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RESEARCH ARTICLE

Hypoxia-induced changes in hemoglobins of Lake Victoria cichlids

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ABSTRACT

In a previous study, broods of the Lake Victoria cichlid *Haplochromis ishmaeli* raised under hypoxic or normoxic conditions showed striking differences in isohemoglobin (isoHb) pattern that were not observed in two other cichlids that do not belong to the Lake Victoria species flock. We therefore hypothesized that the adaptive mechanism seen in *H. ishmaeli* in response to hypoxia constitutes a trait that the Lake Victoria species flock inherited from ancestors that lived in hypoxic environments. We tested this hypothesis by designing split-brood experiments with three other representative species from the same species flock: the insectivorous *Haplochromis thereuterion*, the mollusk-shelling *Platytaeniodus degeni* and the zooplanktivorous *Haplochromis piceatus*, while keeping *H. ishmaeli* as a reference. Split broods were raised, under either normoxia or hypoxia. All hypoxia-raised (HR) individuals of each of the four species exhibited a distinctly different isoHb pattern compared with their normoxia-raised (NR) siblings. The hemoglobin of HR *H. thereuterion* showed higher O_2 affinity compared with NR siblings particularly in the presence of ATP and GTP, indicating that blood of HR juveniles has significantly improved O_2-binding affinity under hypoxic conditions. We also tested the capacity to acclimate at greater age in two species by reversing the O_2 condition after 7 (*H. thereuterion*) and 4 (*H. ishmaeli*) months. After reacclimation for 1 and 2 months, respectively, we found incomplete reversal with intermediate isoHb patterns. As three of the four species do not encounter hypoxic conditions in their environment, this unique trait seems to be a relic inherited from predecessors that lived in hypoxic environments.

KEY WORDS: Isohemoglobins, Hemoglobin affinity, Chronic hypoxia, Acclimation, Species flock, Cichlids

INTRODUCTION

Lake Victoria, located between Tanzania, Uganda and Kenya in East Africa, is the world's largest tropical lake, with a surface area of ca. 68,800 km^2 and a maximum depth of about 70 m. It originated less than a million years ago (Witte et al., 2005) but is considered to have undergone a major desiccation event in the Late Pleistocene period where it may have been dried up completely, refilling again about 14,600 years ago (Johnson et al., 1996). Whether it was completely dry during this desiccation or whether a large marshy area remained is still unclear (Fryer, 2001). Despite its recent age, Lake Victoria has an extremely rich fish fauna, comprising a flock of over 500 cichlid species (haplochromines) (Witte et al., 2007), a large part of which is endemic to the lake. Although some scientists reject this hypothesis (Fryer, 2004), the general opinion is that Lake Victoria did dry out in the Late Pleistocene (ca. 15,000 years ago) and that the species flock developed after this desiccation event (Nagl et al., 2000; Stager and Johnson, 2008; Seehausen et al., 2003), resulting in the fastest large vertebrate radiation known (Schluter, 2000).

The oxygen concentration in Lake Victoria decreases slightly with increasing depth but falls sharply immediately above muddy bottoms, resulting in almost completely anoxic conditions, which are not encountered over sand bottoms (van Oijen et al., 1981). During the rainy season, additional hypoxic zones are formed at varying depths (Wanink et al., 2001). It is therefore likely that species inhabiting these zones developed specific adaptations in order to cope with decreased oxygen availability.

The capacity of fish, which are subjected to far greater vicissitudes of O_2 availability than air-breathing vertebrates, to colonize a wide range of habitats is associated with the evolution of hemoglobin (Hb) systems that display striking diversity in structural and functional properties (Di Prisco and Tamburrini, 1992; Weber et al., 2000). This includes Hb genes that express multiple isohemoglobins (isoHbs), with differing O_2-binding properties (Perutz, 1983; Rutjes et al., 2007; Weber, 2000). The isoHb composition may change during ontogenic development (Maruyama et al., 2004) or during temporal and spatial decreases in O_2 availability (Campo et al., 2008; Feng et al., 2014) such that the multiplicity may safeguard tissue O_2 supply under different operating conditions (Cadiz et al., 2017; Perutz, 1983; Rutjes et al., 2007) and thus extend the inhabitable environment. Fish can be divided into three classes on the basis of isoHb O_2-binding properties, viz class I, which comprises species with single or multiple Hbs that are anodic [have low isoelectric points (pI) and migrate to the anode in standard electrophoresis] and exhibit low O_2 affinities that are markedly sensitive to pH and temperature (e.g. carp, plaice and flounder); class II, whose multiple isoHbs comprise low-pI anodal components with functional properties similar to class I Hbs, as well as high-pI cathodal components with high O_2 affinities that show low sensitivity to pH and temperature (e.g. eels, catfish and salmonid fishes); and class III, whose Hbs are sensitive to pH but not to temperature (e.g. tuna) (Weber et al., 1976; Weber, 1990). In lacking marked pH and temperature sensitivities (that reduce Hb–O_2 binding in the gills), the cathodic isoHbs may function as an O_2 reserve and O_2 carrier under hypoxia and activity-induced acidosis, where oxygenation of anodic Hbs is compromised. This interpretation is supported by the high incidence of cathodic Hbs in active salmonids and catfish, and teleosts from warm and thermally unstable habitats, and their absence in inactive benthic flatfish (Weber, 2000; Brix et al., 1998).

The O_2-binding properties of blood are a product of Hb's intrinsic O_2 affinity, which is determined by its molecular structure, and the intra-erythrocytic levels of effectors that modulate Hb O_2 affinity by allosteric interaction [mainly the organic phosphates ATP and GTP, protons (pH) and chloride ions]. Commonly, exposure to hypoxia

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increases fish blood O_2 affinity directly through a reduction of the red cell levels of ATP and GTP (decreased allosteric interaction with Hb; Weber and Lykkeboe, 1978) as well as indirectly through increased cellular pH, resulting from a linked perturbation of the Donnan equilibrium of protons across the red cell membranes (Wood and Johansen, 1973).

A radically different adaptive mechanism that does not specifically involve increases in the cathodic Hb components or a reduction in nucleoside triphosphates (NTPs) was observed by Rutjes et al. (2007) in the Lake Victoria cichlid, *Haplochromis ishmaeli*, where hypoxia-acclimated specimens exhibited a different isoHb pattern correlated with a higher Hb O_2 affinity compared with normoxia-acclimated specimens. In the same study, two other cichlids (*Astatoreochromis alluaudi*, *Oreochromis mossambicus* × *niloticus*) that do not belong to the Lake Victoria species flock showed no corresponding change in isoHb pattern. As the observed changes in isoHb pattern were unique, it was hypothesized that the adaptive mechanism seen in *H. ishmaeli* in response to hypoxia may be a trait that the Lake Victoria species flock inherited from ancestors that lived in hypoxic environments.

To investigate whether the adaptive response observed in *H. ishmaeli* is a distinguishing characteristic of the Lake Victoria species flock, we designed split-brood experiments with four different cichlid species that belong to the same Lake Victoria species flock, but live in different habitats and are exposed to different oxygen conditions: the mollusc-crushing *H. ishmaeli* (Boulenger, 1906), the insectivorous *Haplochromis thereuterion* (van Oijen and Witte, 1996), the mollusc-shelling *Platytaeniodus degeni* (Boulenger, 1906) and the zooplanktivorous *Haplochromis piceatus* (Greenwood and Gee, 1969). Of these species, *H. thereuterion* and *H. ishmaeli* are unlikely to encounter hypoxia in their natural environment as the former lives high in the water column above rocky substrates (van Oijen and Witte, 1996) and the latter above shallow sandy substrates (Witte, 1981; Witte et al., 1992). *Platytaeniodus degeni* (Boulenger, 1906) and *H. piceatus* (Greenwood and Gee, 1969) are benthic and occur deeper in the water column (Witte, 1987; van Oijen et al., 1981). Unlike *P. degeni*, which lives above sandy as well as soft, muddy bottoms at depths ranging from 2 to 11 m (van Oijen et al., 1981; Witte et al., 1992), *H. piceatus* lives mainly above mud at depths between 7 and 11 m (Witte, 1987), and thus has greater risk of encountering hypoxia or anoxia in its natural habitat.

Broods of all four species were either normoxia-raised (NR; 80–90% air saturation) or hypoxia-raised (HR; 10% air saturation). Hematocrit (Hct) levels were significantly higher in HR fish compared with NR fish in all four species, reflecting a physiological adjustment to safeguard blood oxygen transport capacity. In addition, all four species showed major differences in isoHb composition between HR and NR individuals. Correlated with changes in isoHb pattern, HR *H. thereuterion* showed higher O_2 affinity than their NR siblings. These changes were similar to the response in *H. ishmaeli* described in our earlier study (Rutjes et al., 2007). The results strongly suggest that the hypoxia-induced changes in isohemoglobins of the four selected species have survival value under hypoxic conditions and are a relic inherited from predecessors living in marshy environments.

MATERIALS AND METHODS

Experimental setup

All four haplochromine fish species used in this study were bred in the Institute of Biology Leiden, Leiden University, The Netherlands for 10–20 years after acquiring original specimens from the field. Experimental fish were raised in the same climate room in twelve 45×50×50 cm glass tanks, each with a mixing compartment in which air-saturated (>99% air) or oxygen-poor (6–9% air) water was recirculated (2 l min^{-1}) after admixture with water from the animal compartment to ensure rapid mixing. Water refreshment in each compartment was between 2 and 5 l min^{-1} with filtered tap water (using carbon and sand filters) at the preset temperature of 25°C. A stainless steel plate was placed 3 cm below the water surface to prevent oxygen uptake from the air.

A constant oxygen level was obtained by controlling air flow through a diffuser in the mixing chamber. The oxygen level in each tank was monitored with an oxygen electrode (Applikon model ZZ7\202AP10, Applikon Biotecnology BV, Delft, The Netherlands) and regulated by an Applikon biocontroller (ADI 1030), which regulated the air flow by modulating the activation time of solenoid valves positioned in sequence with the air diffusers. Thus, air flow into the mixing compartment was automatically activated when the oxygen level fell below the set point. The oxygen level in the animal chambers was kept constant within 1% air saturation (AS; at 25°C, 100% AS corresponds to 8.24 mg O_2 l^{-1} at 760 mmHg). The oxygen sensors were calibrated daily with air-saturated water (100% AS) and with 10% sodium sulphite solution (0% AS). The waterflow into the hypoxia setup was degassed using a hollow fiber cartridge (Liqui-Cel® Membrane Contactors) connected to a vacuum pump. The degassing flow-through system produced 3–6% AS water at flow rates around 10 l min^{-1}. Temperature and oxygen level were controlled daily with a portable oxygen/temperature meter (Hach, HQ40D).

Acclimation

All experiments started with broods of fish of approximately 1.5 cm standard length at 4 weeks after release from the buccal cavity of the mother, except the experiments with *H. piceatus*, which started with 4- to 6-month-old juveniles. The experiments with *H. piceatus* could not be added earlier due to limited facilities in combination with the rather long acclimation periods of up to 10 months. The fish were kept at 25±1°C and a 12 h:12 h light:dark cycle. All fish were fed twice daily with flake food (2/3) and spirulina algal powder (1/3) (Landman, The Netherlands). Feeding took place through a funnel placed in a small hole in the top plate of the aquarium, which was otherwise covered with a small metal plate. Cleaning of the tanks and measurement of the growth rate took place on the same day in all aquaria in order to keep the experimental conditions similar and to minimize stress. In addition, PVC tubes were placed inside the animal compartments to provide shelter. The timeline of the five conducted experiments is shown in Table 1. Broods of each species were randomly divided into two groups and raised under normoxia (NR; 90–100% AS) or hypoxia (HR; 10% AS) for 7 months (*H. thereuterion*) or for 3 months (*H. ishmaeli*). For the acclimation reversal experiments, the broods of experiments 3 and 5 were split into four groups: two HR and two NR. After the acclimation period, one of the NR and one of the HR groups were exposed to the reversed condition for 1–2 months (Table 1). To follow growth during the acclimation, the standard lengths (defined as the distance from the tip of the snout to the tail implant) of the fish were measured every month, with the exception of the first month, in which the fish were too small to handle. The experimental protocols and reports were approved by the 'Dierexperimenten Commissie' of the Leiden University.

Blood sampling

Blood was sampled from fish anesthetized by a 2 min submersion in water containing 300 ppm tricaine methanesulfonate (MS222); the animals lost equilibrium within 20 s. For small specimens –

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Table 1. Overview of conducted experiments

Exp.	Species	Days after release	No. fish (broods)	Days first condition	No. sampled after first condition		Days second condition	No. fish sampled after second condition		
					HR	NR		Control	HR>NR	NR>HR
1	*Haplochromis thereuterion*	25	23 (1)	121	6	5	–			
				296	6	6	–			
2	*Platytaeniodus degeni*	25/31	31 (2)	121	7	7	–			
				296	6	7	–			
3	*Haplochromis thereuterion*	34	27 (1)	214	2	2	–			
					–	–	36	–	6	10
4	*Haplochromis piceatus*	120/180	18 (2)	37	9	9	–			
5	*Haplochromis ishmaeli*	23	39 (1)	124	6	6	–			
					–	–	69	7	7	–
					–	–	74	6	–	6

Broods from four different cichlid species were partitioned and siblings were subjected to normoxia [normoxia raised (NR), 80–100% air saturation (AS)] or hypoxia [hypoxia raised (HR), 8–12% AS] from about 1 month after buccal release (days after release) up to 296 days. In experiments 3 and 5, for a subset of animals the acclimatory condition was reversed (HR>NR, NR>HR) for 1–2 months (days second condition). Blood samples were taken at the end of the first and second acclimations to determine the isohemoglobin (isoHb) pattern on thin-layer isoelectric focusing (IEF) gels. Exp., experiment number.

where the blood was used exclusively to analyze isoHb patterns – blood was obtained by cutting off the tail fin. The first blood drop was used for two 5 µl Hct capillaries. The next few blood drops were collected in Eppendorf tubes containing 500 µl heparinized saline. After 3 min centrifugation at 10,000 rpm (8100 ***g***) and 4°C, the supernatant was removed and the erythrocyte pellet was washed twice by resuspension in 500 µl saline and centrifugation to remove contaminants. The erythrocytes in the final pellet were hemolyzed by admixture of twice their volume of deionized water. All experimental animals were very small, certainly at the start: the small blood samples were just enough for Hct and thin-layer isoelectric focusing (IEF)-gel analysis.

For larger specimens that yielded sufficient blood, additional measurements were carried out. The first drops were drawn into capillaries for Hct measurements. The remaining blood was collected and centrifuged (as above) and the erythrocyte pellet was mixed with 2 volumes of heparinized saline to obtain an erythrocyte suspension (blood replicate) with similar Hct and Hb values as in native blood. Hb concentration was measured by mixing and vortexing 5 µl of the erythrocyte suspension with 1 ml of hemoglobin Roche reagent (containing K_3-ferricyanide, KCN, $KHCO_3$), and measuring absorption at 546 nm against deionized water. Hb preparations were stored at −80°C until further analysis.

Thin-layer isoelectric focusing (IEF)

IsoHb patterns of the different species were analyzed by IEF on polyacrylamide gels in the 3–9 pH range, using the PhastSystem of Pharmacia Biotech and following the manufacturer's instructions. Staining of the gels was done manually. Samples were diluted with saline and applied to eight separate lanes of the 43×50×0.45 mm (width×length×thickness) gels using PhastGel Sample Applicators (8×0.5 µl) (see Fig. 1).

A total of 20 gels were analyzed, each with six blood samples in lanes two to seven and two calibration protein markers in lanes one

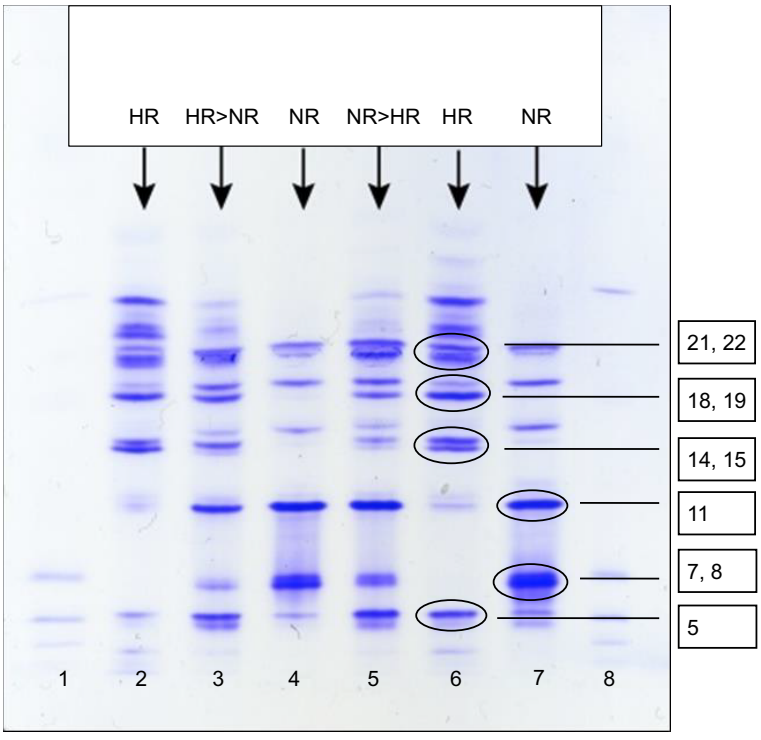

Fig. 1. Thin-layer isoelectric focusing (IEF)-gel analysis. A typical IEF gel showing isoelectric point (pI) calibration proteins (lanes 1 and 8), and the isohemoglobins (isoHbs) of *Haplochromis thereuterion* siblings raised for approximately 10 months under normoxia (NR, 100% AS; lanes 4 and 7) or hypoxia (HR, 10% AS; lanes 2 and 6), and specimens that were subjected to reversed acclimation (HR>NR and NR>HR) for an additional month (lanes 3 and 5). The gels were scanned and the numbers of the ten isoHb bands with the highest intensities are shown.

and eight. Gels were scanned and analyzed with ImageTool and ImageJ software. The height of the gels was used as a reference to calculate the relative distance of each band. Bands were sequentially numbered, starting from the bottom of the gel. The program ImageJ was used to quantify absolute intensity of the bands. For this purpose, a vertical rectangular selection in the middle of the lane was converted into a diagram where the surface area of the peaks represented the absolute intensity of the different bands in the lane. The peaks were expressed as the percentage of the total surface area of all bands in one lane. Band intensity was measured in absolute values and converted into relative values to remove gel-specific effects (differences in the amount of staining) and sample effects (differences in [Hb] on the IEF gel). The average intensity of each band in each experimental group was calculated and used for the graphical representation of the results.

Statistics

The intensity of the bands on the IEF gels was statistically analyzed by a log-ratio analysis of compositional data (Aitchison, 1986). The performed log-ratio analysis took into account gel effects and sample effects, thus allowing application of the statistical results on relative band intensities. Since there were more observations (number of bands) than response variables (number of fish), which was not compatible with the applied test, only the ten bands with the largest intensity could be considered and compared between conditions within one species. All species showed isoHb patterns that differed significantly between NR and HR specimens.

Oxygen-binding measurements

'Stripped' (organic phosphate-free) Hb was prepared at 0–4°C by adding 5 mg mixed bed resin (MB-1 AG501-X8, Bio-Rad) per 100 µl hemolysate, stirring on ice for 1 h and centrifugation at 10,000 rpm at 4°C. After adding buffer (10 mmol l^{-1} Hepes containing 0.5 mmol l^{-1} EDTA, pH 7.61) to bring the total volume to 4 ml, the Hb was concentrated by centrifugation at 10,000 rpm in ultrafiltration in Amicon® Ultra PL-10 units. This step was repeated twice. The concentration and oxidation state of the samples retrieved from the filtration units were checked by recording the absorption spectrum between 350 and 700 nm. Oxygen equilibria of 3 µl samples of the stripped Hb were measured at 25°C and pH 6.9 and 7.6, using a modified gas diffusion chamber setup as previously described (Weber, 1981; Weber et al., 2010). Gas mixtures were prepared by cascaded Wösthoff gas-mixing pumps (Bochum, Germany). Values of half-saturation O_2 tension (P_{50}) and n_{50} (Hill's cooperativity coefficient) were interpolated from linear plots of log [$Y/(Y-1)$] versus log P_{O_2} based on at least four saturation values between 25 and 75%. Using this method, the r^2 determination coefficient for the fitted curve typically exceeds 0.995 (Weber, 2014).

RESULTS

Hematocrit data and growth rates of NR and HR siblings

Size, weight and Hct data are shown in Table S1. Statistical analyses of growth rates are presented in Table S2. Growth rates of NR siblings were always higher than those of the HR siblings. HR fish showed a much more relaxed behavior compared with their NR siblings: they moved less actively and did not fight. The ventilation rate of HR siblings was significantly higher, although their behavior was quite normal. They were fed twice daily like their NR counterparts. Hct levels of all HR individuals were >30% higher than those of the NR siblings. All animals were healthy over the whole period. Some deaths occurred due to the aggressive behavior of dominant males (only in NR groups) at the end of the 10 month conditioning period when fish became sexually mature.

IsoHb pattern of NR and HR siblings of four species

The isoHb patterns of all blood samples were analyzed on IEF gels. Fig. 1 shows an example IEF gel: the isoHb composition of samples from different individuals (*H. thereuterion*) raised under four different conditions, viz NR, HR, NR followed by 1 month acclimation reversal (NR>HR) and HR followed by 1 month acclimation reversal (HR>NR). Data from all four species are summarized in Fig. 2A–D. The variation between individuals within each group was remarkably small.

Data from *H. thereuterion* are shown in Fig. 2A. The gels showed up to 29 bands, and the numbers of the ten major bands were 5, 7, 8, 11, 14, 15, 18, 19, 21 and 22. Seven of these bands show highly significant differences between HR and NR siblings: three were upregulated (bands 14, 15 and 18) and four downregulated (bands 7, 8, 11 and 22). Band 8 was only present in NR fish, while band 14 was only present in HR fish.

Data from *H. ishmaeli* are shown in Fig. 2B. The gels showed up to 27 bands, and the ten bands with the largest average relative intensities are bands 5, 6, 7, 10, 11, 14, 15, 17, 18 and 20. Except for bands 11 and 18, these intensities differed significantly between HR and NR siblings: the intensities of bands 6, 7, 10, 15 and 20 were significantly higher in NR fish, whereas those of bands 5, 14 and 17 were significantly higher in HR fish. Band 7 was only present in NR fish, while bands 14 and 17 were only present in HR fish.

Data from *P. degeni* are shown in Fig. 2C. The gels showed up to 23 bands, and the ten bands with the largest intensities are bands 5, 6, 7, 10, 11, 14, 15, 17, 18 and 20. Except for band 5, the average relative intensities of the bands differed significantly between HR and NR specimens. Bands 6, 7, 10, 15, 18 and 20 were significantly higher in NR fish, whereas those of bands 11, 14 and 17 were significantly higher in HR fish.

Data from *H. piceatus* are shown in Fig. 2D. The gels showed up to 14 bands and, in this case, only nine bands (3, 4, 5, 7, 8, 11, 12, 13 and 14) could be considered since the number of bands should not exceed the number of observations in the statistical analysis. The average relative intensities of bands 3, 4, 5 and 12 differed significantly between HR and NR specimens. Those of bands 4 and 5 were significantly higher in NR fish, whereas those of bands 3 and 12 were significantly higher in HR fish. Bands 3 and 12 were only present in HR fish.

Acclimation reversal experiments

Haplochromis thereuterion

Since there were insufficient specimens at the start of the reversal experiment to include in reference groups, the results were compared to those of HR and NR animals prior to reversal. The most obvious changes occurred in animals that were transferred from normoxia to hypoxia, where five isoHb bands were upregulated and two downregulated. By contrast, there was only one major change in the HR>NR group, viz an increase of band 11, whereas the changes in the other bands were not statistically different (Table 2). It is remarkable that the major changes in the two reversal experiments occurred in different isoHb groups: for the NR>HR acclimation the major changes occurred in bands 5, 7, 8, 15 and 18, whereas, for HR>NR, the changes occurred in band 11.

Haplochromis ishmaeli

In this species, the average intensities of the bands in both reversal groups were compared with the corresponding controls. Except for

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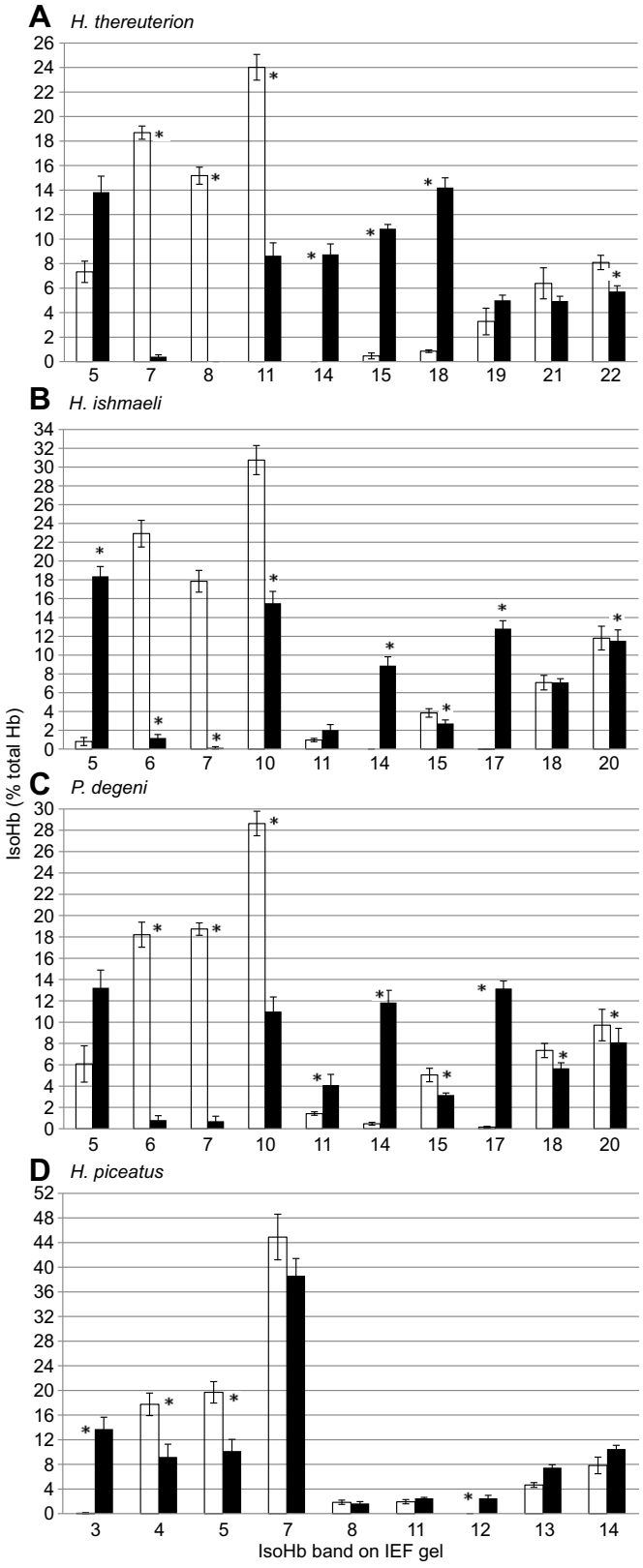

Fig. 2. Average relative intensity of the ten largest isoHb bands of four cichlid species. (A) *H. thereuterion*, (B) *Haplochromis ishmaeli*, (C) *Platytaeniodus degeni* and (D) *Haplochromis piceatus*. Asterisk indicates a significant difference (*P<0.05) between normoxia-raised (NR; white bars) and hypoxia-raised (HR; black bars) siblings. See Table 1 for sample numbers and condition. The *x*-axis gives the isoHb band numbers. Values are means±s.d.

Table 2. Summary of the acclimation reversal experiments with *H. thereuterion* and *H. ishmaeli*

Band no.	Normoxia	NR>HR	P	Hypoxia	HR>NR	P
H. ishmaeli	*n*=12	*n*=6		*n*=12	*n*=6	
5	0.8±1.5	14.0±0.7	**	18.4±3.6	7.6±1.7	ns
6	22.9±4.9	6.0±2.9	***	1.2±1.3	13.5±4.1	**
7	17.8±4.0	3.5±2.8	**	0.1±0.5	8.4±2.6	***
10	30.7±5.4	23.0±6.6	***	15.5±4.4	22.9±10.1	ns
11	1.0±0.6	1.6±0.7	ns	2.0±2.0	2.1±0.8	ns
14	0.0±0.0	6.6±1.3	***	8.9±3.2	5.8±2.9	*
15	3.8±1.6	4.4±1.1	**	2.7±1.3	6.0±2.4	***
17	0.0±0.1	7.4±2.0	***	12.8±2.9	4.6±2.2	ns
18	7.1±2.7	9.8±1.4	**	7.1±1.4	9.1±2.1	**
20	11.8±4.4	10.7±2.5	**	11.5±4.0	10.6±6.7	ns
H. thereuterion	*n*=13	*n*=5		*n*=10	*n*=5	
5	7.3±3.1	17.2±1.7	*	13.8±4.1	14.6±1.6	ns
7	18.7±1.9	4.2±3.2	**	0.4±0.5	3.2±1.6	ns
8	15.2±2.5	2.0±2.6	***	0.0±0.0	0.8±1.1	ns
11	24.0±3.8	22.4±4.2	ns	8.7±3.3	17.0±1.0	*
14	0.0±0.0	2.5±1.3	***	8.8±2.7	4.7±2.6	ns
15	0.5±0.9	5.1±2.4	***	10.8±1.1	9.9±0.5	ns
18	0.9±0.4	9.6±2.8	***	14.2±2.5	12.1±1.6	ns
19	3.3±3.9	7.0±1.8	ns	5.0±1.3	6.7±1.9	ns
21	6.4±4.6	8.2±6.0	**	4.9±1.2	6.1±1.1	ns
22	8.1±2.1	10.4±3.8	ns	5.7±1.5	5.0±1.9	ns

The average relative intensities (means±s.d.) of isoHb bands are shown. Some siblings of the two species were exposed for 1–2 months, respectively, to reversed conditions, i.e. from normoxia to hypoxia (NR>HR) and from hypoxia to normoxia (HR>NR). Values after the reversal are compared with those in the initial acclimation (see details in Table 1). Asterisks indicate significant differences between the initial and reversed conditions: *P<0.05, **P<0.01, ***P<0.001; ns, not significant.

band 11, all bands of the NR>HR group changed significantly (Table 2). Major changes occurred in five isoHb bands: three bands (5, 14 and 17) were upregulated and two bands (6 and 7) were downregulated. The same isoHb bands also changed significantly in the HR>NR group, except that the changes occurred in the opposite direction (5, 14 and 17 were downregulated, and 6 and 7 were upregulated).

Oxygen affinity of *H. thereuterion* hemoglobin

In order to obtain sufficient material for O_2 affinity measurements, additional *H. thereuterion* specimens were acclimated to normoxia and hypoxia. Data for NR and HR siblings are shown in Table 3. The stripped Hb from HR *H. thereuterion* revealed markedly higher O_2 affinities (lower P_{50} values) than Hb from NR fish (P_{50} values at pH 6.97 were 4.73 and 7.69 mmHg, respectively) (Table 3). The difference increased drastically in the presence of saturating levels of ATP (P_{50}=35.6 and 63.4 mmHg, respectively) and GTP (P_{50}=46.3 and 76.6 mmHg, respectively). Corresponding, albeit less pronounced, differences are seen at pH 7.62 (Fig. 3, Table 3). The larger effects of GTP than ATP aligns with results for Hb from other teleosts, where it is ascribed to the formation of an additional hydrogen bond formed between the deoxygenated Hb and GTP (compared with ATP), which stabilizes this low-affinity 'tense' structure of the Hb molecules (Gronenborn et al., 1984; Weber, 2000). In the absence of organic phosphates, the Hb from HR and NR specimens shows similar Bohr effects ($\phi=\Delta\log P_{50}/\Delta pH=-0.43$ and -0.45, respectively). In the presence of ATP and GTP, the Bohr factors increase drastically ($\phi<-1.25$) (Table 3) in accordance with phosphate-induced increases in the pK of proton-binding sites in Hb molecules (Riggs, 1988).

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Table 3. O_2 affinities of hemoglobins (Hbs) from normoxia- and hypoxia-acclimated *H. thereuterion*

		P_{50} (mmHg)		Bohr factor	Phosphate effect (log $P_{50(ATP/GTP)}$–log $P_{50(str)}$)	
Acclimation	Hb	pH 6.97± 0.024	pH 7.62± 0.008	(Δlog P_{50}/ΔpH)	pH 6.97± 0.024	pH 7.62± 0.008
Normoxia	Str	7.69	3.98	−0.43	–	–
	Str-+ATP	63.41	7.51	−1.39	0.92	0.28
	Str+GTP	76.57	8.76	−1.54	1.00	0.34
Hypoxia	Str	4.73	2.46	−0.45	–	–
	Str-+ATP	35.65	4.01	−1.47	0.88	0.21
	Str+GTP	46.34	7.98	−1.25	0.99	0.51

O_2 affinities were indexed as half-saturation O_2 tensions (P_{50}), and their sensitivities to pH (indexed as the Bohr factor) and the organic phosphates ATP and GTP (indexed as the shift in log P_{50} induced by saturating concentrations of these phosphates). Experimental conditions are as specified in the legend of Fig. 3. Str, Hb stripped of organic phosphates.

DISCUSSION

Acclimation effects

To limit the individual variation due to genetic and environmental differences, we chose to work with siblings from one brood (in two cases with two broods). This allows a higher significance of physiological responses with smaller experimental groups. With *H. thereuterion* and *H. ishmaeli*, the broods were large enough for reversal experiments and only with *H. thereuterion* could we also obtain enough blood to include Hb O_2 affinity measurements.

Growth rates of HR individuals were significantly lower compared with NR individuals in all species except *H. piceatus*, which was acclimated for a much shorter period (Table 1), which may explain the difference. A previous study (Rutjes et al., 2007) showed no difference in growth rate between NR and HR cichlids. The reason for this might be due to the fact that the fish in that study were fed once a day, whereas those in the present study were fed twice a day. It is likely that HR individuals were not able to digest as much food per feeding as their NR siblings. HR fish were also less active than NR fish, indicating that hypoxia reduced the activity of HR fish. The ventilation rate was higher in HR fish, which is required to stabilize oxygen uptake. Apart from the reduction in activity level, HR fish displayed normal behavior.

Fighting by dominant males was only observed under normoxic conditions, and after the fish became sexually mature at the end of the 10 month acclimation periods in experiments 1 and 2. The dominant males changed in color, mass and behavior. At the conclusion of the experiments, some *H. thereuterion* females from experiment 1 carried fertilized eggs in their mouth, but this only occurred in NR fish. HR siblings moreover remained smaller and did not reach sexual maturity. Given that these fish are mouth brooders, the carriage of eggs might become problematic during hypoxia-induced hyperventilation.

IsoHb pattern

HR specimens of all four species exhibited strikingly different isoHb patterns compared with their NR siblings. Typical examples are shown in Fig. S1. The observed effects were highly significant. In all experiments, the number of isoHb bands was higher in HR than in NR siblings, indicating that hypoxic exposure induced the expression of different isoHbs. Additionally, ‘normoxia’ bands became weaker, but never disappeared under hypoxia. In *H. piceatus*, the changes were not as prominent, likely due to the shorter exposure period (37 days) and the higher age of the fish at the start of the experiment.

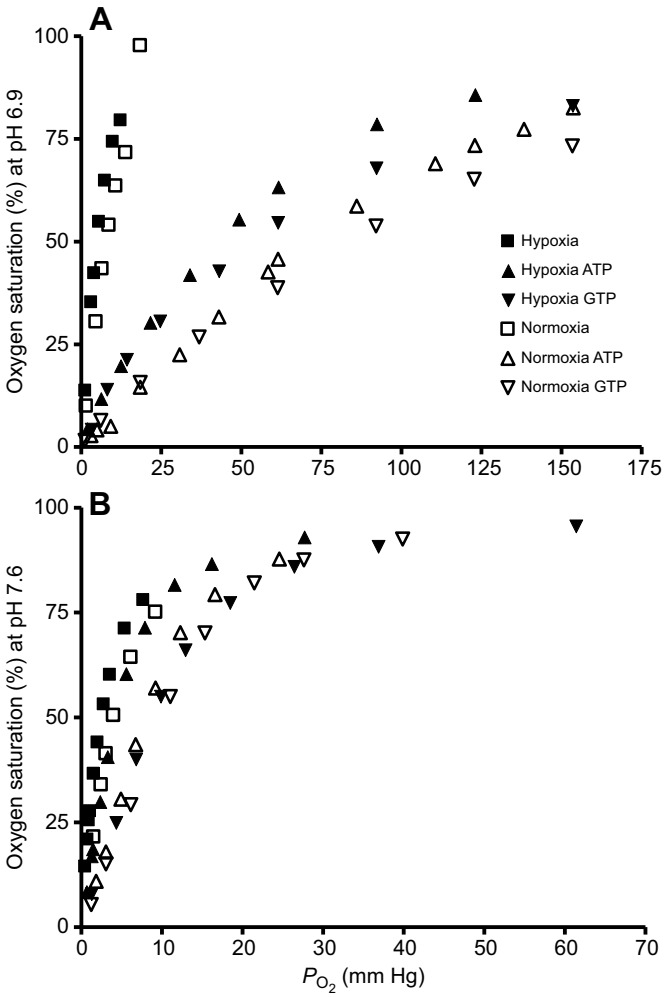

Fig. 3. Oxygen equilibrium curves. Shown are the O_2 equilibrium curves of stripped hemoglobin (Hb) of HR (black symbols) and NR (white symbols) *H. thereuterion* in the absence (squares) and presence of ATP (pyramidal triangles) and GTP (inverted triangles) at pH 6.97 (A) and pH 7.62 (B). Hb samples were prepared from pooled blood samples from at least five animals. Temperature, 25°C; tetrameric Hb concentration, 0.14; ATP/Hb4 and GTP/Hb4 ratio, 19.2.

The differences in isoHb patterns of NR and HR siblings show three isoHbs with marked upregulation and three with downregulation in HR animals: *H. thereuterion* (up: 14, 15, 18; down: 7, 8, 11); *H. ishmaeli* (up: 5, 14, 17; down: 6, 7, 10); and *P. degeni* (up: 5, 14, 17; down: 6, 7, 10). The changes with *H. piceatus* are less pronounced due to shorter and later hypoxia exposure (up: 3; down: 4, 5). Since the pattern change is quite similar, it may be that these new isoHbs are the same in all four species. There is, however, no proof for this hypothesis as the position of the isoHbs on the IEF gels is due to differences in negative and positive charges. Additional proof might be obtained from mass spectrometry, which provides extensive information on molecular structure (Théberge et al., 2015; Kleinert et al., 2008; Woodi et al., 2009).

Multiplicity of isoHbs is quite well known for fishes. The genetic basis for this has recently been extensively described (Cadiz et al., 2017). Teleost fish have a larger number of Hb genes than other vertebrates due to an extra genome duplication during early evolution (Opazo et al., 2013). Hb multiplicity can also be the result of post-transitional changes, such as glycosylation

(Jaisson and Gillery, 2010). Hypoxia is well known as a trigger for large-scale changes in gene transcription (van der Meer et al., 2005). Thus, hypoxia-induced activation of one or more enzymes that modify Hb post-translationally could thus be the underlying mechanisms for the changes in isoHb pattern.

The differences in isoHb patterns between HR and NR fish are not attributable to changes in quaternary structure, i.e. dissociation to dimers or aggregation via disulfide bridges, with the same amino acid composition and isoelectric points (Riggs, 1970; Powers, 1972). Moreover, fish Hbs are very stable, showing an unchanged sedimentation constant (4.3 S), even at extremely low concentrations (micromolar range) and in the presence of high (4–6 mol l^{-1}) concentrations of urea (Brunori et al., 1973; Edelstein et al., 1976; Powers, 1980). To the best of our knowledge, Hb monomers have not been observed in teleosts. Likewise, isoHb banding will not be affected by the formation of the large crystalline aggregates, observed in the red blood cells when fish such as arctic codfish and toadfish are subjected to capture stress and plasma acidosis (Riccio et al., 2011; Koldkjaer et al., 2013), which are insoluble and cannot enter the gel.

A recent paper (Pan et al., 2017) reports transcription changes in the expression of five Hbα and seven Hbβ isoforms in red drum fish exposed for 3 weeks to moderate hypoxia (P_{O_2} 48 mmHg). The study revealed differences in the expression of two Hbα and one Hbβ genes that correlated with increased Hb O_2 affinity in the hypoxia-acclimated specimens. Given that these fish were exposed to oxygen tensions above the P_{crit} (critical P_{O_2}; lowest oxygen tension where the fish can remain in an aerobic state) and did not show increased Hct levels, which typically characterizes hypoxic acclimation, longer exposure to deeper hypoxia may have revealed larger acclimatory responses. By contrast, the juvenile HR cichlids in our study were exposed to oxygen levels (10% AS; P_{O_2} ~16 mmHg) that are lethal for adult fish (data not shown) and exhibited Hct levels that were at least 30% higher than in the NR siblings.

There is no clear understanding of how the oxygen-tension-linked changes in isoform expression observed in red drum or cichlids relate to the overall change in blood oxygen affinity. Oxygen transport is contingent upon correspondence between blood oxygen affinity (which is modulated by interaction with red cell allosteric effectors) and the oxygen tensions for loading and unloading oxygen (in the capillaries of the gills and respiring tissues, respectively), and transport is favored by unloading at high O_2 tension, which increases the gradient for diffusion to the tissues. Apart from short-term changes in the red cell levels of effectors, oxygen transport may be safeguarded by changes in component Hbs (expression of different isoHbs). In this light, the isoHb multiplicity observed in Lake Victoria cichlids may be a strategy to provide Hbs best suited for chronically hypoxic Lake Victoria habitats.

HR *H. ishmaeli* showed striking differences in Hb pattern compared with their NR siblings, as also observed in our previous study (Rutjes et al., 2007). The isoHb patterns in the two studies, however, reveal some minor differences. In the earlier study, all NR and HR individuals (n=17 and 13, respectively) exhibited eight bands for normoxia and 14 bands for hypoxia, while this study shows a larger number of bands in both NR and HR siblings (8–12 and 15–21, respectively). Based in the bright red color of the Hb bands in both studies, there was no evidence for differences in isoHb composition attributable to Hb oxidation (methemoglobin formation). Since the two studies were carried out with siblings from different parents, the difference may relate to different genetic backgrounds. Alternatively, it could result from ontogenetic differences in isoHb composition, post-translational modifications (Giles and Rystephanuk, 1989; Weber, 1990; Rizzotti and Gioppato, 1999) or difference in the duration of the acclimation, which lasted 10 months in this study compared with 18 months in the previous study. Despite these differences the two studies reveal striking similarity in the isoHb patterns of NR and HR siblings.

As adults of the four species did not survive 10% AS in preliminary tests (data not shown), we tested the hypothesis that the hypoxia-induced changes of the isoHb pattern characterizes juveniles by reversing the acclimation conditions (for 1 and 2 months, respectively) in *H. thereuterion* and *H. ishmaeli*. The results showed that the acclimatory adjustment was not complete, i.e. the reversal resulted in an intermediate pattern, confirming our hypothesis that, at this age, the animals may not be able to fully acclimate to the reversed conditions. Still, as there were no mortalities and the fish showed normal behavior, the observed changes in isoHb pattern were sufficient to cope with the new conditions. It should be noted that the transfer from normoxia to hypoxia was a slow and stepwise transition from 90 to 10% AS over 1 week, which may account for the absence of mortalities.

Interestingly, the differences observed in isoHb composition between NR and HR cichlids (Figs 1, 2; Fig. S1) do not pertain to changes in the relative abundances of cathodic or anodic components (that exhibit different oxygen-binding properties in class II fish; see Introduction) but involve components with widely different pI values. Nevertheless our data show distinct differences in Hb O_2 affinity between HR and NR cichlids, contrasting with rainbow trout where significant changes in isomorph profiles induced by acclimation to 'winter' and 'summer' conditions are without critical adaptive significance (Houston et al., 1996).

Hb–O_2 binding affinity

In our former study (Rutjes et al., 2007), the higher O_2 affinity of stripped Hb from HR compared with NR *H. ishmaeli* siblings coincided with a striking change of the isoHb pattern. In this study, affinity for O_2 of stripped Hb from *H. thereuterion* siblings raised at hypoxia or normoxia shows a similar increase in HR individuals that was correlated with a marked change in isoHb pattern. Both species belonged to the same species flock and thus show a similar response to chronic hypoxia, both with respect to isoHb pattern as well as a change in Hb O_2-binding properties. In both studies, the blood was pooled in order to measure the Hb O_2 affinity under different conditions of pH and NTP levels. This means that we are not able to distinguish between the different isoHbs, although it is likely that, in particular, the few bands that are strongly upregulated play an important role in the changed overall Hb affinity.

IEF gels revealed for each of the four species more isoHb bands in HR siblings than their NR counterparts. Additionally, the densities of the individual bands were markedly different compared with the NR siblings. This suggests that the increase in blood oxygen affinity in HR specimens is the result of: (1) the production of new high-affinity Hb types and (2) a shift in band intensity towards the production of more high-affinity Hbs at the expense of the low-affinity Hbs that are more abundantly present in NR specimens. It would be interesting to measure the O_2 affinities of the individual isoHbs under different experimental conditions (pH, ATP and GTP), which, however, would require larger blood samples from at least five animals.

New hemoglobins of the Lake Victoria species flock

Our earlier study (Rutjes et al., 2007) revealed a unique trait in *H. ishmaeli* compared with other cichlids: a markedly different isoHb pattern in HR siblings coinciding with a marked increase of the affinity for O_2 of stripped Hb. This spectacular trait does not appear to have survival value in terms of securing O_2 uptake, as *H. ishmaeli* lives in open water. *Haplochromis ishmaeli* belongs to a species flock in Lake

Victoria that must have survived the geologically recent period when the lake was largely desiccated. It was hypothesized that this event will uniquely have affected the species flock. Testing this hypothesis by investigating four species from the same flock each living in a different habitat, we observed marked differences in isoHb patterns between NR and HR siblings, including new isoHb bands and changes in band densities in each of these species.

Due to limitation in the available experimental animals, Hb O_2 affinities were investigated in only one additional species (*H. thereuterion*). The O_2 affinity data of stripped Hbs from NR and HR *H. thereuterion* siblings corresponded extremely well with earlier findings for *H. ishmaeli* (Rutjes et al., 2007). Both species showed hypoxia-induced changes in Hb O_2 affinity that indicate functional adaptations. The fish species investigated here are good representatives of the species diversity found in the Lake Victoria species flock, their differences in habitat and feeding habits illustrating phylogenetic differentiation within this flock. For *H. ishmaeli*, there was no apparent selective pressure to become hypoxia tolerant, since it is not routinely subjected to hypoxia in their natural habitat.

One may speculate whether the 10–20 years of breeding the different fish species at the Institute in Leiden could have led to changes. Involuntary selection may occur in small populations; however, there was certainly no selection pressure on hypoxia as the fish were bred under normoxic conditions. Still, they did not lose this trait during breeding in the lab. In the same way, these cichlid species did not lose either their unique hypoxia survival capacity in the wild (which is also normoxic) over a very long period of time. It is remarkable that this trait persisted both in the lab for up to 20 years as well as for thousands of years in Lake Victoria.

The unique hypoxia adaptation that occurs in all four tested species suggests the existence of a common adaptive mechanism for all species from the Lake Victoria species flock. The proposed desiccation of Lake Victoria could have favored the development of hypoxia tolerance in the period when the area might have consisted of swamps, although the trait itself could also have been selected much earlier in surrounding swamps. Hence, the current results suggest that the hypoxia-induced changes observed in these four species originating from the Lake Victoria species flock represent traits inherited from their predecessors that lived in hypoxic marshes during the postulated desiccation of the lake approximately 15,000 years ago.

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