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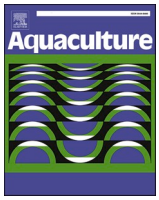

Acid-base disturbances and effects on oxygen uptake rates in Nile tilapia (*Oreochromis niloticus*) following acute and prolonged CO_2 exposure

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ABSTRACT

High levels of dissolved carbon dioxide (CO_2) occur nightly in earthen ponds characterized by high respiration rates. Exposure to high CO_2 conditions (hypercapnia) leads to acidosis in fish, which can be compensated by an accumulation of HCO_3^- to recover intra- and extracellular pH levels, with a capacity that appears to be species-specific. For Nile tilapia, a freshwater tropical teleost traditionally produced in earthen ponds, little information is available on the tolerance to dissolved levels of CO_2 and associated acid-base disturbances. Here, we investigated first the effects of acute and progressively increasing CO_2, from normocapnic conditions to 60 mg CO_2 L^{-1}, on oxygen uptake rates (MO_2). This was followed by exposure to three concentrations of CO_2; 10, 30, and 60 mg L^{-1} (equivalent to pCO_2 of 5.4, 16.2, and 32.4 mmHg) against a normocapnic control (pCO_2 0.3 mmHg), to investigate acute (1 h) or prolonged (24 h) effects on standard (SMR) and maximum metabolic rates (MMR), haematology, and extra- and intracellular acid-base status in adult Nile tilapia (mean BM 435 ± 16 g ± SE). Acute exposure to hypercapnia led to concentration-dependent decreases in both SMR and MMR. Fish were able to fully or partially recover MMR and metabolic scope (MS) after 24 h, while depression of SMR persisted at all CO_2 levels. Acute exposure to CO_2 caused intra- and extracellular pH levels to decrease by up to 0.5 units in a concentration-dependent manner. Only the lowest hypercapnic treatment (pCO_2 5.4 mmHg) was able to fully recover within 24 h. Changes in haematological variables appeared minor, being restricted to increasing haematocrit, haemoglobin concentration, and mean cell volume in the highest CO_2 treatments after 24 h exposure. Although the Nile tilapia is generally considered a species able to tolerate poor water quality, the modest or slow acid-base regulation following hypercapnic exposure suggests sensitivity to hypercapnia.

1. Introduction

Global aquaculture production of Nile tilapia *(Oreochromis niloticus)* predominantly occurs in earthen ponds (Pimolrat et al., 2013) without water treatment. Unlike in recirculating aquaculture systems (RAS), water quality parameters in earthen ponds are allowed to fluctuate daily, which is likely to affect the physiological performance of the cultured fish (Frimpong et al., 2014; Pandit and Nakamura, 2010). In nutrient-rich ponds with high chlorophyll concentrations, the photoperiod creates an intermittent fluctuation of dissolved oxygen and carbon dioxide, resulting in acutely occurring episodes of hypoxia and hypercapnia and carbonate associated pH fluctuations that may last several hours. Dissolved oxygen in the surface layers can reach extreme levels of supersaturation in the middle of the afternoon, while the whole pond approaches anoxic conditions during the night due to the respiration of fish, phytoplankton, and bacteria (Gyamfi et al., 2022; Obirikorang et al., 2020). Dissolved levels of CO_2 have rarely been measured in earthen ponds. However, the CO_2 concentration increases as the rate of CO_2 excretion exceeds the rate of removal by photosynthesis and diffusion across the water surface (Tadesse et al., 2004). As such, the concentration of dissolved CO_2 has been approximated to range from 10 to 15 mg L^{-1} in the early morning (Hargreaves and Brunson, 1996) or above 20 mg L^{-1} in ponds with high feed loading (Boyd, 2008). Recent measurements of water pH and alkalinity in a fertilized fish pond suggest that dissolved CO_2 levels could reach concentrations of up to 50 mg L^{-1} (pers. obs).

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Increasing levels of dissolved CO_2 in the water reduce the pCO_2 diffusion gradient across the gill epithelium, leading to an internal accumulation of CO_2 (Brauner et al., 2019). As hydrated CO_2 (carbonic acid) equilibrates with bicarbonate and H^+ (Truchot, 1994), pH decreases in both the intra- and extracellular compartments, causing a widespread acid-base disturbance (Evans et al., 2005; Shartau et al., 2019). Most fish species can buffer the extra proton load through alterations in ion exchange across the gill epithelium (Shartau et al., 2020), where HCO_3^- is accumulated in exchange with Cl^- at the gills (Brauner et al., 2004). However, these physiological mechanisms are energy-demanding, and compensation may be time-sensitive (Claiborne and Heisler, 1986) and limited by the capacity to accumulate sufficient bicarbonate (Brauner and Baker, 2009; Fivelstad, 2013; Shartau et al., 2019) and environmental ion concentration (Larsen and Jensen, 1997). Therefore, pH compensation may increase the total energy expenditure for acid-base regulation, making less energy available for other metabolic processes such as growth.

The temporal and spatial capacity for pH regulation in fish tends to vary considerably across species (Hayashi et al., 2004; Kaya et al., 2016; Petochi et al., 2011; Ross et al., 2001; Shartau et al., 2019), environment (freshwater vs. marine) (Claiborne et al., 2002), temperature (Shartau et al., 2020), and life stage (Kikkawa et al., 2004; Shartau et al., 2016). It appears to be a normal response among most fish species to employ a strategy of coupled pH regulation, in which intracellular (pHi) recovery occurs faster but cannot be fully compensated until extracellular (pHe) is >50% recovered (Shartau et al., 2016).

Numerous studies have shown that the time required to compensate for CO_2-induced pH disturbances is species-specific, ranging from hours to days (Brauner et al., 2004; Claiborne and Heisler, 1986; Damsgaard et al., 2015; Heuer and Grosell, 2014) The rate of compensation depends on the environmental ion concentrations (Brauner et al., 2004; Claiborne and Heisler, 1986), while the intra- and extracellular compartments also show differences in the recovery kinetics (Brauner et al., 2019; Shartau et al., 2019; Shartau et al., 2020). Currently, no information is available regarding the degree to which the intra- and extracellular acid-base status in Nile tilapia is impacted by increasing environmental CO_2 concentrations, nor is the time required or capacity to compensate for the acidification.

In addition to disturbing acid-base balance, CO_2 has a range of other physiological effects on fish (Cupp et al., 2017; Good et al., 2010; Heuer and Grosell, 2014; Ross et al., 2001). Cruz-Neto and Steffensen (1997) reported decreased oxygen uptake rates (MO_2) in freshwater European eel, *Anguilla anguilla*, subjected to water pCO_2 of 4.0 kPa. Reduced oxygen consumption upon exposure to increased CO_2 has been hypothesized to contribute to the re-distribution of metabolic capacity to facilitate ion regulation (Baker and Brauner, 2012; Claiborne et al., 2002), where both standard (SMR) and maximum metabolic rate (MMR) are impaired and the capacity for aerobic metabolic scope become reduced. This CO_2-induced reduction in metabolic scope demonstrates the reduced ability of fish to consume oxygen that can be made available for activities above those considered maintenance (feeding, swimming, growth, and reproduction). CO_2 can also simultaneously induce changes at the intra and extracellular levels through changes in HCO_3^-, pH, Cl^- and pCO_2 (Montgomery et al., 2019). To some degree, the acid-base disturbance in fish is regulated by internal buffer systems in the blood and extracellular space, serving as an initial line of defence against acidosis (Munday et al., 2016; Shartau et al., 2019).

In aquaculture production, increased CO_2 levels may be problematic and impact the physiology, welfare, and growth performance of captive species when CO_2 content fluctuates during production due to excessive stocking density or poor management (Skov, 2019). Understanding the significance of this and determining the capacity and mechanisms of individual species in aquaculture are important in optimizing production and to decide on the necessity of applying water treatment technology.

The objective of this study was to evaluate the response of oxygen consumption rates (MO_2) of Nile tilapia to determine changes in response to acute changes in dissolved [CO_2] similar in magnitude to what might occur in an earthen pond, and to quantify changes in metabolic rates during short- and long-term CO_2 exposure, in the anticipation that oxygen extraction becomes compromised with increasing hypercapnia. We furthermore evaluated haematological compensation and cellular acid-base regulation process when exposed to elevated CO_2 in the short and long term. The overall study was aimed at identifying any CO_2 threshold levels and compensation mechanisms relevant to ensure good welfare in Nile tilapia that experience cyclic hypercapnic episodes.

2. Materials and methods

2.1. Experimental fish

All-male Nile tilapia (*Oreochromis niloticus*) were acquired from a laboratory stock originally procured from a commercial supplier (TilAqua International, Velden, Netherlands). Fish were held in 500 L fiberglass tanks connected to a recirculation system. Water temperature was maintained at 26 °C, and oxygen saturation levels were kept above 80% by direct aeration in tanks. Approximately 10% of the water was replaced daily. Fish were hand-fed every morning using a commercial diet (Efico Cromis832F, BioMar, France). Tanks were regularly cleaned, and the photoperiod regime was maintained at 12 h light: 12 h dark. All trials were conducted at an experimental temperature of 26 °C.

2.2. Effect of progressively increasing [CO_2] on MO_2

MO_2 was measured in 12 Nile tilapia with an average mass of 435 ± 16 g (SE) in response to progressive increases in [CO_2] using intermittent flow-through respirometry (Steffensen, 1989). pO_2 was measured in the recirculation loop using fibreoptic sensors connected to an OXY-4 m (PreSens, Regensburg, Germany) and transferred to a PC running AutoResp software (Loligo Systems, Viborg, Denmark). Oxygen sensors were calibrated to 0% saturation in a saturated solution of Na_2SO_3 and to 100% in air-saturated water at 26 °C. Tilapia were fasted for 24 h before being transferred to a respirometer with a volume of 5.6 L, where they were allowed to acclimate overnight prior to experimentation. The following morning, fish were exposed to increasing CO_2 concentrations from normocapnic (< 1 mg L^{-1}) to severely hypercapnic (60 mg CO_2 L^{-1}) by ramping CO_2 concentrations at a rate of 5 mg L^{-1} h^{-1} over 12 h. CO_2 levels were controlled as previously described (Hamad et al., 2023). In brief, pH was used as a proxy for CO_2 concentration based on a standard curve for the correlation between pH and dissolved CO_2 specific for the water used (ca. 4 mEq alkalinity L^{-1}). pH was monitored on a pH meter (Radiometer PHM220, Copenhagen, Denmark), with a voltage output collected via a digital acquisition device (U6, LabJack, USA) to a PC running a custom script (DAQFactory Express, AzeoTech, USA). The desired CO_2 levels were defined in the script using extrapolation between time-points to achieve a steadily increasing CO_2 concentration, rather than a step-like ramping. Dissolved CO_2 levels were monitored using a handheld CO_2 meter (Oxyguard, Farum, Denmark) and logged on a PC.

For each exposure level, the average of the last 3 consecutive MO_2 measurements was used. Each measurement loop lasted 8 min, consisting of a 3-min flush, a 1-min wait, and a 4-min measurement period. Experiments were conducted on 4 fish simultaneously, using identical respirometers submerged in an external reservoir with a total volume of 250 L. A small recirculation pump ensured continuous water mixing in the reservoir tank. To avoid CO_2 degassing, O_2 saturation was maintained at 100% by bubbling pure O_2 controlled by a solenoid valve connected to a programmable relay (PR Electronics, Rønde, Denmark) with a galvanic oxygen sensor (Handy, Oxyguard).

2.3. Effect of CO_2 exposure concentration and time on standard (SMR) and maximum metabolic rate (MMR)

Standard (SMR) and maximum metabolic rate (MMR) measurements were performed to evaluate the response in oxygen consumption rate following acute (1 h) and prolonged (24 h) exposure to four CO_2 concentrations (0, 10, 30, or 60 mg CO_2 L^{-1}). SMR and MMR were determined for 8 individual fish each for control, at each CO_2 concentration, and for each exposure duration ($n = 8$, $N = 56$, mean body mass 397 ± 15 g. During the experiment, CO_2 concentration was controlled in a similar way as described in Section 2.2, i.e. ramped up progressively, with the modification that once the target CO_2 concentration was reached it was then maintained for the duration of the exposure. For both acute and prolonged exposure MO_2 was recorded, SMR was determined as the mean value of the last 3 consecutive MO_2 measurements from an individual fish at each CO_2 concentration. MMR was then determined by removing the fish from the respirometer and subjecting it to aerial exposure for 4 min. After this, the fish was returned to the respirometer and the first MO_2 reading after exhaustion was recorded as MMR.

2.4. Effects of exposure level and duration on haematology and acid-base status

Fish with average body mass 247 ± 6 g ($n = 8$) were exposed to one of three elevated CO_2 concentrations described above ($N = 24$), during which fish were individually confined in non-transparent PVC tubes (L 500 × Ø 105 mm). Fish were transferred after 24 h fasting and allowed to recover for a further 24 h before the onset of experiments. At the desired time point, fish were gently but quickly anesthetized in a buffered solution containing 0.1 g L^{-1} benzocaine (ethyl-p-aminobenzoate). When fish became unresponsive to tactile stimulation, they were euthanized by pithing, and a 2 mL blood sample was taken from a caudal vessel using a heparinized syringe. Blood samples were divided into 2 aliquots. One aliquot was centrifuged at 13000*g* for 5 min, plasma was transferred to a new aliquot, and the plasma and red blood cell (RBC) fractions were frozen on dry ice. A sample of white muscle (~1 g) was taken from the left dorsal aspect of the fish at the level of the dorsal fin and frozen on dry ice. Plasma, RBCs, and muscle samples were transferred to −80 °C until analysis. Whole blood pH was measured in the second aliquot using a pH microelectrode (MI-410, Microelectrodes, Inc., USA) attached to a multimeter (HI 98185, Hanna Instruments, Sweden). Haematocrit (Hct) was determined in duplicate using wax-sealed micro-capillary tubes centrifuged at 8700*g* for 5 min. Plasma chloride was determined using a chloride analyser (M926, Sherwood Scientific, Cambridge, UK). Muscle pHi was measured using a glass pH electrode in muscle tissue homogenised in an equal volume of distilled water (Lima dos Santos et al., 1981; Vyncke, 1981) for one min at room temperature using an Ultra Turrax homogenizer (IKA-Werke, Staufen, Germany). RBC pHi was determined on thawed RBC at room temperature using a glass pH electrode. Haemoglobin concentration [Hb] was determined using the cyanmethemoglobin method (Drabkins). RBC counts were determined using a Neubauer haemocytometer (C-Chip, NanoEntek, Massachusetts, USA) loaded with 10 μL blood diluted 200 × with Ringer solution (containing in mmol 124 NaCl, 3 KCl, 0.75 $CaCl_2$, 1.30 $MgSO_4$, and 12 $NaHCO_3$).

Total plasma CO_2 concentration was measured on a custom-built CO_2 setup, inspired from Lee et al. (2018). The CO_2 setup consisted of a custom-made 6 ml gas-sparging column containing 2 mL 10 mmol L^{-1} HCl, that converted the plasma HCO_3^- into CO_2. The gas-sparging column was continuously bubbled with pure N_2 gas at a flow of 40 ml min^{-1} to release the generated CO_2 into the gas phase. The outflowing gas was dehydrated in a column of $CaCl_2$, and CO_2 gas content was measured on a LiCOR LI-7000 gas-analyser using LiCOR software (LI-COR, Cambridge, UK). To measure total plasma carbon dioxide concentration ($[CO_2]$), plasma samples were first thawed on ice. Then, 5 μL plasma sample was injected into the gas-sparging column followed by a 5 μL injection of freshly made 20 mmol L^{-1} $NaHCO_3$ solution. The volume of expelled CO_2 was calculated by integrating the CO_2 signals of the two injections, and the $[CO_2]$ was calculated as the ratio between the two integrations multiplied by 20 mmol L^{-1}.

The partial pressure of blood CO_2 was calculated from measured CO_2 and pH values using the Henderson-Hasselbalch equation, plasma CO_2 solubility constant, and pK' from Siggaard-Andersen (1976). HCO_3^- was then subtracted from the same equation using the actual value of plasma CO_2 with the same pK'.

2.5. Analytical methods and data analysis

Data were verified for normality using the Shapiro-Wilk test, and for equal variance using the Brown-Forsythe test. Changes in MO_2 during progressive increases in dissolved CO_2 and effects of short and prolonged exposure on SMR and MMR was tested using one-way ANOVA and all pairwise multiple comparison (Holm-Sidak method). Changes in haematological parameters and plasma chemistry during short (1 h) and long-term (24 h) exposure to hypercapnia were tested using two-way ANOVA with $[CO_2]$ and time considered as factors, with multiple comparison against the pre-exposure values that served as control for both exposure times (Holm-Sidak). For all statistical tests, a probability below 0.05 was considered significant. All data are presented as mean ± standard error of the mean. Statistics and plots were produced using SigmaPlot (v. 14.5 Systat Software, Inc.).

3. Results

3.1. Changes in MO_2 in response to progressive increases in CO_2

Exposure to progressively increasing $[CO_2]$ resulted in a concentration-dependent reductions in MO_2 in Nile tilapia (Fig. 1, $p < 0.001$). The mean MO_2 of Nile tilapia was 87.02 ± 0.27 mg O_2 kg^{-1} h^{-1} under normocapnic control conditions (~ 1 mg L^{-1} CO_2). MO_2 immediately declined by 10% when $[CO_2]$ increased to 5 mg L^{-1}, and with increasing CO_2 concentrations, MO_2 continued to decrease linearly by an average of 1.4 mg O_2 kg^{-1} h^{-1} (1.6%) for every 5 mg L^{-1} CO_2 increase. At the highest tested CO_2 concentration, the mean MO_2 had declined by 30% to 61.65 ± 0.91 mg O_2 kg^{-1} h^{-1} compared to the normocapnic control condition.

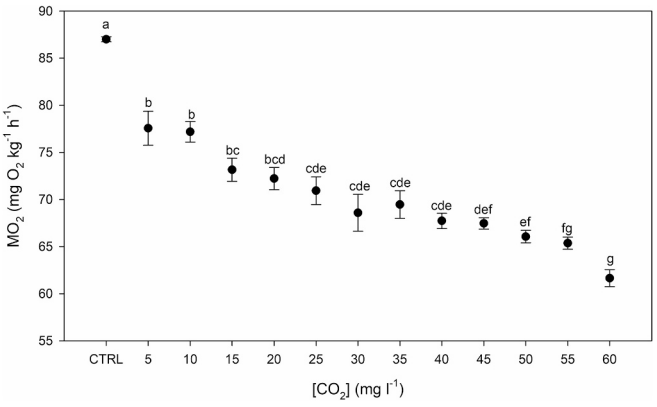

Fig. 1. Changes in mean oxygen uptake rate (MO_2) in Nile tilapia during exposure to hypercapnia, with dissolved CO_2 levels progressively increasing at a rate of 5 mg L^{-1} h^{-1}. Each data point represents the mean value (± SE) from a group of fish ($n = 12$). Differences in superscript letters denote significant changes in MO_2 at the different CO_2 concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Effects of acute and long-term CO_2 exposure on MO_2

Exposing Nile tilapia to different CO_2 concentrations acutely (1 h) or prolonged (24 h) significantly affected SMR and MMR. Following 1 h exposure, fish at all tested CO_2 concentrations showed a significant decrease in SMR by 11, 22, and 29% in the 10, 30, and 60 mg L^{-1} CO_2 treatments compared to the normocapnic (control) group respectively, while only the 30 and 60 mg L^{-1} treatments showed significant reductions in MMR. SMR showed little signs of recovery in any of the CO_2 treatment groups, as all remained significantly decreased by 10, 16, and 28%, respectively. In a similar manner, exposure to 30 and 60 mg L^{-1} CO_2 (Fig. 2B) for 1 h also resulted in significant decreases in MMR by 30 and 31%, respectively, compared to normocapnic control conditions. After 24 h, all CO_2 treatments had recovered their MMR to an extent that there were no longer significant differences from control values (Fig. 2B). Consequently, MS was significantly reduced after 1 h exposure to CO_2 concentrations of 30 and 60 mg L^{-1}, but were recovered after 24 h (Fig. 2C).

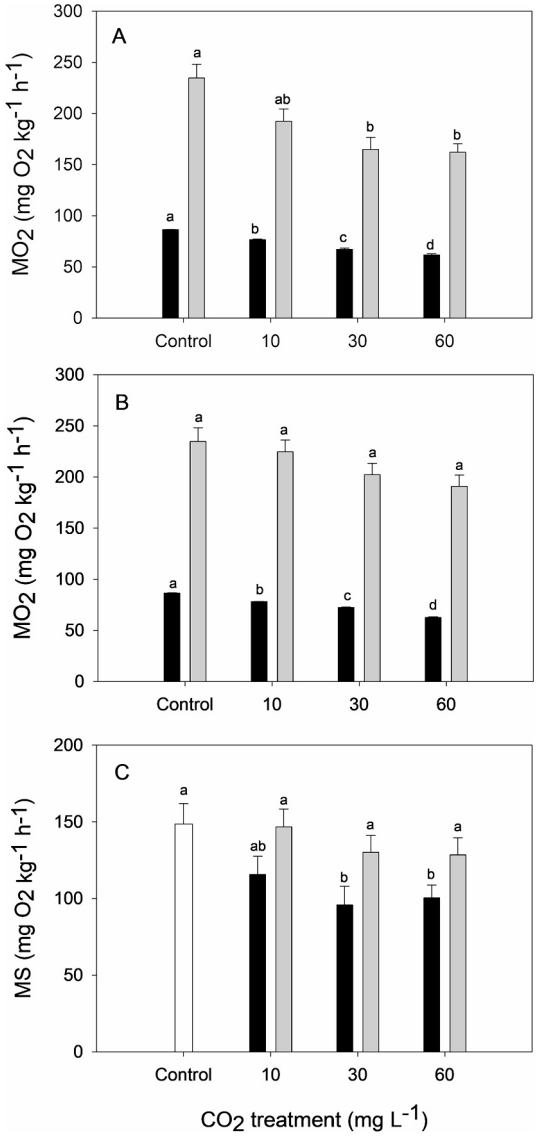

Fig. 2. Standard metabolic rate (SMR; black bars) and maximum metabolic rates (MMR; grey bars) of Nile tilapia exposed to 10, 30 and 60 mg L^{-1} CO_2 for (A) 1 h ($n = 8$, $N = 32$) or (B) 24 h (n = 8, N = 32), and (C) Metabolic scope (MS) of tilapia exposed to normocapnic control (no fill), 10, 30 and 60 mg L^{-1} CO_2 for 1 h (black bars) and 24 h (grey bars). Letters that differ indicate significant differences between concentrations $p < 0.05$.

3.3. Haematological parameters

The haematological parameters at 1 h and 24 h following exposure to different CO_2 concentrations are shown in Table 1. Overall, minor changes were observed in response to CO_2 concentration during both acute and prolonged exposure. There were no effects of time, nor were there interacting effects between [CO_2] and time. There was a dose dependent increase in Hct and haemoglobin concentration. No changes were observed in RBC count (RBCc) or mean cell volume (MCV). Because Hct and [Hb] increased proportionally, there was no overall effect of mean cell haemoglobin concentration (MCHC).

3.4. Blood and muscle pH

Changes in intracellular and extracellular pH at different CO_2 concentrations and exposure times are shown in Fig. 3. Extracellular pH showed a progressive decline in proportion to increasing CO_2 concentration at 1 h of exposure, decreasing by up to 0.52 units in the 60 mg CO_2 L^{-1} treatment. All treatment groups showed an ability to partially, but significantly, restore extracellular pH after 24 h exposure, but all groups remained significantly lower than the normocapnic control group (Fig. 3A). Intracellular pH measured in RBCs decreased significantly in all treatment groups during short term exposure by 0.21, 0.41, and 0.55 units in the 10, 30, and 60 mg L^{-1} CO_2 concentration, respectively, from the normocapnic control (Fig. 3B). Following 24 h of exposure, the 10 mg L^{-1} CO_2 group had recovered RBC pHi. While RBC pHi remained significantly lower than the control group at 24 h in the 30 and 60 mg CO_2 L^{-1} treatments, they were significantly increased from 1 h exposure. Muscle pHi followed this trend of recovery at 10 mg CO_2 L^{-1}, and while pHi in the 30 mg CO_2 L^{-1} treatment was significantly increased at 24 h compared to 1 h, the 60 mg CO_2 L^{-1} showed no signs of recovery (Fig. 3C).

3.5. Total plasma carbon dioxide, bicarbonate and chloride

Changes in plasma [TCO_2], [HCO_3^-], and [Cl^-] between acute (1 h) and prolonged (24 h) exposure to CO_2 are presented in Fig. 4. Following 1 h exposure, all CO_2 treatment groups had significantly increased their plasma [TCO_2] and [HCO_3^-] compared to the normocapnic control group. After 24 h the 60 mg L^{-1} CO_2 treatment had further increased the plasma TCO_2 to 31.2 mmol L^{-1} (Fig. 4A) and HCO_3^- to 29.2 mmol L^{-1} (Fig. 4B), which was a significant increase from the 1 h time point. At 1 h exposure, there were no significant changes in plasma Cl^- concentrations in any of the CO_2 treatment groups compared to the control group, while at 24 h, all CO_2 treatments had significantly decreased their plasma [Cl^-] (Fig. 4C). The absolute average changes in [TCO_2], [HCO_3^-], and [Cl^-] between the normocapnic control and 60 mg L-1 CO2 were 19.1, 17.4 and 12.6 mmol L^{-1}, respectively.

4. Discussion

Nile tilapia acutely exposed to progressively increasing concentrations of dissolved CO_2 respond with a decrease in MO_2 in a dose-dependent manner. Following acute (1 h) exposure to 10, 30, or 60 mg CO_2 L^{-1} fish show significant decreases in both SMR and MMR, and a loss in MS. After prolonged exposure, Nile tilapia were able to recover their MMR to levels not significantly different from normocapnic controls, ultimately recovering their MS. Exposure to 10, 30, or 60 mg CO_2 L^{-1} was associated with large intra- and extracellular acidosis that fish were not able to restore in 24 h, despite accumulation of HCO_3^- through a chloride shift.

The dose-dependent decrease in MO_2 following acute exposure of Nile tilapia to increasing levels of CO_2 observed in the present study appears to differ from most species studied. In the extensive review by

Table 1
Haematological parameters of Nile tilapia exposed to 10, 30, and 60 mg L^{-1} CO_2 after 1 h and 24 h. Data expressed as mean ± SE (n = 8, N = 56), different superscripts in each row indicates a significant effect of CO_2 concentration for the given time of exposure. Haemoglobin concentration ([Hb]) is expressed in mM, red blood cell count (RBCc) is in millions of cells per mL, mean cell volume (MCV) is expressed in femtoliters (fL), while mean cellular haemoglobin concentration (MCHC) is expressed in mM (MW 64,458 g mol^{-1}). Calculations were performed as described in Wells and Baldwin (1990).

	Time		[CO_2]			*P*-value		
Parameter	(h)	Control	10 mg L^{-1}	30 mg L^{-1}	60 mg L^{-1}	[CO_2]	Time	[CO_2] × Time
Haematocrit (%)	1	17.9 ± 1.6^{a}	19.5 ± 1.4^{a}	20.1 ± 1.6^{a}	21.4 ± 1.1^{a}	0.002	0.418	0.277
	24		17.5 ± 1.7^{a}	21.7 ± 1.1^{ab}	25.2 ± 1.6^{b}			
[Hb] (mM)	1	1.07 ± 0.09^{a}	1.19 ± 0.12^{a}	1.39 ± 0.10^{a}	1.45 ± 0.05^{b}	<0.001	0.898	0.568
	24		1.05 ± 0.12^{a}	1.35 ± 0.09^{ab}	1.59 ± 0.11^{b}			
RBCc (10^{6} μL^{-1})	1	2.53 ± 0.22^{ab}	1.99 ± 0.14^{a}	1.93 ± 0.11^{a}	2.67 ± 0.20^{b}	0.013	0.859	0.239
	24		2.21 ± 0.18^{a}	2.19 ± 0.13^{a}	2.28 ± 0.17^{a}			
MCV (fL)	1	74.7 ± 9.3^{a}	99.4 ± 6.7^{a}	107.2 ± 10.9^{a}	81.5 ± 5.2^{a}	0.014	0.666	0.085
	24		82.5 ± 12.1^{ab}	103.2 ± 11.0^{ab}	114.0 ± 9.9^{b}			
MCHC (mM)	1	6.08 ± 0.42	6.16 ± 0.42	6.93 ± 0.36	6.90 ± 0.42	0.280	0.159	0.655
	24		6.05 ± 0.36	6.05 ± 0.16	6.33 ± 0.11			

Lefevre (Lefevre, 2019, Table 1) nearly all studies investigating the effects of elevated pCO_2 show that SMR (denoted as MO_{2min}) are either unchanged or elevated. While our previous observations on Atlantic salmon (*Salmo salar*) have shown a decrease in SMR at a CO_2 concentration above 25 mg L^{-1}, this species maintained an unchanged MO_2 up to this concentration (Khan et al., 2018), underlining the importance of the degree of hypercapnia being tested on the response by the fish. A notable exception in the overview provided by Lefevre (2019) is the response of freshwater European eel (*Anguilla anguilla*), which shows a response similar to that of Nile tilapia, by decreasing MO_2 linearly with increasing pCO_2 from 10 to 40 mmHg (Cruz-Neto and Steffensen, 1997). Presently, we have no clear explanation for why these 2 species stand out. Uncompensated acidification of the blood is likely to occur immediately following exposure to CO_2, and Cruz-Neto and Steffensen (1997) suggested that this might be the cause of the reduction in MO_2. Mechanistically, this could be caused by a Root effect, or by impaired cardiac performance in response to extracellular acidosis, as has been shown for a number of fish species (Farrell et al., 1983, 1986). However, 2 observations from the current study question this. Firstly, while Nile tilapia may have lost 25–30% of their MS following 1 h of exposure, they still have capacity to increase MO_2, and secondly, they still show a depressed SMR following 24 h of exposure, despite having recovered MMR and MS. Thus, it appears to be a strategy to allow SMR to drop in response to hypercapnia. This could be an adaptation to living in environments (lakes and ponds) that experience daily fluctuations in dissolved O_2 and CO_2. Furthermore, it is in alignment with our previous studies on Nile tilapia, that when exposed to severe hypoxia (~15 mmHg) fish decrease their SMR by 50% for up to 24 h as a coping mechanism (Bergstedt et al., 2021). The rate of oxygen consumption (MO_2) is often used to evaluate metabolic performance and indirectly reflects how much energy fish spend to maintain normal physiological functions (Jobling, 1981). Reducing MO_2 in the face of increasing environmental CO_2 could be a protective mechanism, the pathways of which are not clear.

Increasing environmental CO_2 can induce a variety of haematological changes in fish red blood cell number, haemoglobin concentration, and Hct (Kaya et al., 2016) that directly affect blood gas transport capacity (Brauner et al., 2004). In the present study, the haematological changes in Nile tilapia associated with acute (1 h) or prolonged (24 h) increased CO_2 concentrations were minor, and only occurred in the 60 mg CO_2 L^{-1} treatment. During 1 h exposure, Hct was unaffected at all CO_2 concentrations, although there was a tendency for increase. Hct only significantly increased in the 60 mg group at 24 h, which was associated with a significant increase in [Hb]. The increase in Hct did not appear to be caused by changes in RBCc. This would suggest that RBC swelling caused the increase in Hct (Nikinmaa, 1982), which is supported by significantly increasing MCV. However, MCHC did not change, suggesting that some plasma may have been skimmed to the secondary circulation (Gallaugher and Farrell, 1998) causing the rise in Hct rather than swelling. In Mozambique tilapia (*O. mossambicus*) exposed to ca. 14 mg L^{-1} CO_2 showed a decrease in RBCc, increased [Hb], and an unchanged Hct (Kaya et al., 2016) which is the similar trend as observed for the 10 mg treatment in our study. Collectively, there is not much data on the haematological responses to hypercapnia in fish, which might be due to difficulties interpreting which changes are occurring as responses to acute acidification and in subsequent acid-base regulation.

The extent and timing of pH compensation and recovery in fish following exposure to elevated levels of CO_2 vary greatly depending on the regulatory capacity (Brauner et al., 2019; Shartau et al., 2019) and exposure concentration (Claiborne and Heisler, 1986; Shartau et al., 2020). In the present study, Nile tilapia was also able to compensate pH changes in a concentration-dependent manner where pH was partially compensated for at the high CO_2 concentrations of 30 and 60 mg CO_2 L^{-1} and it was fully compensated at 10 mg CO_2 L^{-1}. The majority of studies that have examined the response of fishes to elevated levels of CO_2 have found that the initial acidosis is rapid, arterial pCO_2 equilibrates with water pCO_2 in a matter of moments, and the pH of the blood (pHe) and tissues (pHi) decreases as a function of both the newly equilibrated CO_2 tension and the non-bicarbonate (i.e., intrinsic) buffer capacity of the respective compartment (Shartau et al., 2016). Additionally, most of these studies noted that the pH recovery took between 24 and 96 h and is driven by transferring acid-base-relevant ions with the surrounding water primarily at the gills (Claiborne and Heisler, 1986; Petochi et al., 2011). Nile tilapia showed a similar response to hypercapnia with blood pH reduced in proportion to the increase in pCO_2, where a respiratory acidosis was fully or partially compensated for to control values within 24 h, with subsequent accumulation of plasma HCO_3^- in exchange for Cl^- (Fig. 4).

The pH compensation during hypercapnic acidosis in fish follows a coupled pH regulation pattern where blood pH (pHe) is regulated, at least in part, to muscle pH (pHi) (Claiborne and Heisler, 1986; Claiborne et al., 2002; Damsgaard et al., 2015; Munday et al., 2016) or can be preferential (maintaining constant pHi in the face of lowered pHe) (Baker and Brauner, 2012; Shartau et al., 2019). According to Brauner et al. (2019), fish must regulate pHi to maintain normal cellular functions regardless of the status of pHe. In the present study, Nile tilapia showed a higher capacity for regulating pHi with increased exposure concentration than for pHe. However, intracellular RBC and muscle pHi was significantly reduced at high CO_2, showing that these tissues do not preferentially pHi regulate in response to high CO_2, which is consistent with a previous study on a tilapia hybrid (Shartau et al., 2020). The compensation of pH at higher CO_2 by elevating plasma HCO_3^- in exchange for Cl^- both intra and extracellularly can increase the energy demands for ion-exchanging processes to maintain HCO_3^- at a higher level (Strobel et al., 2012). In the present study, maximum hypercapnia tolerance of Nile tilapia were tested by exposure to dissolved CO_2 levels

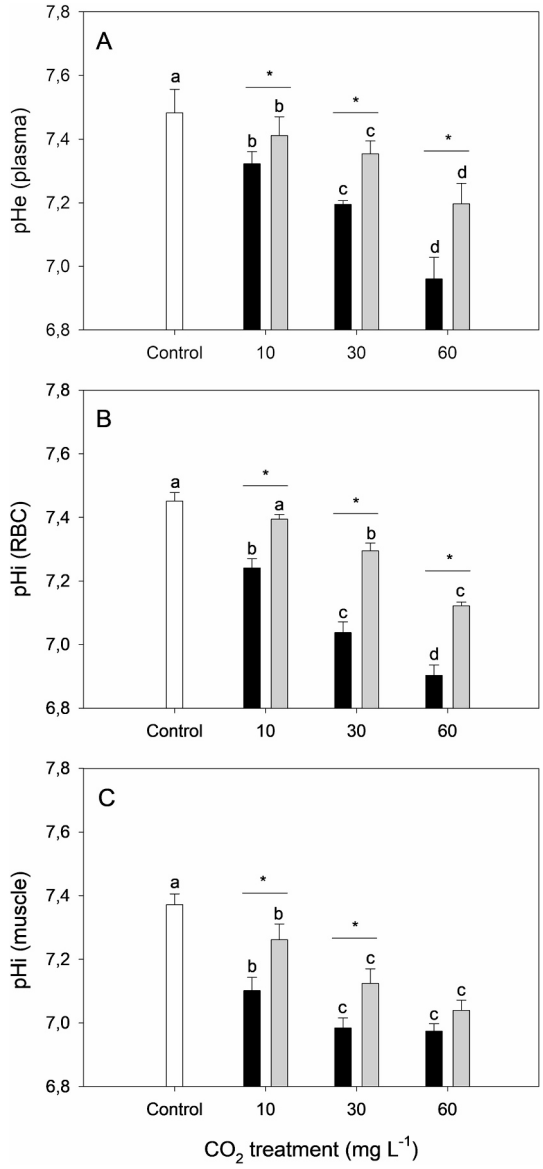

Fig. 3. Extracellular pH (pHe; plasma), red blood cell intracellular pH (pHi RBC) and skeletal muscle intracellular pH (pHi muscle) (C) in Nile tilapia exposed to 10, 30, and 60 mg L^{-1} CO_2 after 1 h (black bars) and 24 h (grey bars), compared to normocapnic control values (no fill). Values are given as means ±SE (n = 8, *N* = 56). Different superscripts indicate significant differences between exposure concentration. Differences between exposure times, within each concentration, are indicated by an asterisk.

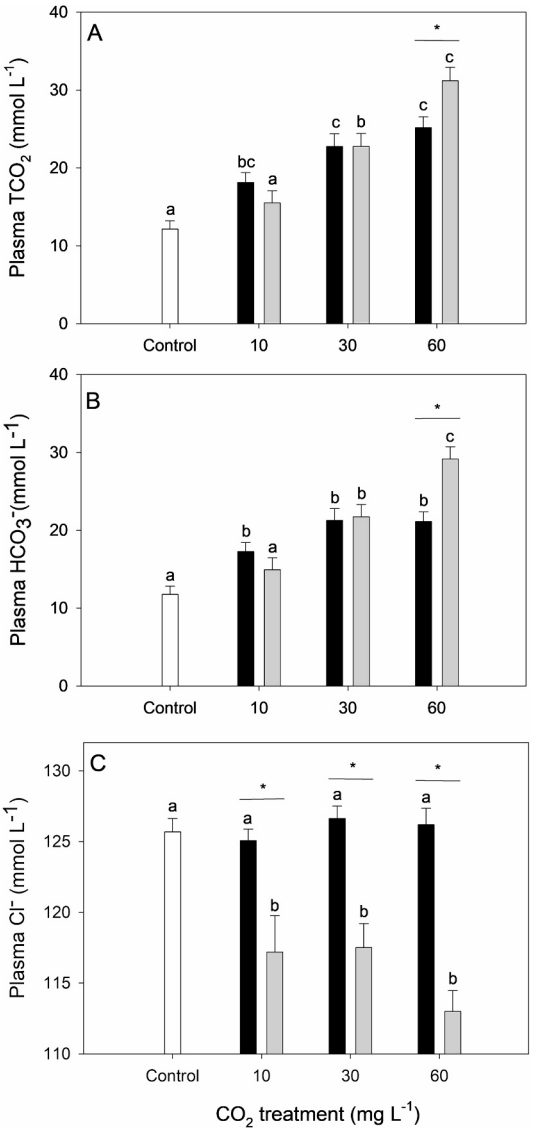

Fig. 4. Total plasma CO_2 (TCO_2 mmol L^{-1}; A), bicarbonate (HCO_3^- mmol L^{-1}; B), and chloride (Cl^- mmol L^{-1}; C) in Nile tilapia exposed to 10, 30, and 60 mg L^{-1} CO_2 after 1 h (black bars) and 24 h (grey bars), compared to normocapnic control values (no fill). Values are given as means ±SE (n = 8, N = 56). Different superscripts indicate significant differences between exposure concentration. Differences between exposure times, within each concentration, are indicated by an asterisk. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

up to 60 mg L^{-1}, during which blood HCO_3^- levels increased by ~20 mM, with an equivalent decrease in plasma Cl^-, which is consistent with the typical reduction in the activity of the HCO_3^-/Cl^--exchanger at the gill (Evans et al., 2005). This compensation limits plasma [HCO_3^-] elevation of Nile tilapia observed during hypercapnia exposure appears to be close to the typical 30 mM threshold of plasma [HCO_3^-] proposed by (Heisler, 1984). These findings suggest that Nile tilapia is unable to compensate pHe during the prolonged exposure to high CO_2, resulting in downstream reductions in intracellular pH.

4.1. Conclusion

Increasing levels of environmental CO_2 leads to significant physiological disturbances in Nile tilapia. Exposure to acutely increasing levels of CO_2 leads to immediate metabolic suppression in a dose dependent manner. Whether this is a coping mechanism, or the result of acidification is unknown, but the response persists over 24 h and MS, although reduced, is not lost, suggestive of a coping mechanism. Exposure to different levels of CO_2 causes a dose-dependent reduction in MMR and associated MS, but fish recovered these after 24 h, despite incomplete recovery of pHe and pHi within this time. A possible compensatory mechanism appears to be an increase in Hct and [Hb] in the 30 and 60 mg CO_2 L^{-1} groups after 24 h. Nile tilapia is generally considered a species that tolerate poor oxygen conditions, but the pattern of acid-base regulation following hypercapnic exposure implies some sensitivity to CO_2 exposure which should be considered in pond management.

CRediT authorship contribution statement

Muumin Iddi Hamad: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft,

Writing – review & editing, Visualization. **Christian Damsgaard:** Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Renalda Nanziga Munubi:** Data curation, Writing – original draft, Writing – review & editing, Supervision. **Peter Vilhelm Skov:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

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