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The effects of elevated environmental CO$_2$ on nitrite uptake in the air-breathing clown knifefish, *Chitala ornata*

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**Key words:** acid-base balance, *Chitala ornata*, hypercapnia, ion exchange, metHb, nitrite

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**Highlights**

- Exposure to Aquatic hypercapnia causes a significant reduction in nitrite uptake in the air-breathing fish, *Chitala ornata*.
- *Chitala ornata* responds to aquatic hypercapnia by reducing the turnover of Chloride and bicarbonate ions at the gill.
- Since nitrite replaces chloride at this ion exchanger, the slow response to hypercapnia slows nitrite uptake.

**Abstract**

Nitrite and carbon dioxide are common environmental contaminants in the intensive aquaculture ponds used to farm clown knifefish (*Chitala ornata*) in the Mekong delta, Vietnam. Here we tested the hypothesis that hypercapnia reduces nitrite uptake across the gills, because pH regulation will reduce chloride uptake and hence nitrite uptake.
as the two ions compete for the same transport route via the branchial HCO$_3^-$/Cl$^-$ exchanger. Fish fitted with arterial catheters were exposed to normocapnic/normoxic water (control), nitrite (1 mM), hypercapnia (21 mmHg CO$_2$), or combined hypercapnia (acclimated hypercapnia) and nitrite for 96h. Blood was sampled to measure acid-base status, haemoglobin derivatives and plasma ions. Plasma nitrite increased for 48 h, but levels stayed below the exposure concentration, and subsequently decreased as a result of nitrite detoxification to nitrate. The total uptake of nitrite (evaluated as [NO$_2^-$] + [NO$_3^-$]) was significantly decreased in hypercapnia, in accordance with the hypothesis. Methemoglobin and nitrosylhemoglobin levels were similarly lower during hypercapnic compared to normocapnic nitrite exposure. The respiratory acidosis induced by hypercapnia was half-compensated by bicarbonate accumulation in 96 h, which was mainly chloride-mediated (i.e. reduced Cl$^-$ influx via the branchial HCO$_3^-$/Cl$^-$ exchanger). Plasma osmolality and main ions (Na$^+$, Cl$^-$) were significantly decreased by hypercapnia and by nitrite exposure, consistent with inhibition of active transport. We conclude that hypercapnia induces a long-lasting, and mainly chloride-mediated acid-base regulation that reduces the uptake of nitrite across the gills.

**Introduction**

In tropical aquatic systems, CO$_2$ levels can reach levels far above atmospheric equilibrium as a result of organic enrichment, poor water exchange or surface cover by floating vegetation, and pCO$_2$ values of up to 50-60 mmHg have been recorded in both natural and aquaculture settings (Willmer, 1934; Wakeman and Ultsch, 1975; Heisler, 1982; Ultsch, 1987; Damsgaard et al., 2015). Environmental hypercapnia (elevated CO$_2$ level) leads to a respiratory acidosis inside fish, which stimulates a compensatory branchial transfer of acid-base equivalent ions (HCO$_3^-$ retention and H$^+$ release) to counteract the acidosis (Heisler, 1984; Claiborne et al., 2002; Evans, et al., 2005; Perry and Gilmour, 2006). In water breathing freshwater fish, pH recovery during hypercapnia is primarily chloride-mediated via the branchial HCO$_3^-$/Cl$^-$ exchanger, affecting a reduction in branchial Cl$^-$ uptake and an elevation of extracellular HCO$_3^-$ (Wood, 1991; Perry and Laurent, 1993; Larsen and Jensen, 1997).
The gills are the main site of acid-base regulation in freshwater fish, accounting for about 90% of the acid-base relevant ion transport during pH compensation, while the kidney and intestine account for the remaining 10% (Heisler, 1984; Evans et al., 2005). Air-breathing fish, which have assumed considerable importance both commercially and as a source of human protein (Lefevre et al., 2014), however, typically show reduced gill surface area, which may limit transepithelial ion exchange and therefore the branchial capacity for acid-base regulation. Thus, it has been proposed that there is a lower capacity for extracellular pH regulation during a respiratory acidosis in the air-breathing fishes (Brauner and Baker, 2009; Shartau and Brauner, 2014).

Nitrite (NO$_2^-$) is a natural intermediate of bacterial nitrification and denitrification processes, and can pose a problem in aquatic ecosystems, because of its well-documented toxicity to animals (Lewis and Morris, 1986; Jensen, 2003). Nitrite is taken up across the gills into the blood via the branchial Cl/HCO$_3^-$ exchanger (Eddy and Williams, 1987; Jensen, 2003). Once in the blood, nitrite can enter the red blood cells by the AE1 Cl/HCO$_3^-$ exchanger and by diffusion of HNO$_2$ through the lipid bilayer of the erythrocyte membrane (Jensen and Rohde, 2010). Inside RBCs, nitrite reacts with haemoglobin (Hb), where it causes haem oxidation (Fe$^{2+}$ to Fe$^{3+}$), generating methaemoglobin (metHb) and nitrate with oxygenated Hb (oxyHb) and metHb and nitrosylHb (HbNO) in the reaction with deoxygenated haemoglobin (deoxyHb) (Kosaka and Tyuma, 1987; Jensen, 2009). Since neither metHb nor HbNO can bind oxygen, these reactions reduce blood oxygen carrying capacity, leading to respiratory disturbance. Both nitrite uptake and toxicity depend on ambient nitrite levels, ambient chloride levels and the rate of active chloride uptake (Williams and Eddy, 1986; Tomasso and Grosell, 2005). The nitrite ion competes with chloride for the Cl/HCO$_3^-$ exchange mechanism in the gills, whereby part of the active chloride uptake is changed to nitrite uptake while the passive Cl$^-$ loss persists, leading to a net Cl$^-$ efflux (Jensen et al., 1987; Eddy and Williams, 1987; Jensen, 2003). In some fish (e.g. carp and rainbow trout) the sum of ions and osmolality stays constant during nitrite exposure (Jensen et al., 1987; Stormer et al., 1996), while in other animals (e.g. ...
crayfish, shrimps, snakehead and clown knifefish) plasma osmolality decreases (Jensen, 1996; Cheng and Chen, 1998; Lefevre et al., 2012; Gam et al., 2017), which may be a result from an expansion of extracellular volume and an increase of the whole body water content.

Since both pH_e regulation during respiratory acidosis and nitrite uptake during nitrite exposure in freshwater fish involve the branchial Cl^-/HCO_3^- exchanger, it can be hypothesized that nitrite uptake may be reduced by hypercapnia (Jensen et al., 2000; Hvas et al., 2016). The idea being that hypercapnia induces an acid-base regulatory decrease in Cl^- uptake (increasing internal [HCO_3^-]) and since nitrite and chloride compete for transport via the branchial Cl^-/HCO_3^- exchanger, nitrite uptake should decrease. This hypothesis was originally tested in crayfish (Astacus astacus), where the data supported that aquatic hypercapnia protected against nitrite toxicity via a significantly lower nitrite uptake (Jensen et al., 2000). However, the effect was only transient in the air-breathing fish Pangasianodon hypophthalmus, and after pH regulation the Cl^-/HCO_3^- exchanger, whose turnover rate depends on blood [HCO_3^-] (Goss and Wood, 1991), functioned at a higher rate and actually increased nitrite uptake (Hvas et al., 2016). These partly opposite responses make it relevant to explore the influence of hypercapnia on nitrite uptake in additional species to reach a consensus. P. hypophthalmus is unusual among air-breathing fish in possessing very large gills (Phuong et al., 2017b; 2017c) and a very well developed capacity for pH_e regulation (Damsgaard et al., 2015). Air-breathing fish seem to possess high nitrite tolerance and unusual temporal profiles of methaemoglobin, with an initial increase as generally seen in fresh water fish, which reverts to a decline after 24-48h of exposure (Lefevre et al., 2011b; 2012; Gam et al., 2017).

The economically important facultative air-breathing knifefish, Chitala ornata is cultured throughout South East Asia, exemplifies this by being one of the most nitrite tolerant freshwater fish species ever recorded (96 h LC_50 of 7.82 mM, Gam et al., 2017). In part, this tolerance is a result of the ability of this fish to up-regulate erythrocyte methaemoglobin reductase activity, allowing it to rapidly reduce methemoglobin back to functional oxygen carrying Hb (Gam et al., 2017). Thus, C.
ornata can effectively convert nitrite to non-toxic nitrate in the reaction of nitrite with oxygenated Hb, while the formed metHb can be efficiently reduced to functional Hb. C. ornata has small gills as is common among air-breathing fish and we therefore hypothesised that it would be slow (>>96h) in compensating for extracellular pH disturbances.

The present study therefore tested the hypothesis that pH regulation under a respiratory acidosis will reduce Cl− and nitrite uptake via the branchial HCO3−/Cl− and thereby protect against nitrite toxicity. Since we expect hypercapnia to develop gradually in tropical freshwater systems (Damsgaard et al., 2015) and C. ornata to be slow in compensating for the resulting acidosis, our study included 4 exposure groups: normocapnia (control), hypercapnia (pCO2 = 21 mmHg), 1 mM nitrite, and combined hypercapnia (96h acclimated hypercapnia) with nitrite, and the hypothesis was evaluated by measuring Hb derivatives (functional Hb versus metHb and HbNO), plasma nitrite and nitrate, plasma ions, plasma glucose and acid-base status.

4.3 Materials and methods

4.3.1 Animal holding

Chitala ornata (body mass 570.95 ± 56.33 g) from a local intensive farm were transported to Can Tho University. They were held at ambient laboratory temperature 27-28ºC in 4 cubic meter tanks with constant aeration (dissolved oxygen >90%) for 2 weeks before experimentation. Fish were fed by commercial feed (shrimp feed with 38% protein, Tomboy Aquafeed company, Vietnam). Thirty percent of tank water was changed every second day to maintain optimal environmental condition (NO2− < 1µM, NO3− < 40µM and NH3 < 40 µM). Feeding was stopped 2 days before starting the experiment. The experiment was performed in accordance with national guidelines on the protection and care of experimental in Vietnam.

4.3.2 Experimental protocols
A total of 24 fish (570 ± 56.33g) were used. They were anaesthetized in 0.05 g l⁻¹ benzocaine and a polyethylene PE40 catheter (Smiths Medical International Ldt., Kent, UK) was inserted into the dorsal aorta through the dorsal side of the mouth (Soivio et al., 1975), while the gills were irrigated with well-oxygenated water containing 0.025 g l⁻¹ benzocaine. Fish recovered in well-aerated water for 24h before starting experimentation to allow post-operative normalization of blood gasses (Phuong et al., 2017a). The experimental set-up included a large 500-L tank from which water was recirculated to 6 smaller 120L tanks with 1 cannulated fish in each. The water pCO₂ was controlled with an Oxyguard Pacific system coupled with a G10ps CO₂ probe and a K01svpld pH probe (Oxyguard International A/S, Farum, Denmark), which supplied CO₂ to the water when pH changed above a value corresponding to the desired PwCO₂. There were 4 exposure groups: (i) normocapnia (pCO₂ < 0.7 mmHg); (ii) hypercapnia (pCO₂ = 21 mmHg); (iii) 1 mM nitrite in normocapnic water and (iv) combined hypercapnia (acclimated hypercapnia) and 1mM nitrite. In this combined group, the fish were cannulated then acclimated to hypercapnia (21mmHg CO₂) for 96 h before adding 1 mM nitrite. Water temperature was controlled at 27-28°C throughout experiments and water Po₂ was above 120 mmHg. Nitrite was added as NaNO₂ and tested after each sampling time. During the exposures, a volume of 0.8 mL blood was withdrawn from the catheter at 0, 3, 6, 24, 48, 72 and 96 h. The blood was divided into two parts. Half was used immediately for measurements of haematocrit, extracellular pH (pHₑ), carbon dioxide tension (PaCO₂), and haemoglobin derivatives (see below). The remainder of the blood was centrifuged (6 min at 6,000g), and the plasma was stored at -80°C for subsequent analysis of ions and osmolality.

### 4.3.3 Analytical procedures

Plasma nitrite was determined spectrophotometrically at 540 nm (Varian Cary 50 Spectrophotometer, Varian Inc.) using the Griess reaction (Lefevre et al., 2011a; Hvas et al., 2016). Plasma nitrate was measured similarly after reducing nitrate to nitrite with vanadium (III) chloride (Hvas et al., 2016; Lefevre et al., 2011a; Miranda, 2001). Plasma Na⁺ and K⁺ were measured by flame photometry (Sherwood Model
Plasma Cl\textsuperscript{-} concentrations were measured with a chloride titrator (Sherwood model 926S MK II Chloride analyzer, Sherwood Science Ltd., Cambridge, UK). Total plasma osmolality was measured on a Fiske one-ten osmometer (Fiske\textsuperscript{®} Associates, Two Technology Way, Norwood, Massachusetts, USA).

For haemoglobin derivatives, 15 \( \mu \)L of blood was mixed with 1.5 mL phosphate buffer (0.02 M, pH 7.3) and centrifuged in 3 min at 18,000g. The supernatant was transferred into a cuvette and measured with a spectral scan in 0.5 nm steps from 480 to 700 nm. The concentrations of oxyHb, metHb, HbNO and deoxyHb were determined by spectral deconvolution, following the procedure described in Jensen (2007), Lefevre et al., (2012), Hvas et al., (2016), using reference spectra prepared from \textit{C. ornata} blood (Gam et al., 2017). Total Hb was calculated from the sum of the derivatives, and functional [Hb] was calculated as \( \{[\text{oxyHb}] + [\text{deoxyHb}]\}/\text{total [Hb]} \).

Blood pH\textsubscript{e} and pCO\textsubscript{2} were measured with an iSTAT analyzer (iSTAT Corporation, Princeton, USA) with CG3+ cartridges. The in vitro non-bicarbonate buffer curve and buffer value (\( \beta_{NB} \)) were determined in separate experiments by equilibrating 5 mL blood from fish (ten fishes for ten replicates) in an Eschweiler tonometer with different mixtures of air and CO\textsubscript{2} using a gas-mixing pump (Wösthoff, Bochum, Germany). The oxygenated blood was equilibrated with 7, 14 and 21 mmHg CO\textsubscript{2} for 45 minutes to reach complete equilibration of the blood with gas mixture. The values for pH\textsubscript{e} and pCO\textsubscript{2} generated by the iSTAT analyzer were temperature compensated to the fish temperature, using the equations from the iSTAT manual. Total plasma CO\textsubscript{2} concentration ([CO\textsubscript{2}]\textsubscript{total}) was measured by the Cameron micro method (Cameron, 1971). It should be noted here that while the temperature corrected iSTAT values were found to be accurate for pH in trout blood at 10 and 20°C, it was found to be highly inaccurate for other blood gas values including pCO\textsubscript{2} under these conditions, particularly at low pCO\textsubscript{2} (Harter et al., 2014). In contrast the system has been found to provide accurate readings of pCO\textsubscript{2} at higher temperature (30°C) in \textit{P. hypophthalmus} blood equilibrated with known pCO\textsubscript{2} levels using tonometry (Damsgaard et al., 2015). The differences in accuracy probably reflect differences in temperature since the system is designed for mammalian blood gas analysis.
Bicarbonate concentrations were subsequently calculated using the following equation:

$$[\text{HCO}_3^-] = [\text{CO}_2]_{\text{total}} - p\text{CO}_2 \ast \alpha\text{CO}_2,$$

where $\alpha\text{CO}_2$ is CO$_2$ solubility in trout plasma (Boutilier et al., 1985).

4.3.6 Statistics

All figures were made in Sigma plot 12.5. All data were analyzed with PASW statistics (SPSS 18). A two-way ANOVA (the Holm-Sidak multiple comparison method, pair-wise comparison) was used to identify differences between sampling times and treatments and sampling times. Normal distribution was tested using the Shapiro-Wilk test. A $p$ value of less than 5% ($p<0.05$) was judged significant. All data are shown as means ± S.E.M.

4.4 Results

Acid-base parameters and plasma ions

Hypercapnia caused arterial pCO$_2$ to increase, leading to an acute decrease of arterial pH by 0.35 units after 3 h, where after pH slowly recovered to attain half-compensation by 96 h ($p<0.05$) Fig. 1A). This pH recovery was associated with a significant increase in plasma HCO$_3^-$ by 10 mM (Fig. 1B). Exposure to nitrite alone induced a temporary small decrease in arterial pH that recovered to control values at 96 h (Fig. 1A). This temporary acidosis was caused by a small increase in arterial pCO$_2$ (Fig. 1D), and was modulated by a small increase in plasma HCO$_3^-$ (Fig. 1B). In the combined hypercapnia and nitrite group, arterial pH, pCO$_2$ and [HCO$_3^-$] at time 0 were similar to values in the hypercapnia group at 96 h, and while pH and pCO$_2$ remained relatively stable, there was a significant increase in HCO$_3^-$ with time (Fig. 1A,B,D). Plasma [Cl$^-$] decreased strongly during pH regulation in the hypercapnia group, and was also significantly decreased during exposure to nitrite (Fig. 1C). In the combined hypercapnia and nitrite group, on the other hand, [Cl$^-$] started out at a
lowered value and subsequently stayed relatively stable (Fig. 1C). In the normocapnic control groups all acid base and ionic parameters stayed constant over the 96 h experiment (Fig. 1).

Plasma sodium decreased significantly for 48 h in the nitrite group, where after a small incomplete recovery of [Na⁺] was observed (Fig. 1E). A similar change occurred in the combined hypercapnia and nitrite group, but the decline in [Na⁺] was smaller (Fig. 1E). Hypercapnia alone also caused a small decrease in [Na⁺] (Fig. 1E). These decreases in plasma [Cl⁻] and [Na⁺] were accompanied with a significant drop in plasma osmolality (Fig. 1F). Thus both exposure to hypercapnia and exposure to nitrite were associated with decreased osmolality. Plasma potassium concentrations were significantly elevated at intermediate exposure times (24 and 48 h) in all exposure groups, but the values recovered to control values at 96 h (p<0.05) (Table 1). A similar tendency was present for plasma glucose (Table 1).

The changes in acid-base status in the different exposure groups are illustrated in a Davenport diagram depicting the in vitro buffer line with a βNB of 21.4 slykes (Fig. 2). Hypercapnia led to an acute pH decrease along the buffer line followed by metabolic pH compensation via bicarbonate accumulation along the pCO₂ ~ 21 mmHg isocline, reaching half-compensation by 96 h. In the combined hypercapnia and nitrite group a further increase in bicarbonate occurred. During exposure to nitrite alone there was a minor respiratory acidosis for some 24 h that subsequently became rectified by a small elevation of bicarbonate (Fig. 2).

**Nitrite uptake and levels of haemoglobin derivatives**

Nitrite exposure was associated with nitrite uptake in the plasma, but plasma [nitrite] increased significantly less during nitrite exposure in hypercapnia than in normocapnia (Fig. 3A). Plasma [nitrite] was maintained below ambient [nitrite] throughout the exposures, and the maximal plasma nitrite values of 0.6 mM (nitrite group) and 0.39 mM (hypercapnia + nitrite) were reached after 48 h, after which plasma [nitrite] decreased towards controls (Fig. 3A). The nitrite uptake induced a rise
of blood metHb to 26% (nitrite group) and 14% (hypercapnia + nitrite) of total Hb after 48 h, whereupon metHb levels slowly decreased (Fig. 3B). Despite lower maximal metHb levels during exposure to combined hypercapnia and nitrite than nitrite alone, the rate of metHb formation was highest during the initial hours of nitrite exposure in hypercapnia (Fig. 3B). HbNO levels rose to 5% and 4% of total Hb (Fig. 3C) in the two nitrite exposure groups. The increases in metHb and HbNO led to a significant reduction in functional Hb to 70% and 83% of total Hb in the nitrite and combined hypercapnia and nitrite groups, respectively (Fig. 3D) ($p<0.05$).

Plasma nitrate significantly increased, reaching 3.8 mM and 2.5 mM in 96 h in the nitrite and combined hypercapnia and nitrite groups, respectively (Fig. 3E). The sum of plasma nitrite and nitrate (Fig. 3F) is a good indicator of the total uptake of nitrite, as nitrate is formed by oxidation of nitrite (e.g. in the reaction between nitrite and oxyHb). The sum of plasma nitrite and nitrate increased continuously with time during the exposures, but the total uptake was significantly lower in the hypercapnia plus nitrite group than in the nitrite group (Fig. 3F). There were only moderate changes in Hct, total blood haem concentration, and MCHC (mean corpuscular haemoglobin concentration) in the four experimental groups (Table 1).

**Discussion**

This study supports our hypothesis that environmental hypercapnia will reduce branchial nitrite uptake via the branchial Cl\(^{-}\)/HCO\(_3\) exchanger, since regulation of a respiratory acidosis causes a slowing of Cl\(^{-}\) uptake via the exchanger and hence also reduces nitrite uptake. Thus the response of *C. ornata* to this combined exposure resembles that of the crayfish *Astacus astacus* (Jensen et al., 2000), but is different to that seen in the air-breathing teleost *P. hypothalamus*, where nitrite uptake is only transiently decreased and subsequently increases (Hvas et al., 2016). These authors argued that when *P. hypothalamus* pH regulates, the resultant elevated plasma [HCO\(_3\)] could drive a supra-normal Cl\(^{-}\)/HCO\(_3\) exchange rate and hence an elevated nitrite uptake rate.
To evaluate the present data it is necessary first to evaluate general aspects of acid-base regulation of the extracellular space in *C. ornata*. It has been suggested that air-breathing fishes are generally unable to effectively regulate extracellular pH during respiratory acidosis (Shartau and Brauner, 2014), which the authors argued was a result of reduced gill surfaces and reduced branchial irrigation resulting from the shift to air-breathing. The exception to this trend to date is *P. hypothalamus*, which shows an extreme capacity for extracellular acid-base regulation, where complete regulation was seen after 72 h in aquatic hypercapnia with a pCO$_2$ of 34 mmHg (Damsgaard et al., 2015). This species incidentally possesses very large gills and hence presumably a large ion-exchange surface area (Phuong et al., 2017b; 2017c). Further, *P. hypothalamus* shows for freshwater fish, an unusual mechanism of acid-base regulation during the initial stages of compensation of respiratory acidosis. Hvas et al., (2016) argued that during the initial compensatory phase, modulation of branchial Na$^+$/H$^+$ exchange dominated over Cl$^-$/HCO$_3$ exchange. There is no evidence of such initial Na$^+$-mediated acid-base regulation in the present study. Further, in terms of prowess at compensating respiratory acidosis, *C. ornata*, which shows 50% pH compensation after 96h in an aquatic PCO$_2$ of 21 mmHg is less proficient than *P. hypothalarnus* (Damsgaard et al., 2015) which can fully compensate after 72h. However, *C. ornata* is more proficient than other air-breathing fish including the south American lungfish *Lepidosiren paradoxa* (Sanchez et al., 2005) the bowfin *Amia calva* (Brauner and Baker, 2009), the armoured catfish *Lipocar pardalis* (Brauner et al., 2004) and the marbled swamp eel *Synbranchus marmoratus* (Heisler, 1982). Heisler, 1986 argued that freshwater fish are unable to increase plasma HCO$_3^-$ beyond 25-35 mM due to limits in the capacity for accumulation of bicarbonate. This has also been argued as the reason that most water breathing fish are unable to completely regulate a respiratory acidosis from environmental hypercapnia in excess of 10-15 mmHg CO$_2$ (Brauner and Baker, 2009). The degree of pH compensation varies among species, but is also dependent on water ionic composition and on time, resulting in slower and less complete acid-base compensation in soft and ionic poor water than in hard and ionic rich water (Larsen and Jensen, 1997). The present water has relatively low ionic content, although not extremely soft (Li and Bush, 2015), and
the pH_e compensation in *C. ornata* was 50% after 96 h, but extracellular [HCO_3^-] seems to be on a slow rising trajectory beyond that point (Fig. 1), meaning that acid-base regulation continues. It is therefore likely that there is a continued suppression of Cl^-/HCO_3^- exchange during the combined exposure. Indeed, in the combined hypercapnia and nitrite exposure group, plasma [HCO_3^-] increased to higher levels than seen in the hypercapnia alone group, supporting our hypothesis that pH_e regulation was slow in this species and that hypercapnia caused a reduction in HCO_3^-/Cl^- exchange well beyond 96h. While, reduced Cl^-/HCO_3^- exchange activity at the branchial surfaces provides the most parsimonious explanation for the reduction in nitrite uptake observed during exposure in the combined group, it is worth considering other possibilities. Reductions in branchial ventilation or metabolic rate might have reduced nitrite uptake as would a shift to air-breathing. While high levels of methemoglobin will inevitably cause respiratory distress, there is currently no evidence for nitrite causing the necessary reduction in metabolic rate to explain the reduced nitrite uptake here. Thus, similar levels of methemoglobin formation from nitrite exposure did not change metabolic rate in zebrafish *Danio rerio* (Jensen, 2007) or in *P. hypothalamus* (Lefevre et al., 2011a), nor is there consistent evidence for hypercapnia causing reduced metabolic rate (Lefevre et al., 2016). In most air-breathing fish hypercapnia will initially stimulate increased gill ventilation and in some species, as levels of hypercapnia increase further, an inhibition with an associated increase in air-breathing (Boijink et al., 2010). It remains to be investigated how *C. ornata* responds in this respect.

All three groups showed a significant decrease in plasma osmolality, and the decrease was similar in the nitrite alone and the hypercapnia alone groups (Fig. 1F). Osmolality typically stays constant during nitrite exposure in water-breathing fish (Jensen et al., 1987; Stormer, et al., 1996) but has previously been shown to decrease in facultative air-breathing *C. ornata* (Gam et al., 2017) and *Channa striata* (Lefevre et al., 2012). Slowing of the Cl^-/HCO_3^- exchanger would not normally be expected to give rise to changes in osmolality since the exchange is 1:1. The decrease in osmolality could result from a general inhibition of active transport processes in the gills and possibly
the kidney, whereby the active ion uptake required to balance passive ion losses becomes insufficient, leading to a net ion loss and a decline in plasma \([\text{Cl}^-]\) and \([\text{Na}^+]\). We speculate that a possible common denominator for this could be production of nitric oxide (NO), as NO can inhibit active epithelial ion transport in fish (Gerber et al., 2016). It is well-known that nitrite can be converted to NO (Jensen, 2009), and it is possible that hypercapnia may down-regulate arginase activity and thereby enhance NO generation via nitric oxide synthases (Belik et al., 2009). Alternatively, it is possible that the osmolality decrease relates to changes in gill structure or an increased reliance on air-breathing during exposure to hypercapnia and to nitrite. The idea here would be that the increased reliance on air-breathing would result in a sub-optimal irrigation of the branchial surfaces for the purposes of ion balance with resulting drop in osmolality. Evidence for this possibility can be found in the Davenport diagram (Fig. 2) where exposure to nitrite causes a respiratory acidosis as would be expected with transfer to air-breathing (Shartau and Brauner, 2014), but also in the hypercapnia group, where the blood pCO\(_2\) takes a relatively long time to equilibrate with the environmental pCO\(_2\) after the onset of hypercapnia (i.e. about 3h). More research is, however, needed to reach a deeper understanding of the underlying mechanism(s) behind the osmolality reductions observed here.

MetHb and HbNO levels in the nitrite alone group are very similar to those seen previously in \textit{C. ornata} at the same nitrite exposure concentration (Gam et al., 2017). There was an increase in [metHb] during the first two days of exposure to nitrite followed by a decline, which is partly a result of an up-regulation of metHb reductase activity inside the RBCs (Gam et al., 2017). MetHb and HbNO levels were lower during exposure to combined hypercapnia and nitrite than during exposure to nitrite alone (Fig. 3), which is in line with the reduced nitrite uptake in the combined group. It is notable that the initial increase in [metHb] is faster in the combined group than in the nitrite alone group (Fig. 3B). This is to be expected because of the lower initial pH in the combined group than in the nitrite alone group. Hydrogen ions are required in the reactions between nitrite and Hb (both oxyHb and deoxyHb), and a reduced pH will therefore speed up the reaction rates (Jensen and Rohde, 2010). Initially, this pH
effect overrides the reduced metHb formation expected from the lower nitrite concentration in the combined group.

Concluding remarks

*C. ornata* achieved incomplete pH compensation during 96h exposure to 21 mmHg CO$_2$, and environmental hypercapnia caused a decreased nitrite uptake by an apparent reduced transport rate of the branchial Cl$^-$/HCO$_3^-$ exchanger. This is in line with the response seen in crayfish (Jensen et al., 2000), but contrary to the response seen in another facultative air-breather, *Pangasionodon hypophthalmus*, which acquired an extremely high HCO$_3^-$/Cl$^-$ exchange rate, leading to an increase in nitrite uptake and mortality during hypercapnia (Hvas et al., 2016).

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**Figure 1:** Time-dependent changes in extracellular pH (A), plasma bicarbonate (B), plasma chloride (C), pCO₂ (D), plasma sodium (E), and plasma osmolality (F) during exposure to normocapnia (open circles), hypercapnia (21 mmHg CO₂, closed circles), 1 mM nitrite (open triangles), and acclimated hypercapnia and nitrite (closed triangles). Data are means ± S.E.M. Asterisks show significant difference from 0 h within treatment and plus signs show significant difference to controls at a sampling time.
**Figure 2:** Davenport diagram showing changes in acid-base status during exposure to normocapnia (open circles), hypercapnia (21 mmHg CO₂, closed circles), 1 mM nitrite (open triangles), and acclimated hypercapnia and nitrite (closed triangles). The dashed line is the non-bicarbonate buffer curve determined in vitro. Data are means ± S.E.M.

**Figure 3:** Time-dependent changes in plasma nitrite (A), metHb percentage (B), HbNO percentage (C), functional Hb (D), plasma nitrate (E), and the sum of plasma nitrite and nitrate (F) during exposure to normocapnia (open circles), hypercapnia (21 mmHg CO₂, closed circles), 1 mM nitrite (open triangles), and acclimated hypercapnia and nitrite (closed triangles). Data are means ± S.E.M. Asterisks show significant difference from 0 h within treatment and plus signs show significant difference to controls at a sampling time.
Table 1: Plasma potassium, plasma glucose, haematocrit, blood haem concentration and cellular haem concentration (MCHC) in the four exposure groups of fish. Data are mean ± S.E.M. Asterisks show significant difference from 0 h within treatment and plus signs show significant difference to controls at a sampling time.

<table>
<thead>
<tr>
<th></th>
<th>K⁺ (mM)</th>
<th>0h</th>
<th>3h</th>
<th>6h</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
<th>96h</th>
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</thead>
<tbody>
<tr>
<td>Normocapnia</td>
<td>3.63 ± 0.07</td>
<td>3.59 ± 0.20</td>
<td>3.68 ± 0.21</td>
<td>3.53 ± 0.25</td>
<td>3.65 ± 0.09</td>
<td>3.55 ± 0.11</td>
<td>3.58 ± 0.14</td>
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<tr>
<td>Hypercapnia (21 mmHg)</td>
<td>3.46 ± 0.14</td>
<td>3.90 ± 0.09</td>
<td>4.53 ± 0.10*</td>
<td>5.49 ± 0.12**</td>
<td>5.13 ± 0.13**</td>
<td>4.19 ± 0.12**</td>
<td>3.89 ± 0.13</td>
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<tr>
<td>1 mM nitrite</td>
<td>3.69 ± 0.21</td>
<td>3.54 ± 0.25</td>
<td>3.97 ± 0.31</td>
<td>4.51 ± 0.38*</td>
<td>4.41 ± 0.39*</td>
<td>3.31 ± 0.44</td>
<td>3.10 ± 0.37</td>
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<tr>
<td>Acclimated hypercapnia+nitrite</td>
<td>3.85 ± 0.11</td>
<td>3.80 ± 0.09</td>
<td>5.33 ± 0.17**</td>
<td>5.96 ± 0.23**</td>
<td>4.62 ± 0.21*</td>
<td>3.44 ± 0.20</td>
<td>3.44 ± 0.12</td>
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<td>Glucose (mM)</td>
<td>19.34 ± 0.21</td>
<td>17.86 ± 0.16</td>
<td>20.65 ± 0.18</td>
<td>18.88 ± 0.14</td>
<td>17.99 ± 0.16</td>
<td>17.85 ± 0.16</td>
<td>18.78 ± 0.16</td>
<td>18.67 ± 0.32</td>
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<table>
<thead>
<tr>
<th></th>
<th>Haem (mM)</th>
<th>0h</th>
<th>3h</th>
<th>6h</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
<th>96h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normocapnia</td>
<td>3.05 ± 1.12</td>
<td>3.05 ± 0.23</td>
<td>3.05 ± 0.13</td>
<td>3.05 ± 0.13</td>
<td>3.05 ± 0.18</td>
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<tr>
<td>Hypercapnia (21 mmHg)</td>
<td>3.04 ± 0.12</td>
<td>2.84 ± 0.16**</td>
<td>3.13 ± 0.15**</td>
<td>3.33 ± 0.14**</td>
<td>3.05 ± 0.13**</td>
<td>3.01 ± 0.19**</td>
<td>3.10 ± 0.16</td>
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<td>1 mM nitrite</td>
<td>3.08 ± 0.15</td>
<td>2.92 ± 0.15**</td>
<td>2.74 ± 0.14**</td>
<td>2.78 ± 0.19**</td>
<td>2.70 ± 0.20**</td>
<td>2.55 ± 0.18**</td>
<td>2.56 ± 0.15**</td>
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<tr>
<td>Acclimated hypercapnia+nitrite</td>
<td>3.13 ± 0.15**</td>
<td>2.95 ± 0.13**</td>
<td>3.08 ± 0.14**</td>
<td>2.90 ± 0.11**</td>
<td>2.90 ± 0.10**</td>
<td>2.84 ± 0.12**</td>
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<tr>
<td>MCHC (mM)</td>
<td>19.88 ± 0.14</td>
<td>19.64 ± 0.19*</td>
<td>19.51 ± 0.30</td>
<td>18.99 ± 0.16</td>
<td>18.75 ± 0.16</td>
<td>18.78 ± 0.16</td>
<td>18.67 ± 0.32</td>
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* Asterisks show significant difference from 0 h within treatment and plus signs show significant difference to controls at a sampling time.