictions may occur. Theoretically, there are several possibilities to accommodate the larger gills: (1) use of the free space within the head; (2) reduction of surrounding structures; (3) increasing the head volume; (4) a combination of the previous possibilities (*e.g.* Witte *et al.*, 1990; Smits *et al.*, 1996 a, b). Apart from the use of free space, solutions 2-4 may have a negative impact on the performance of the animal through transformations of anatomically surrounding structures and decreased streamline (Barel, 1993). In our study, only external features of the head were investigated (possibility 3). We did not investigate whether free space, or a decrease in size of surrounding structures, was involved in allocating larger gills (possibilities 1 and 2). Evidence for combined responses were found in the study of Smits *et al.* (1996) and Chapman *et al.* (2000). In a study on *A. alluaudi* from different locations, two morphs were found (Smits *et al.*, 1996b). Animals that fed on insect larvae and other soft bodied prey had non-hypertrophied pharyngeal jaws, while animals that fed on snails had hypertrophied pharyngeal jaws. In addition to changes in bar lengths 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 a split-brood experiment with NR and HR *Pseudocrenilabrus multicolor victoriae*, an increase in the gills and muscles of the respiratory apparatus was found, together with an increase in head length and a decrease of the *m. sternohyoideus* depth, the *m. retractor dorsalis* depth and the lower pharyngeal jaw depth (Chapman *et al.*, 2000). The studies of Smits *et al.* (1996) and Chapman *et al.* (2000) show that respiratory system and pharyngeal jaw apparatus are phenotypically quite plastic and support the hypothesis that in hypoxia raised cichlids, structures surrounding the enlarged respiratory apparatus may decrease in size or change in shape to provide space.

Barel (1983) recognised two core functions of the oral jaws in food uptake, namely biting and suction, the *m. adductor mandibulae* (*mAM*) being larger in the biters. It has been hypothesised that a thicker *mAM*, which increases biting force (Van Leeuwen and Spoor, 1987), causes a broader head width (Barel, 1983; De Visser and Barel, 1996). In a later study (De Visser and Barel, 2000) it was proven that, in the external framework, the ventral width (bar $[5_L-5_R]$) was the most important measure that distinguished between biters and suckers. This suggests a correlation between width of the *mAM* and the ventral head width. Indeed, Smits *et al.* (1996 b) found that an increase in head width (bars $[5_L-5_R]$ and $[4_L-4_R]$), to allocate larger pharyngeal jaws, resulted

in an increase of the size of the *mAM*. They suggested that this could be an example of an epiphenomenon (i.e. the *mAM* increased in size, not because of functional demands but because space became available). In our study the ventral width also increased most in size. Thus it is possible that, in HR animals there is space for a larger *mAM*. If a larger *mAM* were indeed realised in HR fish, it would have consequences for the food types that could be utilised.

De Visser and Barel (2000) found that in 73 cichlid species from different East African lakes, with a different phylogenetic background, morphology and ecology, the ventral width (bar $[5_L-5_R]$) was the most discriminating factor describing inter-specific differences. It seems that, the phenotypic responses to hypoxia that were found in the present study show a large degree of similarity with phylogenetic differences that are found amongst East African cichlids. One could wonder whether phenotypic plasticity in this region was in any way a steering factor in the morphological diversification of East African cichlids through evolution.

Ventral width, a hotspot of plasticity in cichlids?

The species flocks of Lake Victoria and of other lakes, e.g. Malawi, Tanganyika are characterised by rapid speciation, and in addition, extreme inter-specific variation in morphology and consequent functional differentiation. Liem

(1980) states that “ The morphological novelty characterising the family Cichlidae involves the development of: a synarthrosis between the lower pharyngeal jaws, a strategic shift of insertion of the two fourth *levator externi* muscles, and synovial joints between upper pharyngeal jaws and basicranium. This specialized, highly integrated key innovation enables the cichlids not only to transport (deglutination) but also to prepare food, freeing the premaxillary and mandibular jaws to evolve numerous specializations dealing with the collection of dramatically diverse foods.” According to Galis and Metz (1998), this would in turn support the hypothesis of Vermeij (1984) that, an increase in number of independent elements increases the potential for morphological and functional diversification. To our opinion this view should be approached in a more differentiated way. Barel *et al.* (1989) and Witte *et al.* (1990) showed that great levels of dependency exist and can exist between structures within the head of cichlids. Changes in structures within cichlid heads often seem to affect anatomically related structures. In the previous section we have mentioned the close relation between pharyngeal jaw size and gill shape (Smits *et al.*, 1996b) and between gill size and *m. sternohyoideus* depth and lower pharyngeal jaw depth (Chapman *et al.*, 2000). Thus it seems that the fact that many structures are anatomically linked, constrains the possibilities for

change. However, when reviewing the studies on which external framework or comparable measurements were used, there is one recurrent phenomenon: variation in the ventral width (bar [5_L - 5_R]). 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), environmentally related differences (Bouton *et al.*, 2002; present study) and phenotypic plasticity (present study). These show that besides all other variation in anatomy, the ventral width is a hot spot for both phenotypic as genotypic plasticity, within East African cichlids. The present study shows that such variation in the ventral width is conceivable 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.

