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Hypoxia tolerance and metabolic coping strategies in *Oreochromis niloticus*

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**ABSTRACT**

The Nile tilapia (*Oreochromis niloticus*) is widely farmed in tropical and subtropical pond culture. *O. niloticus* is recognized as a species that is tolerant of hypoxic conditions, a trait that may largely be responsible for the success of this species in aquaculture. Until now, neither coping mechanisms nor a comparison of various indices of hypoxia tolerance to characterize the response to hypoxia, have been described. In the present study, Nile tilapia were subjected to hypoxia of increasing severity and duration to examine effects on metabolic rate (MO2) and post-hypoxic oxygen debt. MO2 was measured during periods of severe hypoxia at 2.1 kPa O2 (10% oxygen saturation) lasting between 2 and 24 h at 27 ºC. Hypoxia tolerance was assessed by determining the critical oxygen tension (Pcrit) and the pO2 at which loss of equilibrium (LOE) occurred. We show that the tolerance of Nile tilapia to severe hypoxia is largely achieved through a capacity for metabolic depression. Despite prolonged exposure to dissolved oxygen levels below Pcrit, the fish showed little excess post-hypoxic oxygen consumption (EPHOC) upon return to normoxic conditions. LOE did not occur until conditions became near-anoxic. Blood pH was not affected by severe hypoxia (2.1 kPa O2), but a significant acidosis occurred during LOE, accompanied by a significant elevation in lactate and glucose levels. The results from the present study indicate that Nile tilapia do not switch to anaerobic metabolism during hypoxia until pO2 falls below 2.1 kPa.

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**1. Introduction**

The Nile tilapia (*Oreochromis niloticus*) exhibit a great tolerance of various abiotic stressors (e.g. pH, ammonia), and are renowned for their ability to tolerate low oxygen concentrations, and periods of prolonged hypoxia (Verheyen et al., 1994; Webster and Lim, 2006). The physiological mechanisms that allow Nile tilapia to survive hypoxia are not well described, but recent studies show that Nile tilapia respond by decreasing their oxygen uptake (Obirikorang et al., 2020). Metabolic rate can be influenced by a variety of factors, including body mass, temperature, level of activity, physiological state, and oxygen availability (Chabot et al., 2016). For fish, there is an oxygen threshold, below which the fish can no longer extract sufficient oxygen from the water to sustain standard metabolic rate (SMR) by aerobic metabolism, and oxygen uptake becomes dependent on ambient oxygen levels (Rogers et al., 2016). This threshold is termed the critical pO2 or critical oxygen tension (Pcrit). The oxygen tension at which Pcrit occurs varies considerably inter-and intraspecifically and is affected by several factors including salinity, temperature, routine activity levels, and body mass (Rogers et al., 2016; Schurmann and Steffensen, 1997). Pcrit has been extensively used as a proxy for hypoxia tolerance, as a lower Pcrit indicates that the organism has a high capacity for O2 uptake, ultimately resulting in a reduced reliance on anaerobic processes (Speers-Roesch et al., 2013). At oxygen tensions below Pcrit, fish increasingly rely on anaerobic metabolism, leading to a production of metabolic by-products such as lactate and protons (Wang and Richards, 2011). Oxygen levels below Pcrit are referred to as severe hypoxia (Svendsen et al., 2012). The experimental determination of Pcrit is influenced by methodology, and may not on its own, accurately reflect the hypoxia tolerance of a species. Therefore, loss of equilibrium (LOE) can be a useful concurrent metric (Borowicz et al., 2020; Wood, 2018). Additionally, while Pcrit identifies the level of hypoxia at which MO2 becomes compromised, LOE provides a measure of the maximum capacity for hypoxia tolerance and hypoxic survival (Speers-Roesch et al., 2013).

Experiments conducted on different species of fish exposed to hypoxic conditions show the presence of some common regulatory responses that minimize the accumulated oxygen debt and the post-hypoxic recovery time. The responses are initiated to enhance oxygen uptake and maintain aerobic metabolism, thereby avoiding the consequences of tissue damage caused by oxygen depletion (Richards, 2011). The first-in-line response is a change in respiratory patterns, such as increased gill ventilation, which acts as a very effective compensation...
mechanism if coupled with maintained oxygen extraction levels (Stefensen et al., 1982). When encountering low oxygen levels, O. niloticus tolerant fish such as sculpins and other cichlids engage in aquatic surface respiration (ASR) (Obirikorang et al., 2020; Richards, 2011; Verheyen et al., 1994). This behavioral strategy enhances survival in hypoxic waters, as the fish can utilize the thin zone of oxygenated water close to the surface, where diffusion from air increases the oxygen concentration (Kramer and McClure, 1982). When such physiological and behavioral responses become inadequate to satisfy oxygen demand, energy production in the fish is fueled through anaerobic processes. Anaerobic metabolic pathways lead to an accumulation of metabolites (e.g., lactate, H+), and the build-up of an oxygen deficit (O2 deficit) (Wang and Richards, 2011). The largest challenge associated with recovery from severe hypoxia is essentially the clearance of metabolic by-products and the re-synthesis of energy substrates. This process can be measured as an increase in oxygen consumption, termed excess post-hypoxic oxygen consumption (EPHOC), which is the result of an oxygen deficit built up during anaerobic metabolism (Svendsen et al., 2012). The proportional relationship, the EPHOC: O2 deficit ratio, can be used to evaluate hypoxia tolerance, and when combined with quantification of metabolites, also provide information on the metabolic coping mechanism employed. During recovery from hypoxia, oxygen is required for the oxidation of anaerobic metabolites and re-synthesis of depleted energy stores. A lower post-hypoxic O2 demand reflects a decreased need for metabolic recovery, thus a greater hypoxia tolerance. Consequently, a higher ratio is observed in species that accumulate a large oxygen debt during hypoxic pO2, whereas hypoxia tolerant species are found to have lower ratios (Genz et al., 2013; Svendsen et al., 2012).

Anaerobic metabolism is associated with several complications for the organism, including a reduced ATP yield from substrate utilization, and the depletion of fermentable fuels and metabolic by-products. To avoid this, the animal must resort to other strategies not relying on anaerobic energy production. In general, three mechanisms determine the hypoxia tolerance of an organism: the ability to (1) decrease the metabolic rate (metabolic depression) during periods of low oxygen concentrations, (2) tolerance and ability to clear accumulated metabolic by-products (e.g., lactate and H+) (3) and the ability to avoid and/or repair serious cellular damage caused by the free radicals arising from reoxygenation (Bickler and Buck, 2007; Hochachka, 1986). Finally, the capacity for anaerobic ATP production affects the hypoxia tolerance of fish. The main substrates are glycogen, glucose, and phosphocreatine (PCr), but reserves vary between species, and may not be sufficient for prolonged survival during hypoxia (Wang and Richards, 2011). As such, the anaerobic ATP production capacity is species-specific with some species, such as rainbow trout (Oncorhynchus mykiss), prioritizing a high ATP output which can be upheld for a short duration (12 min), whereas several members of the genus Carassius have a low output that can be maintained for a prolonged period, due to large storages of glycogen (De Zwaan and Thiillart, 1985).

Metabolic depression can serve as a strategy to postpone the onset of anaerobic metabolism by decreasing oxygen demand. Reduction of metabolic rate has been reported in well-known hypoxia tolerant teleost species like the crucian carp (Carassius carassius). Avoiding a build-up of metabolic end-products is another adaption to hypoxic conditions, as the species is able to convert lactate to ethanol and CO2, thereby avoiding lactic acidosis (Bickler and Buck, 2007). Ethanol can be excreted over the gills, allowing the fish to remain active (Nilsson, 2004). Biochemical modifications and changes commonly present in the blood of fish after exposure to hypoxia, and the quantification of those metabolites provides an indication of the severity of distress in the fish. Hyperglycaemia, an increase in blood glucose levels, is a common indicator of stress in fish and other vertebrates. Elevated glucose levels linked to the mobilization of muscle glycogen stores have been documented in fish subjected to hypoxia (Muuze et al., 1998; van Raaij et al., 1996). Increasing plasma levels of lactate and protons mark the onset of anaerobic ATP production, and can be used as an indicator of anaerobic metabolism and to evaluate the post-hypoxic recovery rate of the organism (Rees et al., 2009). The production of H+, and the resultant drop in blood pH, exerts an important effect on the oxygen affinity of hemoglobin, and for most fish hypoxia survival is limited by the amount of produced lactate and H+ (Nikinmaa, 1990, pp. 165–172; Nilsson and Ostlund-Nilsson, 2008). Overall, metabolic depression appears to be one of the most important survival strategies among hypoxia tolerant species (Bickler and Buck, 2007), with the capacity for metabolic depression reflecting survival probability (Hochachka, 1986; Richards, 2011).

Nile tilapia is one of the main fish species currently cultured in pond systems. Its hypoxia tolerance makes it suitable for production under conditions where other species would perish (FAO, 2018; Webster and Lim, 2006). The species is endemic to Africa, occupying a variety of niches such as floodplain pools, swamps, and lakes (Lowe-McConnell, 2000). In stagnant tropical waters, photosynthesis and nocturnal respiration result in large diurnal fluctuations in dissolved oxygen, with prolonged nocturnal periods of severely hypoxic, or even anoxic, conditions (Obirikorang et al., 2020). While the hypoxia tolerance of Nile tilapia is well known, the metabolic response and coping strategies are less clear. The objectives of the study were to (i) examine the role and contribution of metabolic depression as a response to hypoxia, and (ii) provide an improved understanding of the hypoxia tolerance in Nile tilapia and the metabolic response to severe hypoxia.

2. Materials and methods

2.1. Experimental animals

Juvenile all-male Nile tilapia were acquired from Til-Aqua International, (Someren/Velden, Netherlands). The fish were kept in tanks at 27 °C, connected to a filtered recirculating system. Oxygen saturation was kept above 80% by aeration. An automated light system ensured a consistent circadian rhythm of 12 h of light (6 am to 6 pm). The fish were fed daily, with commercial pellets (EFICO Cromis 832F, BioMar). Fish were not fed for 24 h before any experiment. They were randomly assigned to the experimental groups (normoxia, hypoxia, LOE, exhaustion). Experiments were authorized by Danish Veterinary and Food Administration’s (DVFA) Animal Experimental Council (permit: 2018-15-0201-014079), and all procedures complied with the ethical guidelines of Directive 2010/63/EU.

2.2. Oxygen consumption and SMR

The maximum metabolic rate (MMR), standard metabolic rate (SMR), and MO2 during the hypoxia experiments were determined using computerized intermittent-flow through respirometry (Steffensen, 1989), using loop times of 6 min, consisting of 2 min flush, 1 min wait, and a 3 min measurement period (Chabot et al., 2016; Steffensen, 1989). pO2 in each chamber was monitored using oxygen sensors in a recirculation loop connected to an OXY- 4 mini (PreSens, Regensburg, Germany). Oxygen sensors were calibrated with a saturated solution of Na2SO3 (to strip oxygen from the water), and oxygen saturated water at the experimental temperature before trials. Measurements were transmitted to a PC running AutoResp (Loligo, Viborg, Denmark), which recorded and processed data and managed the flush, wait, and measurement phases. Fish used in the present study had a body mass (mean ± SD) of 104.4 ± 16.5 g (n = 74). The volume of the respirometers was 1.159 L, and experiments were carried out at 27 °C. Four respirometers were operated simultaneously, with four fish randomly selected. MMR and SMR were measured for each fish prior to the prolonged severe hypoxia and LOE experiments. The fish were exercised to exhaustion by hand for 3 min and immediately transferred to the respirometer to obtain the value for MMR. Data collection of MO2 was continuously measured for 24 h, according to the recommendations of Chabot et al. for adequate data recording (Chabot et al., 2016), and SMR was determined as described in section 2.4.
2.3. Hypoxia and LOE exposure

The hypoxia and LOE experiments were initiated following MMR and SMR measurements. Oxygen consumption during hypoxic conditions was measured in respirometers as described above. A PC running a script in DAQFactory (AzeoTech, Inc., OR, USA) was used to control and adjust oxygen tensions to pre-determined levels throughout the experiment, by gradually adjusting nitrogen influx or aeration in the holding tank of the respirometers. N₂ was dispersed through an air stone, in a 1-m degassing tower, to increase the speed and efficiency of deoxygenation. The degassing with N₂ was automatically controlled based on continuous input from an O₂ electrode transferred to the PC via a digital acquisition device (U6 DAQ, LabJack, Inc., CO, USA). Severe hypoxia experiments were conducted with durations of either 2, 4, 8 or 24 h at a pO₂ of 2.1 kPa. Deoxygenation from saturation to set point was achieved linearly over 7 h. At the end of hypoxia exposure, normoxia (pO₂ > 15 kPa) was restored within 1 h. Due to a technical issue, 2 fish were excluded from data analysis, and one was excluded from the calculation of oxygen deficit (4-h hypoxia group) because data did not satisfy the predefined Pcrit calculation method.

Loss of equilibrium (LOE) was determined by subjecting the animal to declining oxygen concentrations until the fish lost the ability to maintain a balanced upright position in the water, that is, the animal loses equilibrium (Borowiec et al., 2020). The experiments were conducted in the respirometers, by decreasing pO₂ to 5 kPa over 5 h using the DAQFactory software as described above. At a pO₂ of 5 kPa, flush pumps were disconnected and the oxygen levels inside each respirometer were allowed to decrease, while the water in the external holding tank was re-oxygenated. When the fish reached LOE, they were left for 3 min. This was done to make sure the definite point of tolerance towards minimum pO₂ was reached since tilapia had been observed to tilt over in the respirometers for a short time only to regain an upright position. After 3 min the pump was reconnected, allowing oxygenated water to flush the respirometer. Fish quickly regained equilibrium after re-oxygenation of the respirometers.

2.4. Data processing and calculations

MMR was identified as the highest measurement after exercising the fish to exhaustion. SMR was hereafter estimated for each fish based on the M02 data from AutoResp, using the average of the lowest 10% with the 5 lowest values excluded (Chabot et al., 2016). Only M02 measurements with R² values > 0.95 were included in the analysis.

A regression line was fitted to each fish based on the M02 measurements obtained below 3 kPa O₂, and Pcrit was determined from the intercept between the regression line and SMR. The pO₂ < 3 kPa was chosen due to variations in the pO₂, where the fish started to decrease M02 concurrently with the declining pO₂, with some individuals already beginning to reduce M02 as a function of oxygen at pO₂ of 16.4–14.3 kPa. Visual inspection of the data showed that at or below 3 kPa all individuals were below their Pcrit, and linear regression of those measurements was consistent with the estimated Pcrit. If linear regression was calculated using all M02 < SMR, Pcrit would have been severely overestimated for several individuals. SMR estimates were determined previously during 24 h before hypoxia experiments and obtaining M02 values for Pcrit (Reemeyer and Rees, 2019). Pcrit values were obtained for each of the experimental groups, n = 29 from prolonged hypoxia of different duration and n = 8 from LOE).

Based on the M02 measurements for each individual during hypoxia and the return to normoxia, Pcrit, oxygen deficit (O₂ deficit), and excess post-hypoxic oxygen consumption (EPHOC) were determined. Oxygen deficit of the hypoxia trials (2, 4, 8, and 24 h) and LOE was calculated as

\[
O_2 \text{ deficit} = \sum_{t=0}^{n} (\text{SMR} - M02) t
\]

where n is the number of measurements taken below the Pcrit of the fish until the termination of hypoxia and t is respiration loop time (h). EPHOC was calculated as

\[
\text{EPHOC} = \sum_{t=0}^{n} (M02 - \text{SMR}) t
\]

where n is the number of measurements starting when oxygen deficit ceased until the termination of EPHOC, defined as when two consecutive M02 measurements fell within 5% of the SMR calculated for the individual fish. Upon return to normoxia, M02 increased, and the highest value occurring was identified as M02peak. M02peak is the maximum oxygen consumption after returning to normoxia and is used as a recovery variable, as hypoxia can delay and decrease M02peak. The difference between M02peak and metabolic scope indicates how much of the aerobic metabolic scope is available and what is allocated to recovery from hypoxia (Svendsen et al., 2012).

In the LOE experiments, the R² values for M02 measurements occasionally dropped below 0.95 due to a less steep decline in the oxygen tension in the respirometer during pO₂ < 0.4 kPa. For calculation of oxygen deficit, values with R² > 0.70 were accepted and included in the dataset. The number of values that were removed due to low R² varied between individuals. To minimize the difference in the number of values used for calculations, an average of the M02 measurements R² > 0.70 observed in the severe hypoxic period (<0.4 kPa O₂) was used to replace the removed values.

2.5. Hematological samples and biochemical parameters

The biochemical responses to hypoxia were determined from blood samples. For comparison of the metabolic response to the treatments, a group subjected to exercise-induced stress was included. Samples from O. niloticus were taken from four separate groups following exposure to one of four conditions (1) normoxia (n = 11, 2) 24 h of hypoxia (2.1 kPa O₂, n = 8, 3) loss of equilibrium (LOE, n = 8) and after (4) chasing the fish to exhaustion (n = 9). Methods for hypoxia, LOE, and exhaustion followed the procedure described previously. Normoxia blood sampling was performed after 24 h in fully saturated water. Sampling from the 24 h hypoxia group was initiated 15 min before the cessation of hypoxia, to ensure that the fish were still exposed to severe hypoxia (2.1 kPa). A similar procedure was applied for the LOE group, where blood was sampled after the occurrence of LOE (as described in 2.3). The exhaustion group was sampled 20 min after exercise to exhaustion, to allow lactate to diffuse from the white muscle into the bloodstream (De Zwaan and v.d. Thillart, 1985). The fish were rapidly anesthetized with an overdose of benzocaine (0.1 g L⁻¹ ethyl-paminobenzoate), and blood samples were taken from the caudal vein with a heparin-coated 1 mL syringe and 23 G needle. The blood was transferred to a 1.5 mL Eppendorf tube, and pH, lactate and glucose were measured within 5 min. pH was measured by a pH electrode immersed in the blood sample using Hanna Instruments PH/ORP/ISE Meter HI 98185 (Kungsbacka, Sweden). A drop of blood was applied on a BM-Lactate strip and analyzed with an Accutrend Plus device (Roche, IN, USA). Glucose levels were assessed with HemoCue Glucose 201 RT device (Angelholm, Sweden). One drop of blood was transferred to a hydrophobic surface and collected with a HemoCue cuvette.

2.6. Statistics

Statistical analyses were performed using SigmaPlot (v. 14.0, Systat Software Inc.). Data were tested for normal distribution (Normality Test, Shapiro-Wilk) and homogeneity of variance (Equal Variance Test, Brown-Forsythe). Statistical comparison of the effects of exposure to severe hypoxia between the different treatment groups was performed by using a one-way ANOVA. Significant differences identified between treatments were followed by pairwise multiple comparison procedures (Holm-Sidak). If data were not normally distributed or failed the test for equal variance, Kruskal-Wallis Test was used (All Pairwise Multiple
3. Results

3.1. Oxygen consumption rates

Metabolic rate was elevated after exercising fish to exhaustion for the measurement of MMR. Mean MMR was 441.4 ± 86.8 mg O$_2$ kg$^{-1}$ h$^{-1}$ ($n = 37$). MO$_2$ declined over the following hours and started to stabilize after 8–10 h (Fig. 1). The mean SMR was 141.5 ± 18.0 mg O$_2$ kg$^{-1}$ h$^{-1}$ ($n = 37$).

3.2. Effect of hypoxia on metabolic rate

30 fish were exposed to severe hypoxia (2.1 kPa O$_2$) for a duration of either 2, 4, 8, or 24 h. The fish were able to uphold their SMR until P$_{crit}$, thereafter the metabolic rate reduced with decreasing oxygen concentrations. The fish continued to reduce MO$_2$ until reaching a pO$_2$ of 2.1 kPa (c.10% O$_2$ saturation), at which point the reduced MO$_2$ was maintained throughout the length of the hypoxic period (Fig. 2). In addition to the reduction of MO$_2$, the fish responded to the declining oxygen levels by ceasing spontaneous activity, which is seen as a large decrease in the variation of MO$_2$ measurements (Fig. 2). At stable oxygen levels during severe hypoxia, MO$_2$ was maintained until pO$_2$ increased again after 2, 4, 8, or 24 h, resulting in a rapid increase in MO$_2$. During severe hypoxia (pO$_2$ of 2.1 kPa) the fish were capable of reducing their MO$_2$ more than 50% of SMR, with a mean reduction in oxygen uptake of 53.3 ± 8.7% below SMR (Fig. 3).

3.3. Critical oxygen tension

Mean critical oxygen tension (P$_{crit}$) was 3.8 ± 0.6 kPa O$_2$ ($n = 37$). The fish maintained SMR until P$_{crit}$, below which MO$_2$ decreased linearly with the declining pO$_2$ (Fig. 4). P$_{crit}$ varied between individuals, from the lowest value at 2.8 kPa to the highest at 5.0 kPa, but no significant differences were found between treatment groups (Table 1).

3.4. Loss of equilibrium

Oxygen tension fell below 0.4 kPa before a loss of equilibrium (LOE) occurred, with a mean pO$_2$ for LOE at 0.2 ± 0.1 kPa. The fish were capable of not only tolerating low pO$_2$, but some individuals endured oxygen tensions below 0.4 kPa for up to 66 min, with a mean of 38.3 ± 21.0 min spent in <0.4 kPa O$_2$ before LOE. On average the fish were able to reduce their metabolic rate by 95.0 ± 2.5% below their SMR (Fig. 3). No correlations were observed between individual P$_{crit}$ and the oxygen tension at which LOE occurred.

3.5. Oxygen deficit and EPHOC

Oxygen deficit and excess post-hypoxic oxygen consumption (EPHOC) were measured after severe hypoxia of 2, 4, 8, and 24 h ($n = 29$) and after LOE ($n = 8$). Results for all treatment groups are summarized in Table 1.

After the termination of severe hypoxia, an increase in oxygen consumption was observed as a peak in MO$_2$ (Fig. 2). MO$_2^{peak}$ was highest in the 24 h hypoxia treatment, with a mean of $329.6 ± 55.4$ (mg O$_2$ kg$^{-1}$ h$^{-1}$) but there was no significant difference between treatments. However, the increase of MO$_2^{peak}$ after hypoxia is not as high as the measured MO$_2$ after exercise until exhaustion (MMR), despite the animals being subjected to severe hypoxia. The duration of hypoxia exposure did not affect EPHOC (mg O$_2$ kg$^{-1}$) significantly. The severity of the hypoxia exposure had a significant effect on EPHOC, and for the LOE treatment EPHOC was significantly higher than in 2, 4, and 8 h hypoxia treatments (Fig. 4). The time required to recover from hypoxia (EPHOC duration) was less than the time spent in hypoxia for all treatments, except for LOE where the mean recovery time (EPHOC duration) lasted for 9.1 ± 4.9 h, and exceeded the time spent in severe hypoxia (1.5 ± 0.1 h). Thus, the duration of EPHOC (h) was influenced significantly by the severity of hypoxia.

3.6. EPHOC: O$_2$ deficit ratio

The cumulative elevated MO$_2$ (EPHOC) after exposure to severe hypoxia increased corresponding to the oxygen deficit. However, the magnitude of EPHOC was not equivalent to oxygen deficit, which is reflected in the low EPHOC: O$_2$ deficit ratios. The mean EPHOC: O$_2$ deficit ratio of the hypoxia treatments 2, 4, 8 and 24 h did not exceed 0.35, and no differences were found between the hypoxia treatments (Fig. 4). An EPHOC: O$_2$ deficit ratio of 3.32 ± 1.21 was found in the LOE treatment, which was significantly higher than for the prolonged hypoxia treatments, with mean ratios between 0.24 and 0.38 (Table 1). The severity of hypoxia exposure, therefore, had a significant effect on EPHOC: O$_2$ deficit, while the duration of hypoxia did not.

3.7. Blood metabolites

Blood pH in LOE and exhaustion treatment were significantly lower than the normoxia and hypoxia group, with the lowest values measured in the LOE treatment (Table 2).

Measurements of blood lactate in the normoxia and hypoxia groups were below detection limits. Lactate levels in LOE and stress groups were significantly higher. Blood glucose levels in LOE were hyperglycaemic, with significantly higher glucose levels than in all other groups. The glucose levels in the exhaustion group were significantly higher compared to normoxia and hypoxia (Table 2).

4. Discussion

4.1. P$_{crit}$

The estimated P$_{crit}$ of 3.8 ± 0.6 kPa (c. 19% oxygen saturation) for O. niloticus in the present study agreed with previous findings of 3.7 kPa O$_2$ (18.6% oxygen saturation, 25 °C) (Verheyen et al., 1994) and 3.9 kPa O$_2$ (19.5% oxygen saturation, 30 °C) (Fernandes and Rantin, 1989). These estimates are also in the same range as reported for other
freshwater species inhibiting waters with high temperature (20–36 °C), though not placed among the lowest $P_{\text{crit}}$ estimates (<3 kPa) of other hypoxia tolerant species such as *Anguilla anguilla* and *Astronotus ocellatus* (Rogers et al., 2016). Nile tilapia occupy a wide range of freshwater environments, including areas of shallow confined bodies of water prone to low oxygen levels at night (Lowe-McConnell, 2000). Exposure to periodical hypoxic conditions in their habitats suggests that the species would have a lower $P_{\text{crit}}$ as part of an adaptation to low oxygen levels. The $P_{\text{crit}}$ found in the present study is not in the low range, as would generally be expected for hypoxia tolerant species, and illustrates that the use of $P_{\text{crit}}$ alone, does not necessarily reflect the actual extent of tolerance to hypoxic conditions. Estimates of various fish species often lie within a similar range, despite great differences in hypoxia tolerance. The $P_{\text{crit}}$ values reported for Atlantic salmon (*Salmo salar*) of 4.59 kPa O$_2$ (22% oxygen saturation) (Barnes et al., 2011) and rainbow trout (*O. mykiss*) of 3.0 kPa O$_2$ (15% oxygen saturation, 16 °C) (Moltesen et al., 2016), which are both regarded as hypoxia sensitive species, exemplifies that $P_{\text{crit}}$ is not necessarily a strong indicator of hypoxia tolerance. The shortcoming of exclusively using $P_{\text{crit}}$ as a measurement of hypoxia tolerance is further demonstrated when considering the differences in temperature as $P_{\text{crit}}$ is largely influenced by temperature and estimates can differ significantly between experimental thermal conditions (Barnes et al., 2011; Schurmann and Steffensen, 1997), although exceptions occur (e.g. carp, *Cyprinus carpio*) (Ott et al., 1980). Moreover, the multiple analytical and experimental approaches used to identify $P_{\text{crit}}$, lead to variations in results and contribute to the limitations of the method as an indicator of hypoxia tolerance, when not coupled with other approaches, such as described by Wood et al. (2018).

### 4.2. Metabolic response

Results from the present study show that *O. niloticus* tolerates severe hypoxia at a pO$_2$ of 2.1 kPa (c. 10% oxygen saturation) for up to 24 h without an increase in the levels of metabolites associated with anaerobic metabolism. Accordingly, the fish rely on aerobic energy...
production during the experimental conditions of severe hypoxia of a pO2 of 2.1 kPa. This hypoxia tolerance appears to be the result of a suite of physiological adaptations and mechanisms employed by the species, such as an efficient gas exchange system capable of extracting 75–85% oxygen from the environment (Fernandes and Rantin, 1989). Additionally, when facing hypoxia in environments with access to the surface, the fish resort to aquatic surface respiration (ASR) (Obirikorang et al., 2020; Verheyen et al., 1994). In our experiments, this strategy was prevented due to the closed design of the respirometers and therefore did not contribute to the tolerance of prolonged severe hypoxia. When regulation of ventilatory responses was not sufficient to maintain oxygen demand during decreasing oxygen saturation, the fish responded by reducing their metabolic rate. Depression of MO2 below Pcrit continued in proportion to the decline in oxygen levels until reaching the desired pO2 of 2.1 kPa. At this pO2, the fish maintained their MO2 throughout the hypoxic period. Consequently, the ability to reduce the metabolic rate by 53.3 ± 8.7% below SMR, and to sustain the metabolic depression, permits survival in severe hypoxia for at least 24 h. Moreover, the fish showed an ability to further decrease their metabolic rate when subjected to a pO2 of 0.4 kPa (LOE), although this reduction could presumably not be sustained over a prolonged period. The above results illustrate a broad flexibility to deal with low oxygen concentrations, and a remarkable ability to avoid or postpone the onset of anaerobic metabolism.

4.3. LOE

The hypoxia tolerance of O. niloticus was further explored by exposing fish to decreasing oxygen levels until LOE occurred at a pO2 of 0.2 ± 0.1 kPa. LOE can be a more useful indicator for evaluating hypoxia tolerance in that it reveals the capacity of fish for dealing with pO2 below their Pcrit. At LOE the animal is pushed to its limits, and this approach might better reflect hypoxia tolerance than Pcrit measurements (Wood, 2018). The difference in measured MO2 during severe hypoxia and LOE demonstrates that the ability to depress its metabolic rate is not fully exploited until fish approach LOE. Here, MO2 was reduced by 95.0 ± 2.5% below normoxic MO2, compared to severe hypoxia (2.1 kPa O2) where MO2 was reduced by 53.3 ± 8.7%. The fish that were exposed to prolonged hypoxia at a pO2 of 2.1 kPa showed no reliance on anaerobic metabolism. In contrast, the fish in LOE treatment showed a significant increase in EPHOC and plasma lactate upon return to normoxia, suggesting that the onset of anaerobic metabolism occurs somewhere between a pO2 of 2.1 and 0.2 kPa.

4.4. Oxygen deficit and EPHOC

The significant differences observed in oxygen deficit are attributed to the length of the hypoxic period, therefore the longer the fish is exposed to hypoxia and undergo metabolic depression, the higher the calculated oxygen deficit (cf. calculations in 2.4). The EPHOC values presented in this study showed that during severe hypoxia (2.1 kPa O2), there is no substantial excess post-hypoxic oxygen consumption, and consequently, energy production in O. niloticus is supported solely by aerobic metabolism, even during prolonged hypoxic periods up to 24 h. A reliance on anaerobic metabolism would result in an oxygen debt
occurring from the synthesis of depleted energy stores of glycogen, and phosphorylation of ADP/AMP and creatine (Mandrelovich et al., 2008). In contrast, EPHOC values from LOE showed a noticeable oxygen debt in the recovery period following hypoxia. Comparison of EPHOC: O₂ deficit ratios of hypoxia treatments and LOE showed that values were significantly lower in hypoxia, and are distinctly lower than what has been reported in other species. EPHOC: O₂ deficit ratio of rainbow trout (O. mykiss) was reported to be 30±7.2 during a pO₂ of 2.1 kPa for 0.97 h (Svensen et al., 2012). Plambech et al. (2013) investigated EPHOC in Atlantic cod (Gadus morhua) and found an EPHOC: O₂ deficit of 6.9±1.5 at 3.3 kPa O₂ for 0.66 h. The lower ratio found for Atlantic cod could result from an adaptation to hypoxia, as the species forages in the oxygen-depleted waters near the ocean floor (Plambech et al., 2013).

The ratios illustrate that there are immense differences that exceed the expected variability between hypoxia sensitive and tolerant species. The ratio is applicable as a general indicator of a species hypoxia tolerance, though the method has some drawbacks when applied to species that deviate from the more common metabolic response to hypoxia. The low EPHOC: O₂ deficit ratio in hypoxia treatments originates from the ability of Nile tilapia to reduce its metabolic rate. By using the general method for calculating oxygen deficit, and thereby assuming that oxygen deficit is initiated when MO₂ declines below SMR, the accumulated oxygen deficit will result in an overestimate. This is because Nile tilapia is not experiencing any oxygen deficit until oxygen tension is much lower than Pcrit, due to a depression of its metabolic rate, and accordingly lowering the oxygen demand to match supply. Furthermore, in the absence of anaerobic metabolic end-products to clear, EPHOC is much lower than would be anticipated. A substantial amount of the EPHOC generated in fish can be attributed to the clearance of anaerobic metabolic end-products during recovery from hypoxia, and re-synthesis of ATP and phosphocreatine (PCr) (Genz et al., 2013).

Our general conception of fish metabolism suggests that energy requirements cannot be sustained aerobically at pO₂ below Pcrit, and the organism must turn to anaerobic energy production (Maun et al., 2016). Most species exhibit a response in alignment with this, and for these the general method of calculating oxygen deficit and EPHOC is appropriate. The ability of other species to undergo metabolic depression when exposed to hypoxia may very well lead to an overestimate of the accumulated oxygen deficit. The hypoxia exposed fish in this study needed little recovery, resulting in low EPHOC values, despite large oxygen deficits, causing comparably small EPHOC: O₂ deficit ratios. To use the EPHOC: O₂ deficit ratio as a definite indicator of hypoxia tolerance and allow for comparison of ratios between hypoxia sensitive and tolerant species, will require some revision and consensus on methodology. The temporal scale of the hypoxic period and rate of hypoxia induction must be accounted for, as must any ability to undergo metabolic depression, utilization of other metabolic pathways, or elimination of metabolic by-products.

4.5. Blood metabolites

Increased plasma glucose levels during hypoxia exposure in fish illustrate the demand for a substrate that can be utilized during anaerobic metabolism. The hyperglycaemic blood values of LOE and the exhaustion group emphasize that very low O₂ levels (LOE) act as a stressor on Nile tilapia, in contrast to the hypoxia group (2.1 kPa O₂), where no signs of increased glucose levels were observed. Ishibashi et al. (2002) showed that glucose concentrations increased at a pO₂ of 1.3 kPa (6.4% oxygen saturation) in Nile tilapia, which compared to the glucose values in the current study, suggest that the oxygen threshold that induces a release of substrate for glycolysis, is found in the range from 2.1 to 1.3 kPa O₂.

Animals subjected to LOE and exhaustion displayed blood acidosis, as pH decreased in the two groups. In contrast, a minor increase of pH was observed in the hypoxia group although this was not significant. An increase in blood pH during hypoxia has also been documented in carp blood, another well-studied hypoxia tolerant species. Similar to Nile tilapia, carp (C. carpio) experience diurnal variations in ambient oxygen saturations, and studies of blood respiratory changes in response to environmental O₂ fluctuations showed increases in blood pH as a mechanism to increase oxygen affinity (Lykkeboe and Weber, 1978). The observed differences in blood pH of LOE and the exhaustion treatment can be attributed to changes in blood CO₂ levels, and residual metabolites from anaerobic glycolysis, where an accumulation of lactate and H⁺ leads to a drop in pH (Lykkeboe and Weber, 1978; Richards, 2011).

The high levels of lactate observed following LOE and exhaustion demonstrate the onset of anaerobic metabolism and is a common response in fish (Wang and Richards, 2011). A corresponding pattern is found in the hypoxia tolerant Amazon basin cichlid, Acaara-acu (Astro-notus ocellatus), where blood lactate levels increased when the fish were exposed to pO₂ of 1.2 kPa, although Pcrit occurs before this point at 4 kPa (c.20% oxygen saturation 28°C, which theoretically should initiate anaerobic metabolism (Mauzse et al., 1998). These responses in blood lactate levels to hypoxia are in contrast with the hypoxia sensitive rainbow trout (O. mykiss). When gradually exposed to hypoxia, blood lactate levels increase at oxygen saturations below Pcrit, even though the increased lactate accumulation is coupled with a higher rate of lactate disposal (Omlin and Weber, 2010; van Raaij et al., 1996). The stimulation of lactate disposal is essential for prolonging survival, as it reduces the accumulation of lactate, which is the limiting factor for survival in hypoxia (Nilsson and Ostlund-Nilsson, 2008). Consequently, the ability to avoid lactate load is of great importance for hypoxic survival in fish. Results from present study show that the lactate avoidance strategy employed by Nile Tilapia relies on metabolic depression, enables aerobic ATP production to satisfy demand, and as a result postponing glycolytic activity and the need for increased lactate removal (Dunn and Hochachka, 1986).

5. Conclusion

The high tolerance towards hypoxia is facilitated by the ability of Nile tilapia to reduce its metabolic rate as a response to declining pO₂ and maintain a reduced MO₂ throughout periods of severe hypoxia. The documented depression of metabolic rate is regulated as a consequence of pO₂ and not the period of exposure (up to 24 h). Examining the capacity for hypoxia tolerance in Nile tilapia through Pcrit and LOE, showed that Pcrit and LOE reflect hypoxia tolerance very differently. The estimated Pcrit values were in the same range as found in non-hypoxia tolerant species, while LOE values reflected the extent of tolerance to hypoxic conditions and the ability to survive severe hypoxia. Quantification of EPHOC following severe hypoxia suggests that there is no or little anaerobic energy production during a pO₂ 2.1 kPa and that pO₂ must approach that of those levels leading to LOE before any reliance on anaerobic metabolism occurs. Analysis of blood metabolites supported the results, as a significant difference in the responses to the experimental oxygen conditions was observed, with no differences between normoxia and hypoxia treatments, while LOE treatment led to metabolic responses comparable to the exhaustion treatment, although more profound.

A reduction in metabolic rate in the magnitude of what is observed in Nile tilapia requires downregulation of some of the most energetically expensive processes, such as protein turnover. Further studies addressing the specific mechanism underlying the ability to undergo metabolic depression are required and could contribute to a more profound understanding of the strategies allowing for hypoxia tolerance.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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