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The impact of elevated water nitrite concentration on physiology, growth and feed intake of African catfish *Clarias gariepinus* (Burchell 1822)

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Abstract

The nitrite threshold concentration in rearing water of African catfish (*Clarias gariepinus*) was assessed. African catfish with an initial mean (SD) weight of 219.7 (57.8) g were exposed to an increasing range of water nitrite from 6 (Control) to 928 μM nitrite for 28 days. Mean (SD) plasma nitrite concentrations increased from 5.0 (3.6) to 32.5 (12.6) μM at 928 μM ambient nitrite. The increase in nitrite was accompanied by gradual increase in plasma nitrate from 41.6 (28.4) μM to 420.2 (106.4) μM. Haematocrit, haemoglobin, methemoglobin, plasma concentrations of cortisol, glucose, lactate, osmolality, gill morphology and branchial Na^+/K^+-ATPase activity were not affected. Feed intake, final weight, SGR, FCR and mortality were not affected. We advise not to exceed a water nitrite concentration of 43 μM (0.6 mg L^{-1} NO_2^--N) to prevent the risk of reduced growth and feed intake in African catfish aquaculture.

Keywords: African catfish, nitrite, growth, stress physiology, methemoglobin, recirculating aquaculture systems

Introduction

The aquaculture industry has been expanding rapidly over the past four decades (FAO 2012). In (intensive) recirculation systems (RAS), water is treated to allow the reuse of over 90% of the rearing water (Bovendeur, Eding & Henken 1987; Eding, Kamstra, Verreth, Huisman & Klapwijk 2006). In RAS, fish risk exposure to toxic levels of nitrite. Nitrogenous wastes in RAS are managed by nitrification (biological oxidation of ammonia to nitrate) and denitrification (biological reduction in nitrate to nitrogen gas) (Bovendeur *et al.* 1987; Eding *et al.* 2006; Van Rijn 2010). Both processes can cause nitrite accumulation in the rearing water (Van Rijn & Rivera 1990).

Nitrite is very toxic to organisms, as it converts haemoglobin (Hb) into methemoglobin (MethHb) that does not carry oxygen (Kiese 1974). At high external nitrite levels, fish reduce their overall activity to reduce their oxygen requirement; this goes, however, with the cost of impaired growth and a series of vital functions (reviewed by Lewis & Morris 1986). In freshwater, nitrite enters the organism primarily via the chloride cells in the gills (Bath & Eddy 1980). Nitrite has an affinity for this branchial Cl^-/HCO_3^- exchanger and therefore can compete with the normal Cl^- uptake mechanism. This can lead to (partial) shift from Cl^- uptake to NO_2^- uptake when nitrite is present at high concentrations in the environment (Jensen 2003). Therefore, fish with high branchial chloride uptake rates are more sensitive to nitrite toxicity than those with low uptake rates (Williams & Eddy 1986). This pertains in particular to freshwater

species, where active branchial uptake is the main route of entry of anions (Jensen 2003). Elevated chloride levels in the water are known to alleviate nitrite toxicity, due to competitive effects on branchial nitrite uptake (Crawford & Allen 1977). This protective effect is not uniform between species (reviewed by Lewis & Morris 1986).

African catfish (*Clarias gariepinus*) is commercially farmed in intensive RAS in the Netherlands (Van Duijn, Schneider, Poelman, Van der Veen & Beukers 2010). The global aquaculture production of African catfish has been increasing rapidly since 2000. In 2011, 194 000 tonnes were farmed globally (FAO 2011). The effects of chronic nitrite exposure on African catfish physiology and food intake are not known. Most of the studies on nitrite toxicity determined the median lethal dose, LD_{50}, for several fish species with exposure times up to 120 h. Chronic exposure studies are scarce and mostly limited to purely toxicological effects, with no attention to effects on growth parameters, of interest to farmers (reviewed by Lewis & Morris 1986; Kroupova, Machova & Svobodova 2005). Hilmy *et al.* (Hilmy, El-Domiaty & Wershana 1987) exposed African catfish for 6 months to 228 μM of nitrite (1/10th of their 96 median tolerance limit). They observed a decrease in erythrocytes counts, haemoglobin content and production of methemoglobin. In our experimental design, we exposed African catfish to a range of nitrite concentrations that allowed us to see subtle yet important effect on parameters of interest for fish farmers, without causing mortalities or serious toxic effect.

For channel catfish, *Ictalurus punctatus*, the lowest concentration nitrite leading to growth suppression after 31 days was 115 μM (44% of the minimum concentration to induce mortality) (Colt, Ludwig, Tchobanoglous & Cech 1981). The minimum concentration causing mortality for this species is about half of the 96-h LC_{50} (Bowser, Falls, Van Zandt, Collier & Phillips 1983). Therefore, the minimum ambient concentration of nitrite leading to detectable growth was set up at about one-fifth of the 96-h LC_{50} (Lewis & Morris 1986). This information was taken into account when designing the experimental concentrations range. We exposed African catfish to increasing water nitrite concentrations [6 (Control), 111, 280, 459 and 928 μM] for 28 days to assess the nitrite threshold concentration for physiology, growth and food intake. In addition, we exposed two aquaria to high ambient nitrite concentrations (921 μM) in the addition on sodium chloride (350 mg L^{-1}; 6 mM) to investigate the potential attenuating effect of chloride on nitrite toxicity.

Materials and methods

Experimental conditions

African catfish (*C. gariepinus*) were obtained from Fleuren-Nooijen BV, Someren, The Netherlands. The treatment of the fish in the laboratory was in accordance with Dutch law concerning animal welfare, as tested and approved by the ethical committee for animal experimentation of Wageningen UR Livestock Research.

Fish ($n = 192$) were randomly divided over sixteen 30-L rectangular glass (12 fish per tank), dark covered tanks and acclimatized to the experimental tanks for 15 days. During this acclimatization period, some fish with skin damage (due to aggressive behaviour; $n = 6$) were removed, resulting in some variation in number of fish per tank (10–12). After this period of 15 days, the behaviour of the fish had stabilized, and nitrite exposure commenced. At the start of the 28 days experiment, the overall initial mean (SD) weight was 219.7 (57.8) g. The mean stocking density was 84.7 kg m^{-3}, a value below densities practised at commercial farms for this size class (100–300 kg m^{-3}, Van de Nieuwegiessen, Olwo, Khong, Verreth & Schrama 2009).

The experiment consisted of eight treatments, in duplicate. Treatments were assigned randomly to the 16 tanks. Treatment 1 was included to collect blood at the start of the experiment ($t = 0$). Fish in treatments 3–7 were exposed to one of five different nitrite concentrations in the water: 6, 111, 280, 459 and 928 μM. Fish in treatment 2 (pair-fed) were kept at control nitrite levels and pair-fed to the fish kept at 928 μM nitrite (treatment 7) to discriminate between effects caused by low feed intake due to high nitrite in the water. Fish in treatment 8 (chloride) were exposed to high nitrite (921 μM) in the presence of sodium chloride (350 mg L^{-1}; 6 mM) to evaluate a potential attenuating effect of chloride (and sodium) on nitrite toxicity (Eddy, Kunzlik & Bath 1983; Stormer, Jensen & Rankin 1996; reviewed by Lewis & Morris 1986 and Kroupova *et al.* 2005). During acclimatization and experimental periods, all tanks were supplied with local tap water via a header

tank at a flow of 185 L day^{-1} (chloride concentrations range: 0.4–0.7 μM, Vitens Watertechnologie, The Netherlands). Experimental nitrite concentrations were realized by infusion of $NaNO_2$ (Merk, Hohenbrunn, Germany) stock solutions prepared in tap water (Table 1), which were pumped into the tanks using a peristaltic pump (Watson Marlow 505 S; Rotterdam, The Netherlands) at a flow of 4.75 L day^{-1} per tank; each tank was equipped with an air stone positioned at the point of sodium nitrite inflow to guaranty good mixing of the infused stock solution with the tank water. Flow rates were monitored daily and adjusted when necessary to reach the desired nitrite concentrations. Nitrite concentrations were gradually increased to the desired concentrations during the first 4 days of the experimental period. Fresh stock solutions (Table 1) were prepared daily during the first 11 days of the experimental period and in this period nitrite (NO_2^--N), nitrate (NO_3^--N) and total ammonia (NH_4^+-N plus NH_3-N) concentrations were monitored daily (NitriVer 3 TNT Reagent Set, NitraVer X Nitrogen-Nitrate Reagent Set, Nitrogen-Ammonia TNT, AmVer Reagent Set, tests for NO_3^-, NO_2^- and NH_4^+, Hach Lange, Düsseldorf, Germany, in a Hach DR/890 colorimeter, Hach Lange). During the remainder of the experimental period fresh stock solutions were prepared weekly and nitrite, nitrate and total ammonia concentrations were monitored twice per week. Water temperature, pH and dissolved oxygen concentrations were monitored daily prior to feeding in all tanks (Hach Lange HQ 40 multimeter) throughout the entire experiment. Mean (SD) water temperature was 25.7 (0.5) °C throughout the experimental period. Conductivity was measured with a WTW Cond 315i (WTW GmbH, Weilheim in Oberbayern, Germany), and presented in Table 1.

Blood sampling

On the day nitrite exposure started (day 0), fish in treatment 1 ($t = 0$) were sampled. After 28 days of exposure to nitrite, the fish from the seven remaining treatments were sampled (10–12 fish per tank). Fish were rapidly netted and anaesthetized in 0.1% (v/v) 2-phenoxyethanol (Sigma, St. Louis, MO, USA). Within 2 min, blood (2 × 1.0 mL) was taken by puncture of the caudal vessels with a tuberculin syringe fitted with a 25-gauge needle; Na_2EDTA was used as anticoagulant. One 150 microtiter aliquot was immediately used for the haematocrit determination and haemoglobin/methemoglobin measurement. The remainder of blood was immediately centrifuged for 10 min (14 000 *g*, 4°C) and the plasma so obtained was stored at −20°C until further analyses.

Blood haematocrit, haemoglobin and methemoglobin levels

Immediately after blood puncture, subsamples were drawn into (heparinized) glass capillaries and centrifuged (13 600 *g*; 2 min) to measure haematocrit values. Results were rounded to the closest 0.5%. Blood haemoglobin and methemoglobin were measured with commercially available kits (Instruchemie, Delfzijl, The Netherlands; FAR diagnostics, Verona, Italy).

Plasma concentrations of nitrate, nitrite, cortisol, glucose, lactate, chloride, plasma osmolality and branchial Na^+/K^+-ATPase activity

Plasma concentration of cortisol was determined using radioimmunoassay as described in detail by Gorissen *et al.* (Gorissen, Bernier, Manuel, de Gelder, Metz, Huising & Flik 2012). Plasma osmolality was measured using a cryoscopic osmometer (Osmomat 030, Gonotec, Germany). Plasma concentrations of glucose, lactate, chloride, nitrate and nitrite were measured with commercially available enzymatic kits adapted to 96-well plates as described recently (Schram, Roques, Abbink, Spanings, De Vries, Bierman, Van de Vis & Flik 2010; Schram, Roques, Abbink, Yokohama, Spanings, De Vries, Bierman, Van de Vis & Flik 2012). Branchial Na^+/K^+-ATPase activity was measured as described by Metz *et al.* (Metz, Van den Burg, Wendelaar Bonga & Flik 2003).

Gill morphology

From each fish a second gill arch was removed immediately after blood sampling and placed overnight in Bouin's fixative (75 volumes saturated picric acid, 25 volumes saturated formaldehyde and 5 volumes acetic acid) and then embedded in paraffin. Gill sections were made to include the trailing edge of the filament where in this species the chloride cells reside. Gill sections were immune stained according to Dang *et al.* (Dang, Lock, Flik & Wendelaar Bonga 2000) as described in detail for African catfish (Schram *et al.* 2010, 2012).

Table 1 Composition of the treatment-specific stock solutions, the predicted* nitrite and sodium concentrations, the predicted salinity in all treatments and the measured values per treatment for nitrite concentration, conductivity and the pH range

		Predicted* water quality			Measured water quality			
NO_2^- Treatment	Stock solutions [$NaNO_2$] (g L^{-1})	[NO_2^-] (μM)	[Na^+] (μM)	[Cl^-] (μM)	[NO_2^--N] (mg L^{-1})	[NO_2^-] (μM)	Conductivity (mS cm^{-1})	pH range
2 – Pair-fed control	0	0	0	0	0.93	66	448.4 (9.9)	7.27–7.80
3 – Control	0	0	0	0	0.10	6	488.0 (23.2)	7.06–7.73
4 – NO_2^-	2.58	89	89.3	0	1.55	111	492.5 (21.5)	7.01–7.80
5 – NO_2^-	5.15	179	178.6	0	3.92	280	484.5 (23.1)	7.28–7.79
6 – NO_2^-	10.30	357	357.1	0	6.43	459	514.4 (30.1)	7.40–7.87
7 – NO_2^-	20.60	715	714.3	0	13.0	928	545.8 (14.4)	7.28–7.80
8 – NO_2^- + NaCl	20.60	715	6361.1	5646.8	12.9	921	1300.3 (78.3)	7.01–7.88

*Based on equal flow rates per tank of 5 L day^{-1} for the stock solutions and 500 L day^{-1} for the tap water flow.

Specific growth rate, feed intake and feed conversion rate

On day 0 and day 28, the fish in each tank were individually weighed (Mettler PM 34 Delta range) to the nearest 1 g, to calculate the specific growth rate (SGR) as follows:

$$\mathrm{SGR} = (\ln(W_t) - \ln(W_0)) \times \frac{100}{t},$$

where SGR = specific growth rate (% day^{-1}), W_t = mean weight at day 28 (g), W_0 = mean weight at day 0 (g) and t = number of days.

Floating feed (Catfish type Me-3; Skretting, Boxmeer, The Netherlands) with 49% crude protein and 11% crude lipids was given twice daily at 09:00 and 15:00 hours until apparent satiation (no more feed was taken for at least 5 min following administration of the feed). Feed loads per tank were recorded daily. All uneaten pellets were collected from each tank 1 h after each of the two daily feeding sessions. Feed loss per tank was calculated as the total number of uneaten feed pellets multiplied by 0.0966 g per pellet, the average weight of a pellet, determined by weighing 100 feed pellets. Daily feed intake per tank resulted from the difference between daily feed load and daily feed loss. Daily feed intake per tank was divided by the number of fish in the tank to calculate the daily feed intake per fish in each tank (to account for different numbers of fish per tank). For each tank the total feed intake per fish (TFI) was determined by summation of daily feed intake per fish in each tank. Total feed intake per fish and biomass increase per fish were used to calculate feed conversion rate (FCR) as follows:

$$\mathrm{FCR} = \frac{\mathrm{TFI}}{(W_t - W_0)}$$

where FCR = feed conversion rate (g/g), TFI = total feed intake (g per fish), W_t = mean individual weight at day 28 (g) and W_0 = mean individual weight at day 0 (g).

Statistics

Physiological parameters

Physiological parameters are expressed as mean (SD) of the individual measurements per treatment. For each tanks, 10–12 fish had been sampled; in some instances not all samples were analysed because of insufficient volume or fish mortality. Data were log transformed (if required) to obtain residuals that were normally distributed and to obtain homogeneity of variance of residuals across treatment levels. Mean values for physiological parameters were tested for differences among the treatments using linear mixed models (REML) with treatments as fixed effects and tank as a random effect (F-tests with Kenward–Roger approximation to the residual degrees of freedom; Kenward & Roger 1997). Statistical analyses were performed in SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Only when significant treatment effects were detected, a least significance difference (LSD) post hoc analysis was used to estimate the level of significance between mean values. For both REML and LSD analysis the fiducial limit was set at 5%.

Table 2 Results of linear regression analyses

		Regression coefficient		Intercept		
Response variable	**Explanatory variable**	**Estimate**	***P*-value***	**Estimate**	***P*-value**	**95% CI**
Plasma nitrite (μM)	Water nitrite (μM)	0.026	<0.0001	8.94	<0.0001	6.0–11.8
Plasma nitrate (μM)	Water nitrate (μM)	0.41	<0.0001	59.9	<0.0001	34.6–85.1

*Equals model *P*-value.
CI, confidence interval.

Plasma nitrite and plasma nitrate concentrations were related to water nitrite concentrations using linear regression analyses (Table 2).

Feed intake and growth

Initial and final individual weight, TFI, SGR and FCR are presented as mean values per treatment (Table 5). Mean values per treatment were log transformed to obtain residuals that were approximately normally distributed and to obtain homogeneity of variance of residuals across treatment levels and then tested for significant differences among the treatments using one-way ANOVA in SAS 9.2 (SAS Institute Inc.). For both ANOVA and LSD analysis the fiducial limit was set at 5%.

Concentration–effect curves

Nitrite concentration–effect curves were fitted for SGR and TFI using a log-logistic model (Seefeldt, Jensen & Fuerst 1995). As a blank could not be included, the effects are expressed as absolute values. Curve fitting was carried out with the Marquadt and Levenberg algorithm (Moré 1978) in R (the 10% effect concentrations (EC_{10})) and their 95% confidence limits were calculated (Miller & Miller 2000).

Results

Plasma nitrite and nitrate concentrations

We observed a significant linear increase in plasma nitrite concentration with increasing ambient nitrite concentrations (Tables 2 and 3). Basal values for the control group were 5.0 (3.6), increasing up to 32.5 (12.6 μM) in the highest NO_2^- treatment. Similarly, a significant linear increase in plasma nitrate was observed with increasing ambient nitrite concentrations (Tables 2 and 3). Basal plasma nitrate concentrations for the control group were 41.6 (28.4 μM), increasing up to 420.2 (106.4 μM) in the highest NO_2^- treatment. The addition of sodium chloride to high ambient NO_2^- (treatment 8) did not show an attenuating effect on both plasma NO_2^- and NO_3^- concentrations.

Blood haematocrit, haemoglobin and methemoglobin

No significant differences in haematocrit and methemoglobin levels after 28 days of exposure to any of the ambient nitrite concentrations were detected (Table 4). Significant differences in haemoglobin were detected among treatments (Table 4), but within the same (narrow) biological range, marginal differences could not be related to treatments.

Plasma cortisol, glucose, lactate, chloride, plasma osmolality and Na^+/K^+-ATPase activity

No significant differences in plasma concentration of cortisol, glucose, lactate, chloride, plasma osmolality and branchial Na^+/K^+-ATPase activity were observed (Table 4). All values were within normal ranges previously reported for African catfish, *C. gariepinus* (Schram *et al.* 2010, 2012).

Gill morphology

Gills stained for Na^+/K^+ ATPase-rich cells (chloride cells) are presented for the control group (Fig. 1a), 928 μM NO_2^- (Fig. 1b) and 921 μM NO_2^- in addition with NaCl (Fig. 1c). Gill morphology was not affected by elevated NO_2^- exposure.

Total feed intake, specific growth rate, feed conversion rate and mortality

Daily feed intake appeared to cumulate to marked treatment effects on TFI (Fig. 2). However, no significant treatment effect on TFI could be detected.

Table 3 Mean (SD) values at the start (t = 0) and per treatment for the end (t = 28 days) of the nitrite experiment for plasma NO_2^-, plasma NO_2^- to water NO_2^- ratio, plasma NO_3^-, plasma Cl^-, plasma osmolality and branchial Na^+/K^+-ATPase activity

NO_2^- Treatment	Water NO_2^- (μM)	Plasma NO_2^- (μM)	*n*	Plasma NO_2^- to water NO_2^- ratio	*n*	Plasma NO_3^- (μM)	*n*	Plasma Cl^- (mM)	*n*	Plasma osmolality (mOsmol kg^{-1})	*n*	Na^+/K^+-ATPase activity (μmol Pi h mg per protein)	*n*
1 – T = 0	–	1.6 (1.0)	12	–	12	52.6 (19.1)	13	113.6 (8.5)	16	269.3 (11.1)	22	–	–
2 – Pair-fed control	–	8.8 (3.8)^{ab}	16	0.13 (0.06)^{a}	16	108.4 (21.6)^{ab}	16	118.2 (12.6)	24	269.7 (6.6)	24	–	–
3 – Control	6	5.0 (3.6)^{a}	19	0.71 (0.51)^{b}	19	41.0 (28.4)^{c}	18	114.4 (10.0)	23	275.0 (13.2)	23	3.11 (0.83)	10
4 – NO_2^-	111	12.0 (5.1)^{abc}	16	0.11 (0.05)^{a}	16	88.6 (44.9)^{bcd}	16	112.0 (12.5)	24	272.3 (17.8)	24	–	–
5 – NO_2^-	280	23.7 (9.7)^{abc}	16	0.08 (0.03)^{ac}	16	220.2 (71.9)^{ae}	16	112.9 (14.8)	21	266.0 (5.4)	21	–	–
6 – NO_2^-	459	18.8 (7.2)^{abc}	13	0.04 (0.02)^{ac}	13	266.1 (112.1)^{ae}	11	113.4 (20.9)	22	269.6 (28.2)	22	–	–
7 – NO_2^-	928	32.5 (12.6)^{c}	17	0.04 (0.01)^{cd}	17	420.2 (106.4)^{e}	9	111.1 (14.0)	22	266.3 (8.9)	22	2.52 (0.57)	10
8 – NO_2^- + NaCl	921	12.5 (10.7)^{abc}	15	0.01 (0.01)^{d}	15	198.4 (98.6)^{bde}	13	118.0 (11.8)	23	271.7 (12.5)	23	3.28 (0.76)	9
P-value	–	0.049		0.003		0.0053		0.41		0.63		–	

Mean values with different superscripts are significantly different (REML, P values as shown).
SD = standard deviation of mean values per treatment, n as indicated in the table. T = 0 values were not considered in the statistical analysis.

Table 4 Mean (SD) values at the start (t = 0) and per treatment for the end (t = 28 days) for blood, haematocrit, haemoglobin and methemoglobin, plasma cortisol, plasma glucose and plasma lactate concentrations

NO_2^- Treatment	Water NO_2^- (μM)	Haematocrit (%)	*n*	Haemoglobin (g dl^{-1})	*n*	Methemoglobin %	*n*	Plasma cortisol (nM)	*n*	Plasma glucose (mM)	*n*	Plasma lactate (mM)	*n*
1 – T = 0	–	32.3 (4.0)	22	12.2 (2.7)	13	8.8 (5.9)	14	91.6 (46.4)	13	2.9 (0.9)	22	3.4 (0.8)	18
2 – Pair-fed control	–	39.1 (2.7)	22	9.2 (1.7)^{abcd}	19	8.1 (2.6)	16	83.9 (63.9)	24	4.5 (1.0)	24	5.5 (1.3)	24
3 – Control	6	38.7 (2.9)	18	8.5 (2.4)^{acd}	13	6.6 (4.3)	15	67.7 (53.0)	23	3.8 (1.1)	23	4.7 (1.2)	23
4 – NO_2^-	111	37.7 (3.8)	22	8.2 (1.6)^{ac}	15	7.6 (3.5)	15	43.7 (35.9)	24	5.7 (1.0)	24	6.7 (1.7)	23
5 – NO_2^-	280	35.8 (4.3)	21	8.7 (1.1)^{ce}	18	8.7 (4.3)	15	88.7 (71.0)	21	3.6 (0.8)	21	4.5 (1.2)	21
6 – NO_2^-	459	38.8 (4.2)	22	10.0 (0.9)^{bd}	14	9.3 (4.3)	16	65.7 (89.0)	22	5.7 (1.4)	22	6.1 (0.7)	22
7 – NO_2^-	928	37.5 (3.1)	22	9.0 (2.0)^{cd}	20	10.8 (3.8)	18	55.9 (27.1)	21	4.4 (1.3)	22	4.7 (1.1)	21
8 – NO_2^- + NaCl	921	38.2 (3.5)	21	9.6 (1.7)^{de}	13	9.8 (4.8)	15	56.6 (32.3)	22	4.4 (1.3)	23	5.2 (1.2)	23
P-value	–	0.14		0.041		0.37		0.71		0.12		0.12	

Mean values with different superscripts are significantly different (REML, P values as shown).
SD = standard deviation of mean values per treatment, n as indicated in the table. t = 0 values were not considered in the statistical analysis.

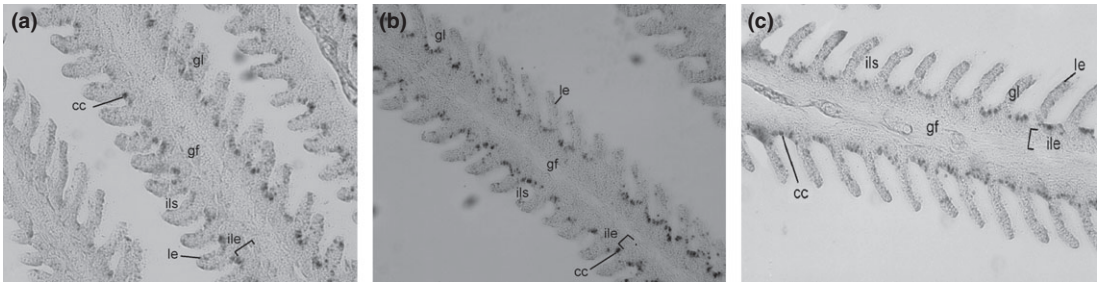

Figure 1 Histology of gill epithelium immunohistochemically stained for Na^+/K^+ ATPase-rich cells (chloride cells) of the control (a), 928 μM NO_2^- (b) and 921 μM NO_2^- in addition to NaCl (c) treatment groups. No effects on the gill's morphology with increasing water nitrite level were observed. Legend: ile, interlamellar epithelium; le, lamellar epithelium; ils, interlamellar space; cc, chloride cell; gf, gill filament; gl, gill lamellae.

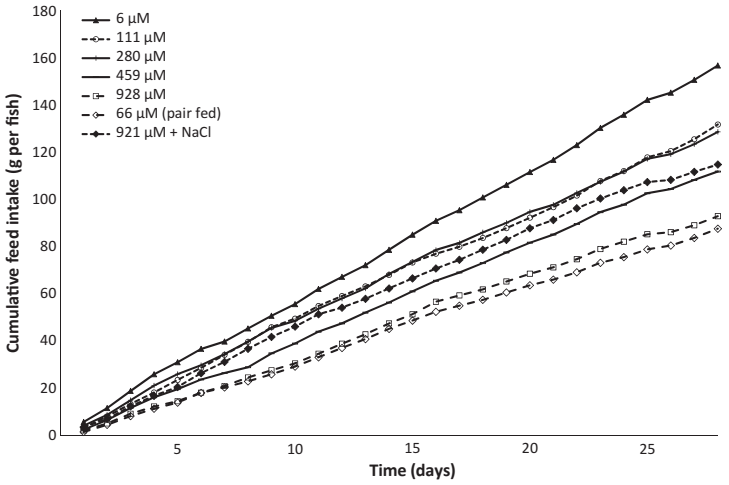

Figure 2 Mean ($N = 2$) cumulative feed intake per fish during the experimental nitrite exposure. The arrow indicates the first day at which all treatments reached their designated nitrate concentrations.

Table 5 Mean (SD) values per treatment ($N = 2$) for initial weight, final weight, TFI, SGR, FCR and survival

NO_2^- Treatment	Water NO_2^- (μM)	Initial weight (g)	Final weight (g)	TFI (g per fish)	SGR (% BWd^{-1})	FCR	Survival (%)
1 – $T = 0$	–	234.4 (17.9)	–	–	–	–	–
2 – Pair-fed control	–	221.8 (4.8)	334.2 (25.0)	87.6 (6.9)	1.46 (0.19)	0.79 (0.08)	100
3 – Control	6	233.3 (19.9)	445.4 (2.6)	156.9 (8.4)	2.32 (0.28)	0.74 (0.02)	100
4 – NO_2^-	111	226.2 (4.0)	409.1 (40.4)	131.8 (18.4)	2.11 (0.29)	0.72 (0.04)	100
5 – NO_2^-	280	223.5 (17.0)	390.8 (33.1)	128.7 (5.7)	1.99 (0.03)	0.77 (0.04)	100
6 – NO_2^-	459	210.0 (13.8)	349.5 (64.1)	111.8 (40.3)	1.79 (0.42)	0.80 (0.00)	91.7
7 – NO_2^-	928	187.2 (1.2)	307.4 (6.1)	92.9 (2.2)	1.77 (0.09)	0.77 (0.03)	91.7
8 – NO_2^- + NaCl	921	220.8 (28.5)	360.3 (83.6)	114.8 (39.7)	1.71 (0.37)	0.83 (0.04)	100
P-value	–	0.17	0.19	0.19	0.19	0.34	–

SD = Standard deviation of mean values per treatment.
TFI, total feed intake; SGR, specific growth rate; FCR, feed conversion ratio.

Also no differences among mean values for SGR, final weight and FCR could be detected after 28 days of exposure to nitrite (Table 5). Two fish of a total 186 died during the course of the experiment. Two fish were euthanized at day 4 (treatment 7) and day 10 (treatment 6) reaching the humane end point. Those two fish had low haematocrit levels (20%), high levels of

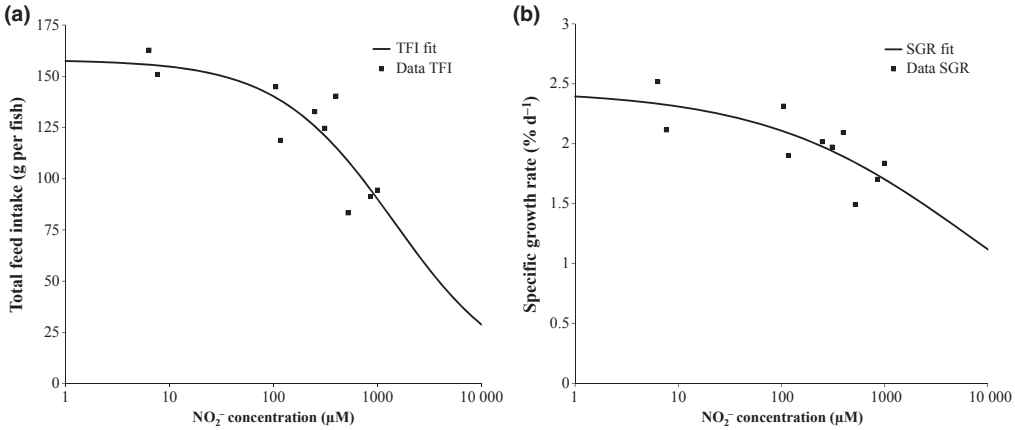

Figure 3 Concentration–effect curves for total feed intake (TFI) (a) and specific growth rate (SGR) (b) in relation to the water NO_2^- concentration. TFI = 158*(1−1/(−log[NO_2^-] − 3.1574)/1.2906) (r^2 = 0.72). SGR = 2.4466*(1 − 1/(−log[NO_2^-] − 3.8324)/2.3018) (r^2 = 0.55).

methemoglobin (96% and 76% respectively) and high plasma nitrite levels (1540 and 510 μM respectively), accompanied by reduced activity (personal observation).

EC_{10} for total feed intake and SGR

The concentration–effect curves for total feed intake and specific growth rate in relation to water nitrite concentration (Fig. 3a and b) reveal a significant effect of nitrite. For total feed intake the EC_{10} for nitrite is 84 μM with a 95% confidence interval from 2 μM to 3.7 mM. For specific growth rate the EC_{10} for nitrite is 43 μM with a 95% confidence interval from 0 to 68 M (data not shown on the figures).

Discussion

Blood haematocrit, haemoglobin and methemoglobin levels

A primary toxic action of nitrite is the conversion of haemoglobin into methemoglobin, which is not able to carry oxygen (Bodansky 1951). Basal levels of methemoglobin in some fish species can reach 10%, and as a rule of thumb, levels below 50% generally do not result in mortality (Lewis & Morris 1986). At higher levels of methemoglobin (70–80%), the behaviour of fish is affected as they become less active to reduce their oxygen demand. In some species, defence mechanisms exist and acclimatization can occur after a certain exposition time (reviewed by Lewis & Morris 1986; Kroupova *et al.* 2005). Pike-perch (*Sander lucioperca*) appears unable to acclimatize when chronically exposed to 250 μM NO_2^-, as mean methemoglobin levels remained at 67% after 32 days of exposure (Wuertz, Schulze, Eberhardt, Schulz & Schroeder 2013). African catfish (*C. gariepinus*; 166 g) exposed to 2.3 mM NO_2^- for 96 h showed blood methemoglobin levels to increase from 10 to 50% after 24 h, and reaching 90% after 96 h (Hilmy *et al.* 1987). Moreover, chronic exposure of African catfish to 1/10th of this dose (0.23 mM) over a period of 6 months led to a slight increase in methemoglobin measured after 1 month (below 15%), reaching a peak after 4 months (40%) followed by a decrease to 25% after 6 months exposure (Hilmy *et al.* 1987). Our data indicate similar acclimatization process occurring in our fish, with acute formation of methemoglobin at high ambient nitrite concentrations (seen in the euthanized fish) and basal levels reached after 28 days of exposure.

Plasma nitrite and nitrate concentrations

Plasma nitrite concentrations gradually increased with increasing ambient nitrite concentrations (ranging from 1.0 to 13.7 μM in the control groups and 13.1 to 56.6 μM in the 928 μM group).

Plasma nitrite concentration varies among species (Table 6). In some species, plasma nitrite concentration can reach build up to levels of up

Table 6 Overview of nitrite plasma concentrations in several fish species under different acute and chronic nitrite exposures. When original data were presented in mg L^{-1} NO_2^--N or mg L^{-1}, we converted them into the international system unit (molar). Data presented in mg L^{-1} NO_2^- were multiplied by 21.63 and data presented in mg L^{-1} NO_2^--N were multiplied by 71.14 to obtain result in micromolar

Species	Water NO_2^- (μM)	Water Cl^- (mM)	Mean plasma NO_2^- (μM)	Exposure time (days)	Reference
African catfish	1736	0.62	5456	1	Ekwe *et al.* 2012
Clarias gariepinus					
Nile Tilapia	1736	0.62	4361	1	Ekwe *et al.* 2012
Oreochromis niloticus					
Goldfish	0.43	0.28	0.75	2	Hansen & Jensen 2010
Carassius auratus					
Largemouth bass	6929	0.62	484	1	Palachek & Tomasso 1984
Micropterus salmoides	13865	0.62	1971	1	
	865	0.62	76	1	Tomasso 1986
Channel catfish	1736	0.62	5471	1	Palachek & Tomasso 1984
Ictalurus punctatus	865	0.62	2784	1	Tomasso 1986
Tilapia	1736	0.62	4361	1	Palachek & Tomasso 1984
Tilapia aurea					
Pike-perch	0	1.13	7.1	32	Wuertz *et al.* 2013
Sander lucioperca	249	1.13	540	32	
	711	1.13	3629	42	
	711	12.41	22.1	42	
Rainbow trout	0.2–2.2	0.28	Not detectable	28	Kroupova *et al.* 2008
Onchorhynchus mykiss	13	0.28	0.65	28	
	65	0.28	8.2	28	
Walleye	900	0.15	2100	1	Madison & Wang 2006
Sander vitreus			3000	2	

to 10 times higher than ambient (Eddy *et al.* 1983). Juvenile African catfish exposed to 1736 μM NO_2^- for 24 h had plasma concentrations on NO_2^- reaching almost 5.5 mM (Ekwe, Nwakpa & Nweze 2012). Exposure of adult African catfish to high ambient nitrite concentration seems to elicit a strong initial increase in plasma nitrite, but fish seem to acclimatize when chronically exposed to high levels of ambient nitrite. This is confirmed by the fact that addition of sodium chloride (6 mM) does not show a significant attenuating effect on plasma nitrite concentration. African catfish seem to acclimatize to the range of concentration studied; therefore, the addition of sodium chloride does not add more protective effect.

Plasma nitrate concentrations gradually increased with increasing ambient nitrite concentrations. This can be explained by the intrinsic defence mechanism of conversion of plasma nitrite into less toxic nitrate (Doblander & Lackner 1996, 1997). African catfish are very tolerant to nitrate exposure, as only their growth was affected when ambient concentrations reached 27.04 mM [plasma: 6.6 (0.9) mM] (Schram *et al.* 2012). In the current experiment, plasma nitrate concentrations were well below the millimolar level, the toxic effects observed should therefore be attributed to nitrite rather than nitrate.

During the whole experimental period, no abnormal swimming behaviour was observed as in other nitrite-exposed fish species (personal observations; Lewis & Morris 1986). The absence of abnormal swimming behaviour may be explained by the air-breathing nature of African catfish. Branchial nitrite uptake could then be limited by limiting oxygen uptake from the water in favour of air breathing. Quantitative observation of air-breathing behaviour of nitrite-exposed African catfish would be required to assess this hypothesis.

Stress physiology, plasma osmolality, plasma chloride and gill morphology and Na^+/K^+-ATPase activity

Plasma glucose, lactate and cortisol levels were in the normal biological range measured in previous studies for this species (Schram *et al.* 2010, 2012). A chronic exposure of 28 days to elevated ambient nitrite concentrations did not affect these

parameters, underpinning once again the tolerance of the African catfish towards high nitrogenous waste compounds. Plasma osmolality and plasma chloride, a major determinant of plasma osmolality, were not affected by high nitrite concentrations. The Na^+/K^+-ATPase activity was in the range previously observed for this species exposed to ammonia (Schram *et al.* 2010). The Na/K-ATPase activity was assessed only in the control group (treatment 3), high nitrite (treatment 7) and high nitrite in presence of sodium chloride (treatment 8). As no differences between those extreme groups were observed, the intermediate groups were not measured. This enzyme does not play a role in the nitrite uptake or removal of nitrite as it was observed for ammonia (Schram *et al.* 2010). The branchial Cl^-/HCO_3^- exchanger which normally is involved in chloride uptake and may be disrupted when ambient nitrite concentrations are high, causing a (partial) shift to NO_2^- uptake (Jensen 2003), was not investigated in this study.

In rainbow trout exposed to several increasing nitrite concentrations for 28 days, severe morphological alterations of the gills were observed already from the lowest 0.22 μM NO_2^-, culminating in the highest concentration (65.2 μM NO_2^-) (Kroupova, Machova, Piackova, Blahova, Dobsikova, Novotny & Svobodova 2008). Over 32 days of exposure to different nitrite concentrations (0–250 μM NO_2^-), 40–60% of the gills of juvenile pike-perch showed abnormalities. Nevertheless, no changes could be related to treatment effects (Wuertz *et al.* 2013). Toxic effect of gill morphology thus depends on the species.

Feed intake

During the first 4 days of the experiment, when nitrite concentrations were building up, feed intake was similar among all treatments. However, when the desired concentrations of nitrite had been reached (day 5), different pattern in feed intake showed up almost instantly (Fig. 2), with intermediate patterns related to the different concentrations, the minimum feed intake being reached in the highest nitrite concentration group and a potential attenuating effect of the addition of sodium chloride. Feed intake and SGR and final weight patterns seem to be gradually affected by raising ambient nitrite concentrations (Table 5; Fig. 2). However, differences are not significant. A pair-fed group was introduced to discriminate effects of high nitrite exposure from potential effects of reduced feed intake. No significant differences were observed in physiological parameters for any treatment.

Nitrite toxicity

The main mechanism of nitrite toxicity is well documented with the conversion of haemoglobin into methemoglobin that is incapable of carrying oxygen (Bodansky 1951). The comparable methemoglobin levels among treatments after 28 days of nitrite exposure show that African catfish is able to acclimatize to relatively high ambient nitrite concentrations. Similar methemoglobin concentrations were observed in this species exposed to 1/10th of the 96 median tolerance limit (228 μM NO_2^-; Hilmy *et al.* 1987). Plasma nitrite concentrations gradually increased with increasing ambient nitrite concentrations. The increase in plasma nitrate with increasing water nitrite concentrations indicates that African catfish successfully detoxify internally nitrite to less toxic nitrate as described earlier in trout hepatocytes (Doblander & Lackner 1996). The addition of sodium chloride (6 mM) does not show a further attenuating effect both regarding plasma nitrite concentration and growth parameters; African catfish appears to acclimatize to chronic nitrite exposure already without sodium chloride; the addition of this compound has no real beneficial effect in the concentration range studied.

The nitrite concentration range was designed based on actual nitrite exposure in commercial African catfish aquaculture combined with values from the literature, and the aim of this experiment was to measure subtle changes that could impair the fish welfare. In our opinion, the set of data obtained allows to draw conclusions regarding the nitrite toxicity to African catfish and the threshold concentrations for safe aquaculture production. Previous studies on nitrite toxicity mainly focused on acute LC_{50} up to 96 h (reviewed by Kroupova *et al.* 2005). The parameters traditionally measured are mainly related to the nitrite effect on blood haemoglobin and methemoglobin formation. In this study, we investigated, in addition to those parameters, the effect of chronic nitrite exposure on stress physiology and growth, parameters of interest from the welfare and commercial aquaculture points of view.

Nitrite threshold concentrations

African catfish chronically exposed to 1 mM ambient nitrite appears capable of mitigating the adverse effects of nitrite. The concentration effect curves revealed a significant effect of nitrite exposure on total feed intake and specific growth rate. The concentration–effect curves do not provide a clear-cut nitrite threshold concentration for African catfish as the calculated EC_{10} values for nitrite of 84 for feed intake and 43 μM for growth both have very large 95% confidence intervals. Therefore, the reported EC_{10} values should be treated as indicative. All observations jointly taken we advise for African catfish not to exceed a nitrite concentration of 43 μM. As stated earlier, several studies investigated the acute lethal concentration of nitrite for numerous species, but data regarding nitrite toxicity for chronic exposure, combining physiology and growth are scare. As an example, the NOEC and LOEC for juvenile rainbow trout after 28 days of nitrite exposure were estimated at 0.22 μM NO_2^- (0.01 mg L^{-1} NO_2^-) and 4.34 μM NO_2^- (0.2 mg L^{-1} NO_2^-) respectively (Kroupova *et al.* 2008); which indicates that this species is more sensitive to nitrite than African catfish.

Conclusions

This study demonstrates that African catfish, *C. gariepinus*, is tolerant to high ambient nitrite concentrations over a period of 28 days. Nitrite accumulates mildly in the plasma with increasing ambient nitrite concentrations. The greater increase in plasma nitrate with increasing ambient nitrite concentrations provides a good indirect evidence for internal nitrite detoxification into less toxic nitrate. Stress physiology and ionic balance are not affected by high ambient nitrite concentrations. Growth and feed intake show a differential pattern from the first day of exposure, but differences are not significant.

We advise for African catfish not to exceed a water nitrite concentration of 43 μM (0.6 mg L^{-1} NO_2^--N). Below this nitrite concentration physiological and growth disturbances are avoided.

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