



Normoxia exposure reduces hemoglobin concentration and gill size in a hypoxia-tolerant tropical freshwater fish

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Abstract Hypoxia is a widespread environmental stressor that shapes fish physiology and morphology. Plasticity in traits that improve oxygen uptake and delivery or reduce oxygen requirements may be critical for fish to cope with fluctuating dissolved oxygen (DO) conditions in their natural habitat or adapt to new environments. In this study, we characterized a suite of morpho-physiological respiratory traits of a naturally hypoxia-acclimated weakly electric mormyrid fish, *Petrocephalus degeni*, and quantified their plasticity in response to long-term normoxia exposure. We captured *P. degeni* from a hypoxic swamp habitat ($PO_2 = 2.43 \pm 1.85$ kPa) surrounding Lake Nabugabo, Uganda, and acclimated them to normoxia ($PO_2 > 16$ kPa) for up to 75 days. At various time points throughout normoxia exposure, we measured blood hemoglobin and lactate concentration, gill

size, routine metabolic rate (RMR), regulation index (RI), and critical oxygen tension (P_{crit}). We found that 62–75 days of normoxia exposure significantly reduced blood hemoglobin concentration (–17%), gill filament length (–14%), and hemibranch area (–18%), whereas RMR, RI, P_{crit} , and blood lactate showed no significant change. Our results support earlier findings that swamp-dwelling *P. degeni* are well adapted to life in chronic and severe hypoxia and indicate that they possess a limited capacity for phenotypic plasticity in response to a change in their DO environment.

Keywords Mormyrid · Phenotypic plasticity · Weakly electric fish · Respirometry · Lactate · Gill filament length

List of symbols and abbreviations

A_{conf}	Area under the $\dot{M}O_2$ versus PO_2 curve of a perfect oxyconformer
A_{reg}	Area under the $\dot{M}O_2$ versus PO_2 curve of a perfect oxyregulator
A_{trial}	Area under the $\dot{M}O_2$ versus PO_2 curve of a fish during a closed respirometry trial
b	Allometric scaling exponent
DF	Degrees of freedom
DO	Dissolved oxygen
η^2_g	Generalized eta squared for effect size
F	F -Statistic
FL	Gill filament length
FN	Gill filament number

Lauren J. Chapman and Rüdiger Krahe contributed equally to this work.

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HA	Gill hemibranch area
M	Body mass
$\dot{M}O_2$	Oxygen consumption rate
P	Probability
P_{crit}	Critical oxygen tension
PO_2	Oxygen partial pressure
RI	Regulation index
RMR	Routine metabolic rate
SL	Standard body length

Introduction

Water-breathing fish depend on dissolved oxygen (DO) for their long-term survival (Kramer 1984; Richards 2009). The availability of DO can vary considerably on a small spatial scale due to the low solubility and slow diffusion speed of oxygen in water (Graham 1990). Under conditions of low mixing of the water column and/or high rates of oxygen-consuming decomposition processes, oxygen partial pressure (PO_2) in water can fall to levels that negatively affect aquatic organisms, a condition called hypoxia (Greenbank 1945; Kramer 1984; Diaz 2001; Pollock et al. 2007; Farrell and Richards 2009).

Hypoxia is a widespread environmental stressor, and fish have evolved a great variety of adaptations to satisfy their metabolic oxygen demand and mitigate hypoxic stress (Lewis 1970; Kramer 1984; Soares et al. 2006; Richards 2011; McBryan et al. 2013; Abdel-Tawwab et al. 2019). The manner in which fish respond to hypoxia varies among species and individuals (Virani and Rees 2000; Wannamaker and Rice 2000) and is often reflective of the ecological background of an animal (Fu et al. 2014; Borowiec et al. 2015, 2020; Montero-Taboada et al. 2022). Some fish increase oxygen uptake (e.g., through gill remodeling, Sollid and Nilsson 2006; increased gill ventilation rate, Jones 1952; aquatic surface respiration, ASR, Lewis 1970) or storage and supply of oxygen to the tissue (e.g., change of blood hemoglobin concentration and function, Wells 2009; Collins et al. 2015; Pan et al. 2017). Others reduce their metabolic oxygen demand (e.g., through metabolic rate suppression, Richards 2010; behavioral inactivity, Nilsson et al. 1993; Speers-Roesch et al. 2018) or increase their capacity for anaerobic ATP production (e.g., glycolysis yielding lactate or ethanol, Shoubridge and Hochachka 1980; Richards 2009).

The maintenance of respiratory traits can be energetically costly. When oxygen is not a limiting factor, plastic changes in those traits can serve to maintain balance between energy availability and demand and optimize growth rate and use of resources. For example, some fish reduce their respiratory gill surface area at high PO_2 through gill remodelling, which reduces the energetically costly loss of ions from the body that occurs in the gills (Nilsson 1986; Gonzalez and McDonald 1992; Sollid and Nilsson 2006). Thus, phenotypic plasticity enables organisms to cope with environmental change and plays an important role for the adaptation of animals to their environment (McBryan et al. 2013; Seebacher et al. 2015; Norin and Metcalfe 2019).

In this study, we aimed to characterize the plasticity of morpho-physiological respiratory traits and their interaction in a weakly electric mormyrid fish, *Petrocephalus degeni*. This species is found across a wide oxygen gradient in their natural habitat from well-oxygenated lakes to extremely hypoxic areas such as the Lwamunda Swamp, a wetland surrounding Lake Nabugabo in Uganda (Ogutu-Ohwayo 1993; Chapman et al. 1996, 2002; Chapman and Hulen 2001). Previous studies have found that swamp-dwelling *P. degeni* are remarkably hypoxia tolerant (Chapman and Chapman 1998) and that important respiratory traits such as their oxyregulative capacity (i.e., their ability to maintain a stable rate of oxygen consumption in declining PO_2) are subject to phenotypic plasticity when fish are exposed to high environmental DO (normoxia, Clarke et al. 2020). We captured fish from a lagoon in the Lwamunda Swamp and subjected them to normoxic conditions for up to 75 days at a nearby field research station. We measured routine metabolic rate (RMR), critical oxygen tension (P_{crit}), regulation index (RI), gill morphometrics, blood hemoglobin, and blood lactate concentration at various time points of normoxia exposure.

Materials and methods

Study site

All experiments were conducted at the Lake Nabugabo field research site in Uganda. The field research site is located at the edge of Lake Nabugabo and close to the surrounding wetland where fish were captured

(0°21'07"S, 31°52'43"E, Fig. A1). Respirometry experiments, blood sampling, and gill measurements were conducted between July and September 2019; supplemental blood samples were taken between May and August 2018. In the wetland, inundation levels, water temperature, and DO show seasonal and diel variation, although DO remains generally low (Table 1; previously reported night-day range: $0.47 \pm 0.42 - 0.92 \pm 0.72$ mg O₂ L⁻¹; Mucha et al. 2022). Water conductivity and pH remain low throughout the year (Table 1).

Study species

Petrocephalus degeni is a small mormyrid (standard length of 8.1 cm, Kramer et al. 2012) reported from sites within the Lake Victoria basin of East Africa (Ogutu-Ohwayo 1993; Chapman et al. 1996; Kramer et al. 2012). Weakly electric mormyrid fish generate an electric field around their body by discharging their electric organ (Lissmann 1951), which they use to navigate and forage without relying on their visual sense (Lissmann 1958; Lissmann and Machin 1958) and to communicate with conspecifics (Moller 1970; Moller and Bauer 1973). Despite the presumed high energetic costs that are associated with active electrosensation (Salazar et al. 2013; Lewis et al. 2014), swamp-dwelling *P. degeni* are remarkably hypoxia-tolerant. Previous studies have characterized a suite of hypoxia adaptations in this species, such as a large gill surface area, a low RMR, a low P_{crit} , and a strong ability to maintain RMR at low environmental PO₂ (Chapman and Chapman 1998; Clarke et al. 2020). Clarke and colleagues (Clarke et al. 2020) found that long-term normoxia exposure of swamp-dwelling *P. degeni* leads to increases in P_{crit} and decreases in oxy-regulatory capacity.

Fish collection and normoxia acclimation treatment

Petrocephalus degeni were captured from a small lagoon in the Lwamunda Swamp (Petro Lagoon, 0°19'07"S, 31°56'48"E, Fig. A1). Fish were located using an electric fish finder that detects EODs and converts them into acoustic signals via a hand-held speaker (RadioShack Corp., USA). Fish were then captured using dip nets and transported to the nearby field research station for the normoxia acclimation treatment and experiments. For normoxia

Table 1 Water conditions at Petro Lagoon and during the period of the laboratory normoxia acclimation at the research station. DO, dissolved oxygen. Measurements from Petro Lagoon were taken between 8 am and 1 pm on 16 occasions between May and June 2018 ($n=4$) and July and September 2019 ($n=12$). All measurements were taken using handheld measurement devices. Values are expressed as mean \pm sd and range

	pH	Conductivity ($\mu\text{S cm}^{-1}$)	Temperature ($^{\circ}\text{C}$)	DO (kPa)	DO (mg L ⁻¹)
Petro lagoon	5.2 \pm 0.1 (5.0–5.6)	11 \pm 2 (10–20)	23.0 \pm 1.3 (21.4–28.2)	2.43 \pm 1.85 (0.38–7.62)	0.99 \pm 0.75 (0.16–3.16)
Normoxia acclimation	6.6 \pm 1.1 (4.2–7.8)	41 \pm 9 (20–80)	22.6 \pm 1.5 (19.6–25.9)	16.95 \pm 0.89 (11.73–18.07)	7.02 \pm 0.43 (4.89–7.71)

acclimation, fish were housed at the field research station in groups of 4–14 fish in plastic coolers ranging from 30 to 90 L in volume at an approximately equal density of ca. 1 fish per 7 L of water. Each cooler was connected to air pumps that supplied a continuous air-flow into the water to maintain normoxic conditions (Table 1). Water was replenished regularly with water from Lake Nabugabo to maintain pH and conductivity at stable levels. Fish were held at ambient temperature and were fed daily with a small amount of earthworms and live mosquito larvae. Fish that were used for respirometry, blood sampling, and gill measurements were split into three acclimation groups: short-term (2–6 days, $n=6$, body mass, $M=2.4\pm 0.4$ g), medium-term (30–36 days, $n=8$, $M=2.4\pm 0.7$ g), and long-term normoxia exposure (62–75 days, $n=7$, $M=2.4\pm 0.6$ g). Fish that were used only for blood samples were split into five normoxia acclimation groups covering a comparable range of acclimation duration: 1 day ($n=12$, $M=2.7\pm 0.6$ g), 10 ($n=10$, $M=2.3\pm 0.4$ g), 30 ($n=4$, $M=2.3\pm 0.7$ g), 50 ($n=9$,

$M=2.3\pm 0.5$ g), and 70 days ($n=11$, $M=2.2\pm 0.6$ g) of normoxia exposure. In addition to these, blood was sampled from a small subset of fish within 10 min after capture from the swamp lagoon ($n=5$, $M=2.9\pm 1$ g).

Respirometry

A combination of intermittent-flow and closed respirometry was used to measure the metabolic rate and the P_{crit} of *P. degeni*. We used a flow respirometer that creates a current within the respirometry circuit to ensure the mixing of water and the precise measurement of PO_2 (Svendsen et al. 2016). As fish used small movements to maintain their position against the current in the respirometry chamber; we assume that they operated at RMR rather than standard metabolic rate (Chabot et al. 2016a, b; Rosewarne et al. 2016).

The respirometer consisted of a water circuit made of plastic tubing (12 mm inner diameter) connected to an acrylic glass tube (36 mm

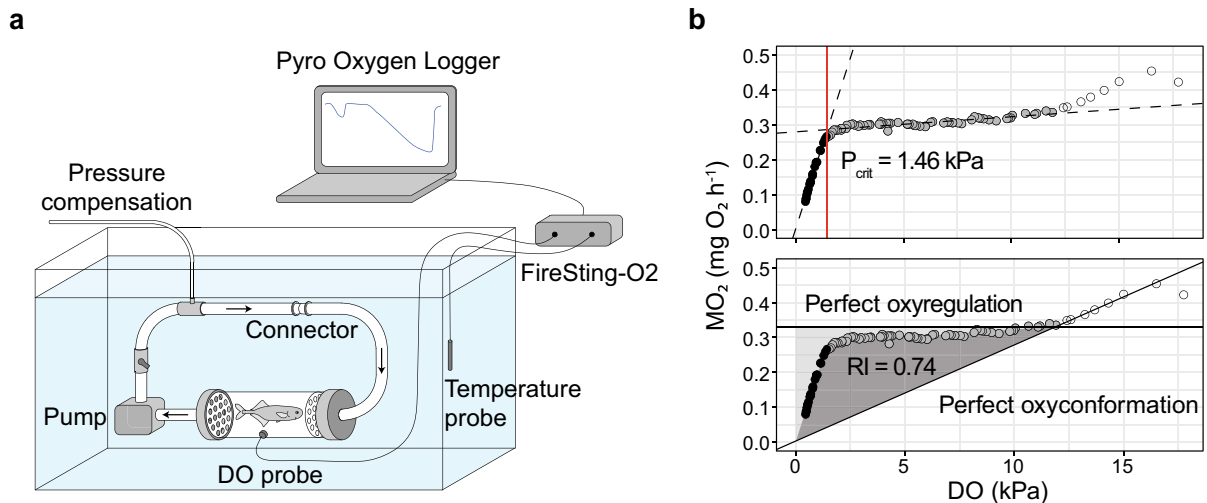


Fig. 1 Schematic of respirometry setup and example measurements. **a** The respirometer consisted of a cylindrical acrylic glass chamber that contained the fish and an optical dissolved oxygen (DO) sensor spot. Water was pumped through the chamber in a recirculation loop that could be opened at a connector to allow water exchange with the surrounding water bath. DO was measured in the respirometry chamber, and temperature was measured in the water bath. Temperature and DO data were recorded on a PC using the software Pyro Oxygen Logger. **b** Examples of critical oxygen tension (P_{crit} , top) and regulation index (RI, bottom) and their computation from respirometry data. White circles show data points that were

excluded from analysis due to high overall respiration at DO above 12 kPa (see methods). Grey circles show oxygen consumption rate ($\dot{M}O_2$) during oxyregulation (before P_{crit}), and black circles show $\dot{M}O_2$ during oxyconformation. Dashed lines represent linear regressions that were fitted to the oxyregulation and oxyconformation sections of the data. The red vertical line at their intersection represents the P_{crit} . Solid lines in the bottom panel represent linear trends for perfect oxyregulation (horizontal) and oxyconformation (diagonal). The RI is derived by dividing the area under the $\dot{M}O_2$ -versus-DO curve of the fish that lies above the trend for oxyconformation (dark grey) by the area for perfect oxyregulation (light grey)

inner diameter) that served as respirometry chamber to hold the fish, and a pump (Universal 1005; EHEIM, Germany; Fig. 1a). Total water volume in the respirometer was 280 ml. Water flow was regulated with a shutoff valve (Absperrhahn; EHEIM, Germany) to a rate of ca. 760 ml per minute. A 3D-printed baffle was inserted into the PVC fittings that connected the plastic tubing to the respirometry chamber to homogenize water flow inside the chamber. The complete setup was submerged in an aerated water bath inside a cooler that was covered with a dark cloth to prevent disturbance and minimize animal stress. A plastic hose connector was used to manually open and close the respirometry circuit to allow for intermittent exchange of water between the respirometry circuit and the water bath. Pressure fluctuations during the opening and closing of the circuit were compensated by a thin plastic tube (4 mm inner diameter, 2 m length and filled with water to prevent diffusion of oxygen into the respirometry circuit) that was connected to the respirometry circuit via a T-connector. Oxygen partial pressure and temperature were measured with an optical oxygen meter (FireSting-O2; PyroScience, Germany) connected to a temperature sensor in the water bath and a fiberglass probe that was fixed to a DO sensor spot inside the respirometry chamber. Values were recorded at a sample interval of 1 s using the software Pyro Oxygen Logger ver. 3.315 (PyroScience, Germany). Fish were not fed for 48 h prior to trials to ensure a post-absorptive state (Chabot et al. 2016b). The respirometry setup was filled with water from Lake Nabugabo in the afternoon before trials and temperature, and pH and conductivity were matched to the fish housing parameters. The respirometry circuit was closed without a fish, and DO was measured for 30 min to quantify pre-trial background respiration. The circuit was then opened to reoxygenate the water, and a fish was captured from the acclimation tank and inserted into the respirometry chamber. The chamber was connected to the plastic tubing, the respirometry circuit was opened to allow water exchange with the water bath, and the fish were left to acclimate overnight to the respirometry chamber. Following 12 h of acclimation to the respirometry setup, RMR was measured the next day using intermittent-flow respirometry: the respirometry circuit was closed for 45 min or until PO₂ dropped below

15.4 kPa, whichever occurred first, and then opened to allow PO₂ to return to normoxic levels. This procedure was repeated for a total of three measurements. After completion of the third measurement, the water circuit remained closed and PO₂ was allowed to drop until the P_{crit} was reached. Afterward, the respirometry circuit was opened to allow PO₂ to return to a normoxic level and the fish were given a recovery time of 60 min, during which all fish regained normal swimming behavior. Then, fish were removed from the respirometry chamber and anesthetized for blood sampling, followed by preservation (see gill morphometrics). The respirometry loop was closed for another 30 min to measure post-trial background respiration. Throughout respirometry experiments, the swimming behavior was observed at regular intervals. The behavior was categorized as stationary (fish maintains position in chamber with small swimming motions), actively swimming (fish changes position/orientation in chamber), and burst swimming. All but one fish showed resting behavior at high PO₂. When DO levels approached the P_{crit} , all fish increased activity (e.g., changed orientation in the chamber); after P_{crit} and before loss of equilibrium, fish showed burst swimming. Two trials were aborted before the fish reached their P_{crit} due to extremely slow decrease of PO₂, and one fish was excluded from analysis due to a high and erratic swimming activity at high PO₂, resulting in a total of 18 respirometry trials that were analyzed.

Routine metabolic rate (RMR)

Oxygen consumption rate ($\dot{M}O_2$) was calculated as the slope of linear DO decline during intermittent respirometry loops at PO₂ above 15.4 kPa. Routine metabolic rate was calculated by averaging $\dot{M}O_2$ values over the number of intermittent respirometry loops for each fish. Subsequently, RMR was adjusted for background respiration assuming a linear increase of background respiration between pre-trial and post-trial control measurements. Routine metabolic rate scaled with body mass and therefore was adjusted to a common mean body mass (M) before assessing the effect of normoxia exposure. An allometric power scaling equation was adapted from Ihssen et al. (1981) for the adjustment:

Table 2 Results of ANCOVA on allometric relationships of log-transformed variables and slope of linear regression

	Effect: log M			Effect: acclimation			Interaction			Slope
	<i>F</i> (1, 12)	<i>P</i>	η_g^2	<i>F</i> (1, 12)	<i>P</i>	η_g^2	<i>F</i> (1, 12)	<i>P</i>	η_g^2	<i>b</i>
log RMR	31.647	<0.001	0.725	1.302	0.308	0.178	1.306	0.307	0.179	1.69
log P_{crit}	7.825	0.016	0.395	2.106	0.176	0.251	0.683	0.524	0.102	0.38
	<i>F</i> (1, 14)			<i>F</i> (2, 14)			<i>F</i> (2, 14)			
log FL	33.396	<0.001	0.705	6.204	0.012	0.470	0.083	0.921	0.012	0.48
log FN	18.273	<0.001	0.566	1.202	0.330	0.147	0.011	0.989	0.002	0.20
log HA	34.018	<0.001	0.708	6.067	0.013	0.464	0.027	0.974	0.004	0.62

b, slope of bilogarithmic regression between trait and body mass; *M*, body mass; *F*, F-statistic; *FL*, gill filament length; *FN*, gill filament number; *HA*, gill hemibranch area; P_{crit} , critical oxygen tension; *RMR*, routine metabolic rate; η_g^2 , generalized eta squared for effect size. Numbers in brackets indicate degrees of freedom in the numerator and denominator. Bold numbers indicate statistically significant outcomes

$$Variable_{adj} = Variable_{obs} * \left(\frac{M_{mean}}{M_{obs}} \right)^b \quad (1)$$

where the scaling exponent *b* is equal to the slope of the bilogarithmic linear regression between RMR and *M*. Prior to adjustment, the effect of normoxia acclimation on the slope of the bilogarithmic relationship between RMR and *M* was tested using ANCOVA with log *M*, acclimation group, and their interaction as effects (Table 2). As this resulted in a non-significant outcome for the interaction term, the slope of the bilogarithmic linear regression was used without blocking for acclimation group. The effect of normoxia exposure was tested on mass-adjusted RMR using ANOVA with hypoxia acclimation group as grouping variable followed by a Tukey post-hoc test in case of a significant result.

Critical oxygen tension (P_{crit})

Critical oxygen tension is defined as the oxygen partial pressure at which an organism can no longer maintain a stable rate of oxygen consumption and begins to conform to environmental DO (Beamish 1964; Ultsch et al. 1978). To determine the P_{crit} , $\dot{M}O_2$ values were calculated repeatedly over short intervals for the duration of the closed phase of the respirometry trial (Fig. 1b). The size of the intervals over which $\dot{M}O_2$ is calculated is an important variable in this procedure as large intervals increase the reliability but decrease the temporal resolution of the results. As trial duration varied based on fish size and $\dot{M}O_2$, the size of intervals over which $\dot{M}O_2$ was calculated was adjusted to one-tenth of trial duration

at the highest PO_2 (to compensate for lower precision of optical DO meters at high DO concentrations), and decreased in 10 equal steps to one-100th of trial duration at the lowest PO_2 . All trials showed elevated $\dot{M}O_2$ at PO_2 above 12 kPa with a high degree of oxyconformation, a phenomenon that has also been observed in previous respirometry studies conducted at this site with *P. degeni* (Clarke et al. 2020) and another mormyrid, *Marcusenius victoriae* (Moulton et al. 2020). We conducted long-term control measurements with no fish present in the respirometer and detected that background respiration followed a similar nonlinear curve with higher respiration rates at high PO_2 (see Supplementary Material, Fig. A2). Thus, we attribute this phenomenon to biological activity in the lake water that was used for respirometry and used only $\dot{M}O_2$ values at PO_2 below 12 kPa for P_{crit} determination. To quantify P_{crit} , we compared broken-stick regression (Yeager and Ultsch 1989), nonlinear regression (Marshall et al. 2013), and the P_{crit} -alpha method (Seibel et al. 2021). Nonlinear regression tended to produce erroneous results when $\dot{M}O_2$ increased toward P_{crit} and yielded the highest P_{crit} values of the three approaches in the medium-term acclimation group (see Supplementary Material, Fig. A3). There was no significant difference between the other approaches, and we chose to use the P_{crit} derived by broken-stick regression because it showed the smallest variability within each acclimation group and was closely correlated to the P_{crit} -alpha. Two linear regressions were fitted to the $\dot{M}O_2$ values corresponding to the phases of the trial when the fish behaved as oxyregulator or as oxyconformer (Fig. 1b). The intersection between the two linear regressions marked the P_{crit} , at which the $\dot{M}O_2$ became strongly dependent on external DO

concentration. Critical oxygen tension scaled with body mass and was adjusted to a common mean body mass as described above using Eq. (1). Acclimation group had no effect on the slope of the bilogarithmic relationship between M and P_{crit} (Table 2); thus, P_{crit} was adjusted without blocking for acclimation group. The effect of normoxia exposure on mass-adjusted P_{crit} was tested using ANOVA with hypoxia acclimation group as grouping variable followed by a Tukey post-hoc test in case of a significant result.

Regulation index (RI)

The RI is a measure of the oxyregulatory ability of an organism at declining PO_2 (Mueller and Seymour 2011). To calculate RI, the area under the $\dot{M}O_2$ versus PO_2 curve of a fish during the closed respirometry trial is estimated (A_{trial}) as well as the area for perfect oxyregulation (i.e., under the horizontal line intersecting $\dot{M}O_2$ at the highest PO_2 , A_{reg}) and the area for perfect oxyconformation (i.e., under a diagonal line through the origin and $\dot{M}O_2$ at the highest PO_2 , A_{conf} , Fig. 1b). The RI is then calculated using Eq. (2). A RI close to 1 indicates that the fish behaves mostly as an oxyregulator, whereas a value close to 0 indicates that $\dot{M}O_2$ mostly conforms to PO_2 . As for P_{crit} determination, we used only $\dot{M}O_2$ at PO_2 below 12 kPa for RI determination. The effect of normoxia exposure on RI was tested using ANOVA with hypoxia acclimation group as grouping variable followed by a Tukey post-hoc test in case of a significant result.

$$RI = \frac{A_{trial} - A_{conf}}{A_{reg} - A_{conf}} \tag{2}$$

Gill morphometrics

Gills were sampled from fish that were used in respirometry trials. Approximately 60 min after respirometry trials, fish were submerged in clove oil solution until deep anesthesia was reached. This was marked by complete cessation of motor functions, which occurred at 3–5 min after submersion. Fish were then weighed (Ohaus Scout Pro SP401), and standard body length was measured with a slide gauge. Fish were sacrificed by drawing blood from the caudal vein for blood parameter measurement (see below). Euthanized specimens were preserved in 4% formaldehyde for 24 h and soaked in distilled water for 24 h prior to gill morphology measurement. Afterward, all four gill arches on the left body side were extracted using a needle scalpel and carefully rinsed with distilled water to remove residual tissue. Both sides of each arch (i.e., two hemibranches) were photographed under magnification using a portable microscope camera (WiFi USB Mikroskop; ROTEK, China).

As some gill arches were damaged during the extraction process, only the first two gill arches (i.e., the four hemibranches on the outer two gill arches) were used for analysis. We used ImageJ ver. 1.53c (<https://imagej.nih.gov/ij/>) and followed standard

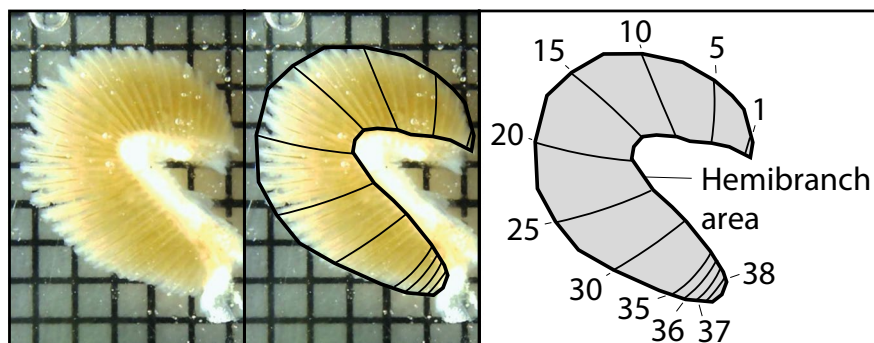


Fig. 2 Procedure for gill morphometric estimation on one hemibranch. Filament length was measured on every 5th filament from the dorsal end of the hemibranch onward and approximated for filaments in between measurements as mean of the two closest measured filaments. Toward the ventral end,

the final filaments (here: filaments 35–38) were measured individually. Hemibranch area was measured by fitting a polygon to the area of the hemibranch that was covered by filaments (grey area). Measurements were performed using ImageJ

procedures to measure a suite of traits on each of the four hemibranches of the first two gill arches: number of filaments (FN), filament length (FL), and hemibranch area (HA; Fig. 2; Hughes 1984; Chapman et al. 1999; Crampton et al. 2008; Potts et al. 2021). Filament length was estimated by measuring the length of every fifth filament from the dorsal end of the hemibranch onward. The length of intermediate filaments (i.e., between two measured filaments) was approximated as the average length of the two closest neighboring filaments that were measured. The last filaments at the ventral end of the arch were measured individually because the length shows large changes at the end of the hemibranch. Finally, the lengths of all filaments from the four measured hemibranches were added together to represent FL. Hemibranch area was measured by fitting a polygon to the area of each measured hemibranch that was covered by gill filaments. The results of the four hemibranches were added together to represent HA. The number of filaments was counted on all four measured hemibranches and added together to represent FN. Due to the missing third and fourth gill arches in our analysis, our measurements do not represent the total FN, FL, and HA of the fish gills. However, the values from the first two arches were strongly correlated to the total values of all gill arches on a subset of five fish (Fig. A4). A similar correlation of morphometrics between gill arches has been found by another study (Sharpe and Chapman 2018). Thus, we assume that our measurements are meaningful indices of total gill FN, FL and HA, which allows us to compare different acclimation treatments. In conformity with findings from other fish species (Palzenberger and Pohla 1992), gill morphometrics scaled with body mass and the resulting values were adjusted to a common mean body mass as described above using Eq. (1). Acclimation group had no effect on the slope of the bilogarithmic relationship between M and gill morphometrics; thus, gill morphometrics were adjusted without blocking for acclimation group (Table 2). The HA measurements violated the assumption of homogeneity of variance across groups (Levene's test: $n=20$, $F=8.71$, $DF_1=2$, $DF_2=17$, $P=0.002$). However, as there are no robust alternatives to ANCOVA with more than two groups available in R and residuals were normally distributed, the effect of acclimation group on the slope of the

bilogarithmic relationship between M and HA was tested with ANCOVA and found to be non-significant (Table 2). The effect of normoxia exposure on FL and FN was estimated using ANOVA on mass-adjusted values with hypoxia acclimation group as grouping variable followed by a Tukey post-hoc test in case of a significant result. The effect of normoxia exposure on HA was estimated using the Kruskal–Wallis test with hypoxia acclimation group as grouping variable followed by a Wilcoxon post-hoc test with Bonferroni correction of P -values in case of a significant result.

Blood sample collection

Hemoglobin and lactate concentrations were measured in fish that were used in respirometry and in fish that underwent undisturbed normoxia acclimation. Due to the field background of our study, we could not employ laboratory gold standards for blood parameter measurement but used handheld devices (hemoglobin: DiaSpect Tm; lactate: Lactate Scout+; both: EKF Diagnostics, UK). Both devices have been evaluated and found to deliver sufficient accuracy and reliability particularly for comparisons of repeated measurements (Bonaventura et al. 2015; Ranjan et al. 2020). Blood samples from undisturbed fish were collected in 2018. Samples were taken directly after capture from the swamp and after 1, 10, 30, 50, and 70 days of normoxia exposure. Fish were captured with a dipping net and immediately submerged in clove oil solution until deep anesthesia was reached, as described above. Fish were then weighed, and standard body length was measured with a slide gauge. The caudal peduncle was severed with a scalpel, and blood was collected from the caudal vein with heparinized capillary tubes. Care was taken to avoid unnecessary handling stress (e.g., chasing), and all blood samples were collected within 5–10 min of initial handling of the fish. Hemoglobin and lactate concentrations were measured in full blood immediately after extraction according to the instructions of the measurement devices.

We additionally sampled blood from the fish that were used in respirometry trials to correlate respirometry data, gill morphometrics, and blood parameters. Blood was sampled ca. 60 min after the respirometry trial using the same anesthesia and measurement protocol as described above but with a different extraction

technique. Previously, severance of the caudal peduncle had in some cases yielded insufficient amounts of blood for measurement. Thus, blood was extracted directly from the caudal vein using syringes that were flushed with a heparin-PBS solution. However, this technique did not increase sampling success but introduced a systematic dilution of measured lactate and Hb concentrations due to residual heparin-PBS solution in the syringe and the small volume of blood that was collected (in many cases <20 µl).

The results from these two cohorts of fish were analyzed separately to account for the different extraction protocols that were used and the possible effect of acute hypoxic stress during respirometry trials on blood hemoglobin and lactate. The effect of normoxia exposure time on full blood hemoglobin and lactate was estimated using linear regression for data from undisturbed fish and ANOVA for data from fish that were used in respirometry trials, followed by a Tukey post-hoc test in case of a significant result.

Correlations between traits

Traits that were measured on the same fish were tested for correlations using Spearman correlation analysis (Fig. A5). As the number of fish on which all traits were sampled successfully was low ($n=11$)

and there was no correlation between blood parameters and other respiratory traits, correlation analysis was repeated with fish that were used in respirometry trials and gill morphometrics ($n=16$).

Statistical analysis

All statistical analyses were conducted using R ver. 4.1.0 (<https://www.r-project.org/>). Data from gill measurements and respirometry trials were aliased and randomized prior to analysis to minimize experimenter bias. If not otherwise stated, all values represent means ± standard deviation. Respirometry data were analyzed using the “respirometry” package for R (<https://cran.r-project.org/package=respirometry>). Figures were created using R and formatted using Adobe Illustrator CC 2018 (Adobe Inc., San Jose, CA, USA). A significance level of $P<0.05$ was used for all statistical procedures.

Results

Respirometry

Across all three acclimation groups, RMR and P_{crit} scaled with body mass and were mass-adjusted to a

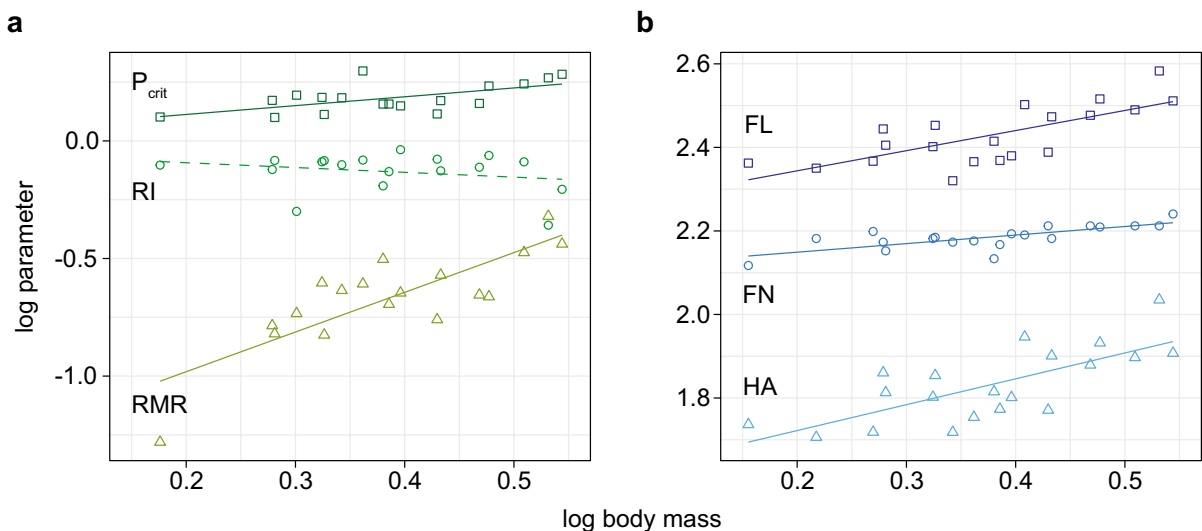


Fig. 3 Allometric scaling of respirometric traits and gill morphometrics. **a** Bilogarithmic relationship among routine metabolic rate (RMR), critical oxygen tension (P_{crit}), regulation index (RI), and body mass. **b** Bilogarithmic relationship

among gill filament length (FL), filament number (FN), hemibranch area (HA), and body mass. All values were log-transformed, and solid lines indicate significant linear regressions ($P < 0.05$); see Table 2 for regression slopes

Table 3 Overview of biometric data and morpho-physiological traits of *P. degeni* measured in 2019

	Acclimation group		
	Short-term	Mid-term	Long-term
Days accl	2–6	30–36	62–75
<i>N</i>	6	9	7
Sex ratio	3:3	4:5	4:3
<i>M</i> (g)	2.5 ± 0.4 (1.9–3.0)	2.4 ± 0.7 (1.4–3.4)	2.4 ± 0.6 (1.5–3.5)
SL (cm)	5.4 ± 0.4 (4.9–5.9)	5.5 ± 0.5 (4.7–6.2)	5.5 ± 0.5 (4.8–6.4)
Respirometry			
RMR (mg O ₂ h ⁻¹)	0.25 ± 0.07 (0.16–0.33)	0.25 ± 0.05 (0.17–0.28)	0.20 ± 0.06 (0.12–0.29)
<i>P</i> _{crit} (kPa)	1.48 ± 0.11 (1.38–1.63)	1.61 ± 0.22 (1.36–2.04)	1.51 ± 0.15 (1.27–1.69)
RI	0.79 ± 0.08 (0.64–0.87)	0.69 ± 0.17 (0.44–0.83)	0.78 ± 0.10 (0.62–0.92)
Gill morphology			
FL (mm) ^a	284 ± 16 (262–304)	288 ± 29 (239–326)	244 ± 21 (220–273)
HA (mm ²) ^a	73 ± 5 (66–78)	74 ± 11 (59–88)	60 ± 3 (56–65)
FN ^a	151 ± 8 (136–157)	155 ± 5 (146–165)	157 ± 7 (147–167)
Blood			
Hb (g dl ⁻¹)	8.1 ± 2.6 (4.6–12.0)	6.2 ± 1.6 (4.2–7.8)	5.3 ± 1.2 (4.1–7.0)
Lactate (mM)	5.0 ± 1.7 (2.6–7.7)	3.3 ± 1.3 (1.8–4.2)	5.3 ± 1.6 (3.3–7.1)

M, body mass; *FL*, gill filament length; *FN*, gill filament number; *HA*, hemibranch area; *Hb*, hemoglobin concentration; *RMR*, routine metabolic rate; *P*_{crit}, critical oxygen tension; *RI*, regulation index; *SL*, standard length (body length excluding caudal fin). Values are expressed as mean ± sd (range) except for sample size *n*, which is expressed as absolute number, and sex ratio, which is expressed as female:male ratio. Traits that scaled with body mass (*RMR*, *P*_{crit}, *FL*, *FN*, and *HA*) were adjusted to a mean body mass. Note that sample sizes differ between measurement types: *RMR*, *P*_{crit} and *RI* were measured on 18 fish; *FL*, *HA*, and *FN* on 20 fish, hemoglobin on 12 fish, and lactate on 11 fish

mean body mass of 2.49 g (Table 2, Fig. 3a). *RI* did not correlate with body mass.

Normoxia acclimation did not affect the respirometric performance of *P. degeni* (Table 3, Table A2, Fig. 4). Across acclimation groups, mass-adjusted *RMR* averaged 0.23 ± 0.06 mg O₂ h⁻¹, mass-adjusted *P*_{crit} averaged 1.56 ± 0.20 kPa, and *RI* averaged 0.72 ± 0.12. The mean water temperature during respirometry trials was 24.0 ± 0.6 °C, and there was no correlation between *RMR*, *P*_{crit}, or *RI* and water temperature (linear regression: *P* > 0.05).

Gill morphometrics

Across acclimation groups, *FL*, *HA*, and *FN* scaled with body mass and were mass adjusted to a mean body mass of 2.44 g (Table 2, Fig. 3b). Mean mass-adjusted *FL* and *HA* were significantly reduced in fish after long-term (62–75 days)

normoxia acclimation compared to fish in short-term (2–6 days) and mid-term (30–36 days) normoxia acclimation (Table A2, Fig. 5a, b). Overall, *FL* decreased by 14% from 283.7 ± 15.6 to 243.9 ± 20.6 mm, and *HA* decreased by 18% from 73.2 ± 4.5 to 60.0 ± 3.7 mm² from short-term to long-term normoxia acclimation. The number of gill filaments did not change during normoxia acclimation, with an adjusted mean of 154 filaments per fish (Table 3, Table A2, Fig. 5c).

Blood hemoglobin and lactate concentrations

Neither hemoglobin or lactate concentration in the blood scaled with body mass. In fish that underwent undisturbed normoxia acclimation, mean hemoglobin concentration was highest shortly after capture and decreased significantly over the course of normoxia acclimation by 17% from 10.8 ± 0.6 to

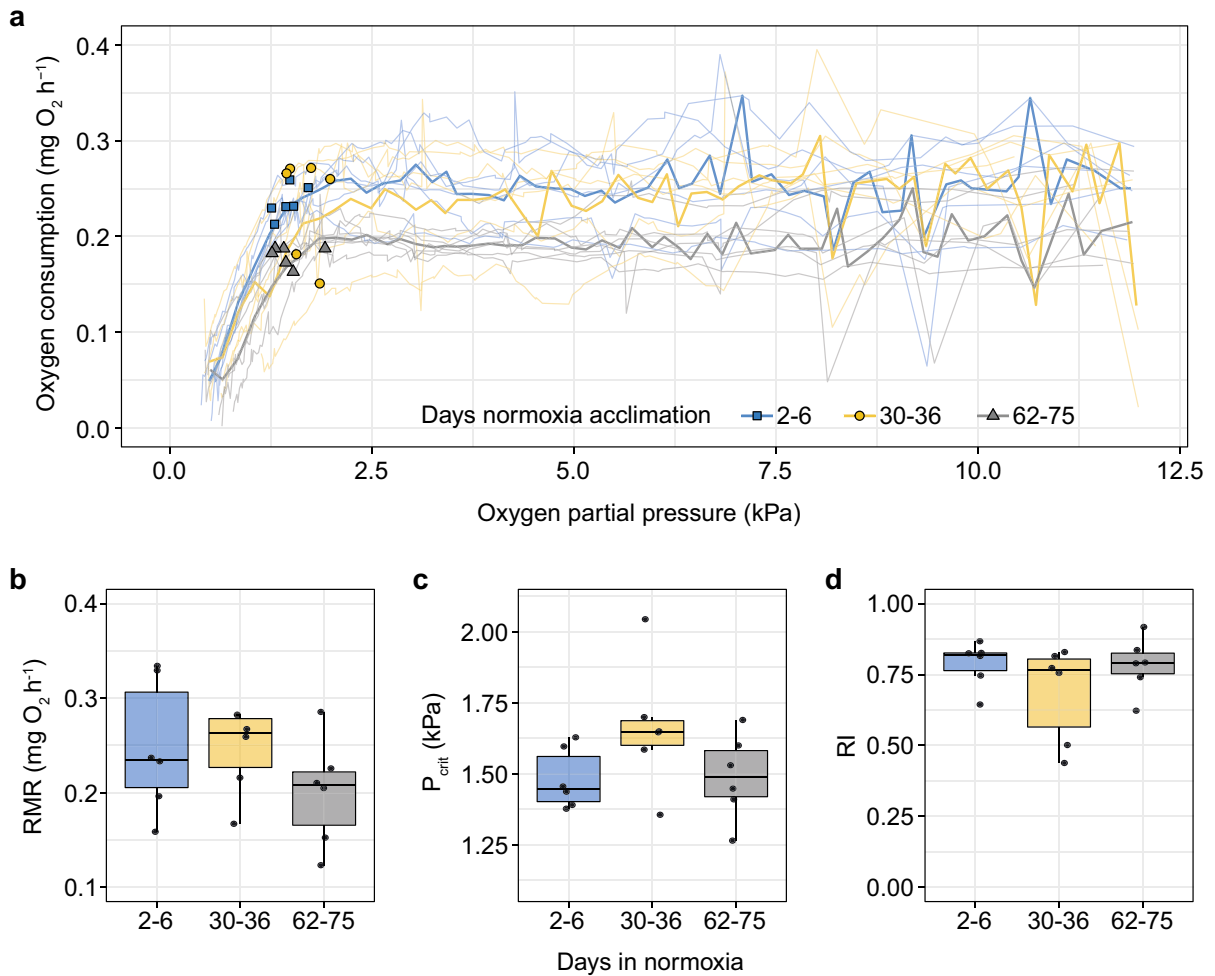


Fig. 4 Respirometry performance of *P. degeni* during normoxia acclimation ($n=18$). **a** Raw oxygen consumption rates as function of PO_2 during closed respirometry trials. Thin lines represent oxygen consumption rates of individual fish, and bold lines represent average oxygen consumption rates per acclimation group, averaged over DO bins of 0.2 kPa. Filled symbols represent critical oxygen tensions of individual fish (P_{crit}). Extreme outliers with a distance of more than 3 times the interquartile range from the 1st to 3rd quartiles were

removed from this plot ($n=3$ data points). In all three acclimation groups, oxygen consumption rate showed a high degree of oxyregulation until P_{crit} was reached. **b–d** Mass-adjusted routine metabolic rate (RMR), mass-adjusted P_{crit} , and regulation index (RI) did not differ between acclimation groups. Note that RMR was measured at $\text{PO}_2 > 15.4$ kPa, whereas closed respirometry data was analyzed at PO_2 below 12 kPa due to nonlinear background respiration at $\text{PO}_2 > 12$ kPa

$8.45 \pm 1.0 \text{ g dl}^{-1}$ (Table A2, Fig. 6a). Fish that were used in respirometry trials showed a similar trend that was not significant (ANOVA: $P=0.121$, Table 3, Table A2, Fig. 6b).

Lactate was detectable at appreciable concentrations in all fish and showed no change over time (Table 3, Table A2, Fig. 6c, d). Lactate concentration averaged $6.58 \pm 1.63 \text{ mM}$ (undisturbed) and $4.67 \pm 1.67 \text{ mM}$ (after respirometry).

Correlation between traits

Blood parameters did not correlate with other traits and were excluded from correlation analysis after preliminary correlation tests (see methods, Fig. A5). Among the remaining traits, there was a strong positive correlation between FL and HA, and a negative correlation between FN and RMR (adjusted to a common mean body mass, Fig. 7).

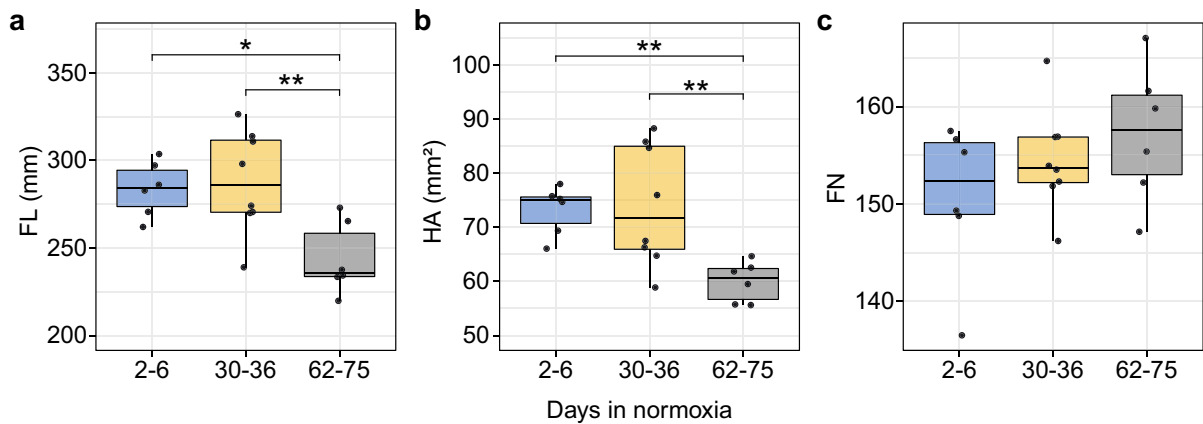


Fig. 5 Gill morphometrics of *P. degeni* during normoxia acclimation ($n=20$). **a**, **b** Mass-adjusted gill filament length (FL) and mass-adjusted gill hemibranch area (HA) were significantly lower in the long-term normoxia-acclimated fish than in the other acclimation groups. **c** Mass-adjusted gill fila-

ment number (FN) remained unchanged throughout normoxia acclimation. All metrics were measured on the first two arches of the left gill (see Table A2 for statistical test outcomes; $P < 0.05^*$, $P < 0.01^{**}$)

Discussion

The goal of this study was to explore the phenotypic plasticity of a naturally hypoxia-acclimated tropical freshwater fish in response to a change in DO environment. We measured routine metabolic rate (RMR), critical oxygen tension (P_{crit}), regulation index (RI), gill filament length (FL), gill filament number (FN), gill hemibranch area (HA), blood hemoglobin, and blood lactate concentrations in the mormyrid weakly electric fish, *P. degeni*, which were subjected to up to 75 days of normoxia acclimation upon capture from a hypoxic swamp. We show that swamp-dwelling *P. degeni* exhibit a strong oxyregulatory capacity, a high blood hemoglobin concentration, and phenotypic plasticity in blood hemoglobin concentration and gill size, both of which were significantly reduced after long-term normoxia exposure. There was no effect of normoxia acclimation on oxyregulatory ability and blood lactate concentration, which remained appreciably high even for the fish acclimated to normoxia the longest.

Hypoxia adaptations of swamp-dwelling *P. degeni*

We found indications for hypoxia adaptation in all measured traits. Blood hemoglobin concentration in fish that were sampled within 1 day of capture was high compared to other teleost fish (10.08 ± 0.61 g dl⁻¹, this concentration lies above

69% of values reported from 69 species from marine and freshwater habitats; Prosser et al. 1957; Filho et al. 1992; Chapman et al. 2002; Timmerman and Chapman 2004; Wells et al. 2005; Cook et al. 2011; Cook et al. 2013; Almeida et al. 2017). High hemoglobin concentration supports aerobic metabolism by enhancing the oxygen-carrying capacity and affinity of blood (Brauner and Val 2006). This finding confirms earlier measurements of high blood hemoglobin concentrations in swamp-dwelling *P. degeni*, which were considered preliminary due to their low sample size ($n=2$; Chapman et al. 2002).

On the oxygen-consumption side of their energy balance, *P. degeni* exhibited low RMR compared to the range of metabolic rates measured in teleost species, indicating a low tissue energy demand (Chapman and Chapman 1998). Further, fish showed a strong oxyregulatory capacity (i.e., a flat $\dot{M}O_2$ vs. PO_2 curve) and a low P_{crit} (compared to values compiled by Rogers et al. 2016), which is in agreement with previous studies that have reported similar values of RMR, P_{crit} , and RI in swamp-dwelling *P. degeni* (Chapman and Chapman 1998; Clarke et al. 2020).

The positive allometric relationship between P_{crit} and body mass indicates that smaller fish might be at an advantage when environmental PO_2 reaches critical levels. Interestingly, RMR scaled more steeply than P_{crit} with body mass, and RI did not change significantly with body mass. This suggests that larger

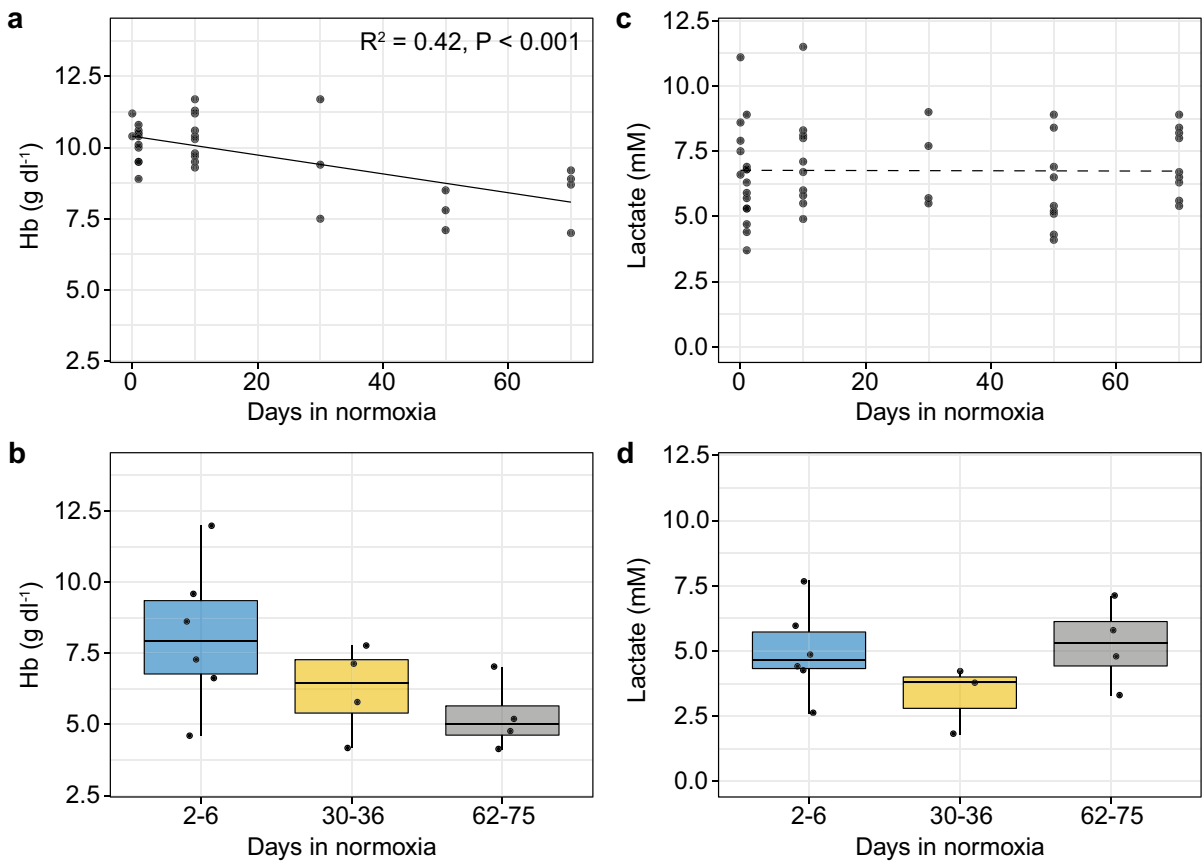


Fig. 6 Blood hemoglobin and lactate concentration during normoxia acclimation. Top row: fish that underwent undisturbed normoxia exposure. Bottom row: fish that were used for respirometry. **a** Full-blood hemoglobin (Hb) concentration decreased significantly during normoxia exposure in fish that were left undisturbed ($n=32$, Pearson correlation). **b** This

trend was similar but not significant in fish that were used for respirometry ($n=14$). **c, d** Lactate concentration was not affected by normoxia exposure in both cohorts (undisturbed: $n=49$, respirometry: $n=13$). Solid lines indicate significant linear regressions; dashed lines indicate non-significant linear regressions

fish might be more efficient in taking up and utilizing oxygen for processes other than homeostasis, such as growth and reproduction, and thus might benefit more from higher DO conditions. However, given that *P. degeni* are generally small (Chapman and Chapman 1998; Chapman and Hulen 2001; Chapman et al. 2002; Clarke et al. 2020), within-species variation of body size likely only plays a minor role for the hypoxia tolerance of these fish.

In a recent study, we found that PO_2 in the swamp habitat of *P. degeni* drops to sub- P_{crit} levels, especially during the night when they are most active (Mucha et al. 2022). It is likely that fish employ behavioral responses such as increased gill ventilation rate and ASR to increase oxygen uptake and

mitigate hypoxia during these periods (Chapman and Chapman 1998). Given the prevalence of hypoxia in their habitat and our finding of high blood lactate in fish directly after capture from their habitat, it also seems possible that *P. degeni* routinely utilize anaerobic metabolic pathways (Richards 2009). However, further experiments that control for confounding factors, such as capture stress and natural stressors, are necessary to elucidate the role of anaerobic metabolism for the survival of *P. degeni*.

Effect of normoxia acclimation

Normoxia acclimation had only moderate long-term effects on the respiratory physiology of *P. degeni*.

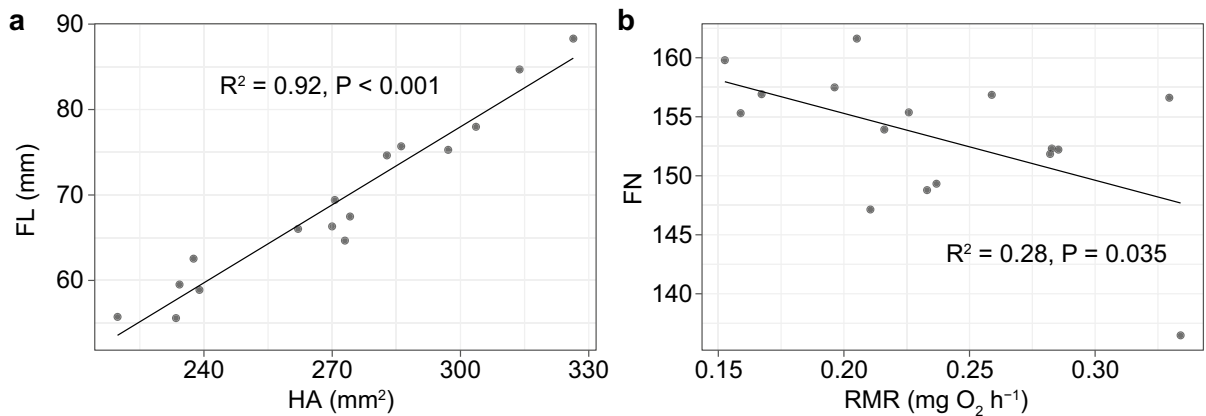


Fig. 7 Correlation between mass-adjusted respiratory traits ($n=16$). **a** Gill filament length (FL) and gill hemibranch area (HA) were strongly positively correlated (Spearman rank cor-

relation). **b** Gill filament number (FN) and RMR were weakly negatively correlated (Pearson correlation)

Exposure to normoxia for 62–75 days led to a significant decrease of blood hemoglobin concentration and gill size (as estimated from the first two gill arches) but did not affect routine oxygen consumption, P_{crit} , oxy-regulatory capacity, or blood lactate concentration.

The observed phenotypic shift could be a mechanism to reduce the metabolic cost of homeostasis in normoxia. Reduced synthesis of hemoglobin frees metabolic energy for other processes and, if it coincides with a reduced hematocrit and red blood cell count, reduces the energetic cost for cardiac output (Gallaughier et al. 1995) and red blood cell maintenance (Wells and Baldwin 2006). The reduction of FL and HA is likely correlated with a decrease in gill surface area (Palzenberger and Pohla 1992; Crispo and Chapman 2010), which benefits the retention of ions in the body and reduces the cost for osmoregulation. As *P. degeni* were captured from and held in almost completely ion-free water, osmoregulation is likely an important factor in their metabolic energy balance. Additionally, the water pH in the holding tanks ranged closer to physiological values than in the natural habitat, which may have resulted in reduced energy cost of acid–base regulation during normoxia acclimation. However, as we would expect an effect of pH to manifest predominantly in the ion permeability of the gill epithelium and not so much in gill size, we suggest that the observed plasticity in gill size was primarily due to varying oxygen levels in *P. degeni*.

The reduction of gill size shown here is smaller but close to the difference in gill size that was measured

in *P. degeni* from the normoxic waters of Lake Kayanja compared to conspecifics from Lwamunda Swamp (21% shorter total gill FL in fish from the Lake, Chapman and Hulen 2001). Thus, phenotypic plasticity is a possible mechanism among others, such as transgenerational plasticity and genetic divergence, underlying these interdemic differences. The gill-oxygen limitation theory posits that gill surface limits growth in larger fish and has far-reaching implications for key aspects of their biology, such as thermal tolerance, feeding, reproduction, and metabolism (Pauly 1981, 2021). In light of this theory, plasticity of gill surface could be an important mechanism for adult fish to buffer environmental fluctuations and survive in their habitats, e.g., during seasonal changes in temperature and DO.

Our results show that P_{crit} , RI, and RMR were not affected by normoxia acclimation, which was somewhat surprising given the observed reduction of gill size and hemoglobin concentration. In other species, P_{crit} has been associated with traits related to oxygen extraction and carrying capacity, such as hematocrit (Cook et al. 2011), gill surface area (Childress and Seibel 1998), and hemoglobin- O_2 -binding affinity (Mandic et al. 2009). It is possible that changes in these and other traits, which were not measured here, compensated for the reduction of gill size and hemoglobin concentration. Alternatively, pre-acclimation gill size and hemoglobin concentration may not have been limiting oxygen uptake but rather fueling energy-intense natural behaviors such

as foraging, migration, and predator avoidance. This is in agreement with the weak negative correlation that we found between FN and RMR, which also suggests that oxygen uptake is not limited by gill size. Unfortunately, we could not include a control group of fish that were housed under hypoxic conditions to control for the effect of laboratory housing-induced stress on the measured traits. Metabolic rate estimates usually positively correlate with stress levels (Clark et al. 2013; Nadler et al. 2016; Norin et al. 2018); thus, housing stress may have prevented changes in P_{crit} , RI, and RMR, masking the effect of normoxia on the medium- and long-term acclimation groups. In a prior study, Clarke and colleagues (Clarke et al. 2020) exposed swamp-dwelling *P. degeni* to similar housing conditions and found that RMR likewise remained unaffected after long-term normoxia. However, contrasting with our results, they also found an increase in P_{crit} of 36% and a decrease in RI of 12% after long-term normoxia exposure. Given that the fish sampled by Clarke and colleagues were on average almost twice as heavy as the fish used here, it is possible that our fish were more susceptible to housing stress or that larger fish respond more strongly to normoxia acclimation regarding their P_{crit} and RI than smaller fish.

Another unexpected finding was that lactate concentration in the blood of *P. degeni* remained high (6.6 ± 1.6 mM) throughout normoxia acclimation. Plasma lactate concentrations in fish typically range between 0 and 2 mM under normoxic or unstressed conditions (e.g., Virani and Rees 2000; Chippari-Gomes et al. 2005; Wells and Baldwin 2006; Regan et al. 2017). However, lactate concentrations similar to the levels we observed have been reported in fish that experience physiological stress from acute hypoxia exposure (e.g., Maxime et al. 2000; Routley et al. 2002; Chippari-Gomes et al. 2005; Cook and Herbert 2012; Regan et al. 2017) or strenuous exercise and capture stress (e.g., Frisch and Anderson 2005; Behrens and Steffensen 2007). Although we collected samples within 5–10 min of initial handling, capture stress is the most likely explanation for our finding. Some fish such as several trout species have shown increased plasma lactate concentration within a similar time frame (Pankhurst and Dedualj 1994; Frisch and Anderson 2005), whereas others such as tropical labrids (*Hemigymnus melapterus*; Grutter and Pankhurst 2000) have not. This would suggest

a relatively rapid onset of the physiological stress response in *P. degeni* after capture. Another possible explanation is that lactate levels were constantly elevated due to chronic stressors in the wild (e.g., hypoxia, predation) and during acclimation (housing stress). A further investigation of lactate fluxes in *P. degeni* could reveal whether these fish maintain a static pool of lactate in their blood and whether lactate might serve a metabolic function other than being an end product of anaerobic ATP production (e.g., as mobile fuel or substrate which has been found in mammals under hypoxic conditions, Brooks 1985; Connett et al. 1990; Gladden 2004).

If the constitutive expression of low P_{crit} and RMR that we observed in *P. degeni* was not due to confounding factors, it could be the result of adaptation to a chronically hypoxic habitat such as Lwamunda Swamp. In fish that inhabit fluctuating DO environments such as killifish, short- and medium-term hypoxia acclimation has significant effects on P_{crit} , RI, metabolic rate, and gill morphology with changes of up to 50% in P_{crit} and oxygen consumption (Borowiec et al. 2015, 2018, 2020). Furthermore, it is possible that *P. degeni* exhibit plasticity in other traits (e.g., behavioral plasticity) or on a longer timescale (e.g., developmental and/or transgenerational plasticity, Chapman et al. 2008), which might respond to longer-term environmental change.

Conclusions

In conclusion, our study highlights the extraordinary hypoxia tolerance of swamp-dwelling *P. degeni* and their moderate phenotypic plasticity in response to long-term normoxia acclimation. Plasticity of gill size and hemoglobin concentration are potentially important mechanisms that may help these fish to cope with seasonal variations in their habitat. Our findings suggest that oxyregulative capacity is not affected by changes in these traits, indicating that gill size and blood hemoglobin concentration do not limit oxygen uptake at low PO_2 in naturally hypoxia-acclimated *P. degeni*. Furthermore, our study emphasizes the importance of field data and the need for researchers to consider the impact of housing conditions on hypoxia tolerance when studying hypoxia-tolerant species in the laboratory. In the light of increasing prevalence of hypoxia in aquatic ecosystems,

acclimation studies will be valuable tools to disentangle how morpho-physiology and environment interact to shape the hypoxia tolerance of animals.

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Author contribution All authors participated in the design of this study. S.M. performed all experiments, field data collection, and data analyses. L.J.C. contributed to field work. S.M. drafted the manuscript. All authors took part in its revision.

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Data availability All datasets and R scripts used for data analysis are available under the following URL: <https://doi.org/10.6084/m9.figshare.21551676>.

Declarations

Ethical approval All procedures involving animals were in accordance with local legislation and with European Union regulations on animal research; approval was granted by the Uganda National Council for Science and Technology (research clearance nr. 10601).

Competing interests The authors declare no competing interests.

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