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

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ABSTRACT

The threshold concentration for NH_3 in rearing water of African catfish (*Clarias gariepinus*) was assessed. African catfish with an initial mean (SD) weight of 141.0 (24) g were exposed to five different T_{amm} [sum of NH_3 and NH_4^+] concentrations: 0.37 (Control), 1.06, 2.12, 5.16 and 19.7 mM, which concurs with NH_3 concentrations of 4 (Control), 14, 38, 176 and 1084 μM. Plasma concentrations of NH_4^+, cortisol, glucose and lactate, plasma osmolality, gill morphology, branchial Na^+/K^+-ATPase activity, feed intake and specific growth rate were monitored. No effect of water NH_3 on plasma NH_4^+ concentrations was detected. Feed intake and specific growth rate were severely affected at exposure to water NH_3 concentrations above 90 μM (calculated EC_{10} values: 89 and 122 μM). No major disturbances in physiological blood parameters were observed at these NH_3 concentrations, but gill morphology (a remarkably sensitive stress indicator) deteriorated significantly. Based on the lower limit of the 95% confidence interval for EC_{10}, we advise for African catfish not to exceed a water NH_3 concentration of 24 μM (0.34 mg NH_3-N/L). This finding is relevant for design and management of African catfish production systems.

1. Introduction

Fish produce nitrogenous wastes through catabolism of amino acids (Wood, 1993). The majority of fresh water and marine teleost fish are ammonioteles and excrete most of their nitrogenous wastes as ammonia across the gills to the water (Wilkie, 2002). The mechanisms involved in branchial ammonia excretion remain controversial. In the most recently proposed model for branchial ammonia excretion, simple NH_3 diffusion down the partial pressure gradient is the predominant mechanism under normal conditions. At high water ammonia concentrations, when NH_3 diffusion is impaired or even reversed, several active NH_4^+ excretion pathways, involving Rhesus (Rh) glycoproteins as membrane transporters, facilitate ammonia efflux (Wright and Wood, 2009).

High water ammonia leads to rapid accumulation of ammonia in plasma and tissues (Wright et al., 2007), where it is mainly present as NH_4^+ at physiological pH (Wilkie, 2002). High internal NH_4^+ causes neurotoxicity (Cooper and Plum, 1987 in Wilkie, 2002).

High water ammonia, caused by high feed loads and high fish densities, is an important limiting factor for intensive aquaculture (Boeuf et al., 1999). Water ammonia should therefore be kept below species-specific threshold levels.

The African catfish (*Clarias gariepinus*) is empirically known to be highly tolerant to ammonia toxicity (Ip et al., 2004a). Several defence strategies allow this fish to cope with increased internal ammonia, for instance during prolonged air exposure or during periods of draught, when the fish survive in mud pools. The defence strategies include active excretion of NH_4^+, reduced ammonia production by reduction of proteolysis and/or reduced amino acid catabolism and a high ammonia tolerance of tissues and cells. Moreover, it appears that this catfish reduces membrane and skin permeability to NH_3 in response to high water ammonia concentrations (Ip et al., 2004a).

The NH_3 threshold concentrations for physiological disturbances, feed intake and growth are unknown for African catfish. As a result it is unclear whether intensive farming of this fish species at high water NH_3 concentrations results in physiological disturbances, reduced feed intake and reduced growth, and thus may impinge on the welfare of the fish. In the present study, African catfish (*Clarias gariepinus*) was exposed to increased water ammonia for 34 days to establish NH_3 threshold concentrations.

2. Materials and methods

2.1. Experimental conditions

African catfish (*Clarias gariepinus*) were obtained from Fleuren-Nooijen BV, Someren, The Netherlands. Fish (n = 168) were randomly divided over 12 30-L rectangular glass tanks and allowed to acclimatise

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to the experimental conditions for 7 days. At the start of the 34-day experiment, the overall initial mean (SD) individual weight was 141.0 (24) g. The resulting mean stocking density was 65.8 kg/m^3, well below fish densities found at commercial farms for this size class (100 to 300 kg/m^3, Van de Nieuwegiessen et al., 2009). The treatment of the fish was in accordance with Dutch law concerning animal welfare, as tested by the ethical committee for animal experimentation of Wageningen UR Livestock Research (number 2009045.a).

We aimed at a threefold ammonia concentration increase for five consecutive treatments and a concentration range around the highest total ammonia concentrations observed at commercial farms (4.2 to 5.0 mM) without exceeding the acute toxic total ammonia concentration (96 h LC_{50}) of 380 mM (Britz, 1988 in Ip et al., 2004b). Five (1 to 5) different total ammonia [T_{amm} = sum of NH_3 and NH_4^+] concentrations in the rearing water were used: 0.37 (Control), 1.06, 2.12, 5.16 and 19.7 mM. These T_{Amm} concentrations concurred with NH_3 concentrations of 4 (Control), 14, 38, 176 and 1084 μM (Table 1). Treatments were executed in duplicate and assigned randomly to the tanks. Treatments are hereafter referred to as 4, 14, 38, 176 and 1084 μM NH_3.

During the acclimatisation and experimental period, all tanks were supplied with local tap water via a header tank at a flow of 185 L/d for each tank. During the experimental period, experimental ammonia concentrations were realised by infusion of ammonium chloride (NH_4Cl) stock solutions (Table 1). Stock solutions were pumped into the tanks by a peristaltic pump (Watson Marlow 505 S; Rotterdam, The Netherlands) at a flow of 4.75 L/d per tank. Each tank was equipped with an air stone to mix the stock solution with the tank water. Flows were monitored and adjusted as required to reach the experimental ammonia concentrations. Sodium bicarbonate ($NaHCO_3$) was added to the stock solutions to adjust the pH. In addition, sodium chloride (NaCl) was added to the stock solutions to compensate for the differences in chloride concentrations arising from NH_4Cl addition. Total predicted sodium concentrations in the tanks from $NaHCO_3$ and NaCl combined were equal among treatments (Table 1). Fresh stock solutions were prepared daily. The salinity of the tank water resulting from the infusion of stock solutions did not exceed 5 g/L. According to Clay (1977) African catfish tolerate salinities up to 10 g/L.

Water quality (Table 2) was monitored by daily (between 1 and 2 pm) measurements of total ammonia (T_{Amm}) concentrations (photometrically, Hach Lange DR2800), water temperature, pH, dissolved oxygen concentrations (Hach Lange HQ 40 multimeter) and conductivity (WTW Cond 315i) in all individual experimental tanks. NH_3 concentrations were calculated from the temperature, pH and salinity dependent molar fraction of NH_3 and the measured T_{Amm} concentrations (Creswell, 1993). Ammonia concentrations were gradually increased to the designated concentrations during the first four days of the experimental period. Mean water temperature was 27.0 °C throughout the experimental period.

2.2. Plasma sampling

One day before ammonia exposure started (day 0), the fish from two tanks were sampled. After 34 days exposure to ammonia, the fish from the 10 remaining tanks were sampled (two tanks for each of the five treatments, 12 fish per tank). Fish were rapidly caught with a net and quickly anaesthetised in 0.1% (v/v) 2-phenoxyethanol (Sigma, St. Louis, USA). Within two minutes, blood had been taken by puncture of the caudal vein using a lithium heparinised Vacuette blood collection system (Greiner Bio-One GmbH, Kremsmünster, Austria). The blood was centrifuged for 10 min (14,000×*g*, 4 °C) and the plasma obtained was stored at −20 °C.

2.3. Plasma NH_4^+

Plasma NH_4^+ was determined using a commercially available test kit (Instruchemie, Delfzijl, The Netherlands), with a protocol adapted for a 96-well microplate application.

2.4. Plasma cortisol

Cortisol was measured by radioimmunoassay (Metz et al., 2005) with commercially available antiserum (Campro Scientific, Veenendaal, The Netherlands). Samples of 10 μl of 1:5 (v/v) water diluted plasma were incubated overnight at 4 °C with 100 μl first antibody (IgG-F-1; 1:400), 2000 cpm ^{125}I-cortisol (Amersham, Buckinghamshire, UK) and 100 μl secondary antibody (GARGG; 1:160). All constituents were dissolved in cortisol RIA buffer [0.063 M Na_2HPO_4, 0.013 M Na_2EDTA, 0.02% (w/v) NaN_3, 0.1% (w/v) 8-anilino-1-naphthalene sulfonic acid (Sigma) and 0.1% (w/v) bovine gamma globulin (Sigma)]. Immune complexes were precipitated by addition of 1 ml ice-cold 5% (w/v) polyethylene glycol and 2% (w/v) bovine serum albumin (Sigma) and subsequent centrifugation (20 min, 2000×*g*, 4 °C). Pellets were counted in a gamma counter (1272 Clinigamma, LKB Wallac, Turku, Finland).

2.5. Plasma glucose and lactate

Plasma glucose and lactate were measured with commercially available enzymatic test kits (Instruchemie, Delfzijl, The Netherlands), with protocols adapted to a 96-wells microplate. For glucose, 10 μl sample or standard (5.55 mM glucose) was mixed with 200 μl reagent and incubated for 10 min at 25 °C. Absorbance was read within 60 min at 495 nm. For lactate, 10 μl sample or standard (4.44 mM lactate) or blank (8% perchloric acid) was mixed with 290 μl of lactate reagent and incubated for 20 min at 37 °C. Absorbance was read at 355 nm.

2.6. Osmolality

Plasma osmolality (sample volumes: 50 μl) was measured with a cryoscopic osmometer (Osmomat 030, Gonotec, Germany). Deionized water (0 mOsmol/kg) and a standard solution (300 mOsmol/kg) were used as reference.

2.7. Gill morphology

One gill arch was removed immediately after blood sampling and placed overnight in Bouin's fixative (75 ml saturated picric acid, 25 ml saturated formaldehyde, 5 ml acetic acid). Gill sections were made to include the trailing edge of the filament where the chloride cells

Table 1
Compositions of the daily prepared treatment specific stock solutions and the calculated[a] TAN, sodium and chloride concentrations in the tanks for all treatments.

Treatment	NH_4Cl (g/10 L)	$NaHCO_3$ (g/10 L)	NaCl (g/10 L)	Total Cl^- dose (g/10 L)	Total Na^+ dose (g/10 L)	Predicted tank [Na^+] (g/L)	Predicted tank [Cl^-] (g/L)
1	0	0	1555	933	622	1.6	2.4
2	15	9	1549	939	622	1.6	2.4
3	45	47	1523	943	622	1.6	2.4
4	135	180	1432	948	621	1.6	2.5
5	404	530	1192	982	620	1.6	2.5

[a] Based on equal flow rates per tank of 4.75 L/day for the stock solutions and 185 L/day for the tap water flow.

Table 2
Mean values per treatment for NH_3, total ammonia (T_{Amm}) and dissolved oxygen (DO) concentrations, water temperature, conductivity and the pH range in the treatments during the experimental period.

Treatment	NH_3		T_{Amm}		DO	Water temperature	Conductivity	pH range
	(μM)	(mg N/L)	(mM)	(mg N/L)	(mg/L)	(°C)	(mS/cm)	
1	4	0.06	0.37	5.2	4.8	27.0	7.18	7.17–7.72
2	14	0.19	1.06	14.8	4.5	27.0	7.07	7.07–7.64
3	38	0.53	2.12	29.7	4.9	27.1	7.44	7.30–7.83
4	176	2.47	5.16	72.2	5.1	27.0	7.68	7.26–8.18
5	1084	15.2	19.7	275.1	5.6	27.0	8.78	7.46–8.66

reside. Gill sections were prepared according to Dang et al. (2000). After dewaxing, blocking of endogenous peroxidase with 2% (v/v) H_2O_2 and blocking of non-specific sites with 10% (v/v) normal goat serum, slides were incubated overnight with a monoclonal antibody against chicken Na^+/K^+-ATPase (final dilution of 1:500; IgGα5, Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, USA). Goat anti-mouse (Nordic Immunology, Tilburg, The Netherlands) was used as a second antibody (1:150). The slides were subsequently incubated with mouse peroxidase anti-peroxidase (1:150) (M-PAP, Nordic Immunology). In the peroxidase reaction 0.025% (w/v) 3,3′-diaminobenzidine (DAB) was used as chromogen in the presence of 0.0005% (v/v) H_2O_2.

2.8. Branchial Na^+/K^+-ATPase activity

The specific, Na^+- and K^+-dependent, ouabain-sensitive ATPase activity was measured in homogenates of gills preserved in SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole: pH 7.4) as described in detail by Metz et al. (2003). Aliquots (5 μl in triplicate) of homogenate (protein content of 1 mg ml^{-1}) were incubated in assay medium for 15 min at 37 °C. The specific activity was calculated by subtracting the K^+-independent, ouabain-insensitive ATPase activity from total ATPase activity. ATP hydrolysis was assessed by the amount of inorganic phosphate formed per minute per mg of protein. Sample protein content was estimated with a commercial protein kit (BioRad, Hercules, CA, USA), and bovine serum albumin as standard.

2.9. Specific growth rate, feed intake and feed conversion rate

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

$$SGR = (\ln(W_t) - \ln(W_1)) \times \frac{100}{t}$$

where SGR = specific growth rate (%/d), W_t = mean weight at day 34 (g), W_1 = mean weight at day 1 (g) and t = number of days.

Feed (Catfish type Me-3; Skretting, Boxmeer, The Netherlands) with 49% crude protein and 11% crude lipids was administered twice daily at 9 am and 5 pm until visually observed satiation. Feed loads per tank were recorded. Uneaten pellets were collected from each tank one hour after the two daily feeding sessions. Feed loss per tank was calculated as the total number of uneaten feed pellets multiplied by 0.0966 g/pellet, determined by weighing 100 feed pellets. Daily feed intake per tank was defined as the difference between daily feed load and feed loss. Total feed intake per tank resulted from the sum of the daily feed intake.

Total feed intake and biomass increase per tank were used to calculate feed conversion rate (FCR) as follows:

$$FCR = \frac{TFI}{(n_t \times W_t - n_1 \times W_1)}$$

where FCR = feed conversion rate (g/g), TFI = total feed intake (g), W_t = mean weight at day 34 (g), W_1 = mean weight at day 1 (g), n_t = number of fish at day 34 and n_1 = number of fish at day 1.

2.10. Statistics

2.10.1. Physiological parameters

Physiological parameters are expressed as mean (SD) of the individual measurements per treatment. For each treatment, 24 fishes were sampled; in some instances not all samples collected were analyzed, either because samples were accidentally lost or because predicted low within treatment variation in readout allowed assessment of significance with lower sample numbers. Where necessary, data were log-transformed 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. Only in case 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%.

2.10.2. Feed intake and growth

Total feed intake, specific growth rate (SGR) and feed conversion rate (FCR) were expressed as mean per treatment (N = 2). Mean values per treatment were tested for significant differences among the treatments by one-way ANOVA. Only in case 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 ANOVA and LSD analysis the fiducial limit was set at 5%.

2.10.3. Concentration–effect curves

NH_3 concentration-effect curves were fitted for specific growth rate (SGR) and total feed intake (TFI) using a log-logistic model (Seefeldt et al., 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) as provided in the PRISM 4.00 software package (Graphpad Software, Inc.). The 10% effect concentrations (EC_{10}) and their 95% confidence limits were calculated (Miller and Miller, 2000).

3. Results

Plasma analyses are shown in Table 3.

3.1. Plasma NH_4^+

Plasma NH_4^+ concentrations did not differ among treatments (REML, P = 0.10). Mean plasma NH_4^+ concentrations ranged between 159.2 and 217.8 μM in catfish exposed to water NH_4^+ levels ranging from 4 to 1084 μM.

Table 3
Mean (SD) values at the start (t = 0) and per treatment for the end (t = 34 d) of the experiment for plasma cortisol, plasma glucose, plasma lactate and plasma NH_4^+ concentrations, plasma osmolarity and branchial Na^+/K^+-ATPase activity. Mean values with different superscripts are significantly different (REML, P values as shown). SD = standard deviation of means values per treatment, n as indicated in the table. t = 0 values were not considered in the statistical analysis.

Treatment	Water NH_3	Plasma cortisol		Plasma glucose		Plasma lactate		Plasma NH_4^+		Plasma osmolality		Na^+/K^+-ATPase activity	
	(μM)	(nM)	n	(mM)	n	(mM)	n	(μM)	n	(mOsmol/kg)	n		n
t = 0		30.0 (18.2)	24	3.4 (1.0)	24	2.74 (0.78)	24	167.9 (27.8)	23	251.2 (10.5)	24	2.5 (1.2)	10
1	4	44.5 (35.1)	24	3.5 (0.6)^b	24	3.84 (1.58)	24	159.2 (39.9)	16	267.3 (8.1)^a	24	3.4 (1.5)^a	12
2	14	43.3 (20.9)	24	3.0 (0.5)^a	24	4.17 (1.58)	24	200.1 (19.3)	15	278.7 (24.3)^a	24	3.9 (1.9)^a	12
3	38	72.9 (52.7)	24	3.1 (0.8)^{ab}	24	3.88 (1.16)	24	205.7 (31.0)	16	276.8 (8.3)^a	24	3.6 (0.9)^a	6
4	176	62.1 (38.6)	24	3.0 (0.8)^a	23	4.02 (0.97)	24	213.4 (13.5)	22	278.3 (13.8)^a	22	5.5 (2.3)^a	12
5	1084	33.6 (26.4)	23	4.8 (0.9)^c	23	6.23 (1.53)	23	217.8 (17.8)	21	347.0 (16.9)^b	23	10.2 (3.6)^b	9
P-value		0.21		0.002		0.25		0.10		0.002		<0.001	

3.2. Plasma cortisol

Plasma cortisol concentrations did not differ among treatments (REML, P = 0.25). The mean (SD) plasma cortisol concentration in the control (4 μM NH_3) was 44.5 (35.1) nM. In the other experimental groups, mean (SD) concentrations ranged between 33.6 (26.4) (1084 μM NH_3) and 72.9 (52.7) nM (38 μM NH_3). The initial concentration (t = 0) was 30.0 (18.2) nM.

3.3. Plasma glucose and lactate

A significant treatment effect was observed for plasma glucose concentrations (REML, P = 0.002). In the control (4 μM NH_3) the glucose concentration was slightly but significantly higher at 3.51 mM than the concentrations seen in fish kept in 14, 38 and 176 μM NH_3; the highest concentration (4.8 mM) was observed in the 1084 μM NH_3 treatment. Plasma lactate concentrations were similar in all groups (REML, P = 0.25). The mean plasma lactate concentration in the control (4 μM NH_3) was 3.84 mM. The concentrations in the 14, 38 and 176 μM NH_3 treatments were within the same range.

3.4. Plasma osmolality

A significant treatment effect was observed for plasma osmolality (REML, P = 0.002). The mean plasma osmolality in the control (4 μM NH_3) was 267.1 mOsmol/kg The osmolality in the 14, 38 and 176 μM NH_3 treatments were within the same range and no significant differences were detected among these. Plasma osmolality in the 1084 μM NH_3 treatment group rose to 347 mOsmol/kg, a significantly higher value than found in any other treatment.

3.5. Gill morphology

Gill morphology deteriorated with increasing water ammonia concentration (Fig. 1a–e): the inter-lamellar and lamellar epithelium thickened and the inter-lamellar space got reduced. The effect was directly visible in the 38 μM NH_3 treatment (Fig. 1c) and most profound in the 1084 μM treatment (Fig. 1e). In the latter group, distal and basal hyperplasia with lamellar fusion, epithelial hypertrophy and enhanced mucus secretion were observed.

Chloride cells in the control (4 μM NH_3, Fig. 1a) were mainly present in the inter-lamellar area and to a lesser extent in the lamellae. With increasing NH_3 exposure, the number of chloride cells increased, as did the number of chloride cells that had migrated to the lamellae (Fig. 1b–e).

3.6. Branchial Na^+/K^+-ATPase activity

A significant treatment effect was observed for branchial Na^+/K^+-ATPase activity (REML, P < 0.001). In the control (4 μM NH_3) the mean branchial Na^+/K^+-ATPase activity was 3.4 (1.5) μmol P_i/h per mg protein. The activity in the 14, 38 and 176 μM NH_3 treatments were within the same range. Na^+/K^+-ATPase activity in the 1084 μM had significantly increased (more than two-fold) compared to the other treatments at 10.2 (3.6) μmol P_i/h per mg protein.

3.7. Feed intake, specific growth rate, feed conversion rate and mortality

No mortality was observed during these experiments. Total feed intake differed among the treatments (one-way ANOVA, P < 0.01, Table 4). The total feed intake was highest in the 14 and 38 μM NH_3 treatments. Total feed intake was lower in the 176 μM NH_3 treatment compared to the control and 14 and 38 μM NH_3 treatments. Total feed intake was strongly reduced in the 1084 μM NH_3 treatment compared to all other treatments. The differences in total feed intake among treatments developed over time (Fig. 2).

Specific growth rate (SGR) differed among treatments (ANOVA, P < 0.01, Table 4). The highest SGR was observed in the 4, 14 and 38 μM NH_3 treatments. The SGR in the 176 μM NH_3 treatment was lower than observed for the 14 and 38 μM NH_3 treatments, but equal to the SGR observed in the control (4 μM NH_3). The SGR in the 1084 μM NH_3 treatment was lower than in all other treatments.

Feed conversion rates (FCR) differed among treatments (one-way ANOVA, P = 0.03), with an approximately 30% higher mean FCR for the 1084 μM NH_3 treatment than for the other treatment (Table 4).

3.8. EC_{10} for total feed intake and SGR

The concentration–effect curves for total feed intake and SGR in relation to the water NH_3 concentration (Fig. 3a and b), demonstrate that for total feed intake the EC_{10} for NH_3 is 89 μM (1.24 mg NH_3-N/L), with a 95% confidence interval from 24 to 321 μM. For SGR, the EC_{10} for NH_3 is 122 μM (1.70 mg NH_3-N/L), with a 95% confidence interval from 44 to 330 μM.

4. Discussion

African catfish successfully control plasma NH_4^+ concentrations within physiological concentrations over a wide range of water ammonia concentrations that would be lethal to many other fishes. However, the high ammonia concentrations did affect the fish, as revealed by other parameters: plasma glucose, plasma osmolality, branchial Na^+/K^+-ATPase activity, gill morphology, specific growth rate (SGR), total feed intake (TFI) and feed conversion rate (FCR) were affected, albeit that this species is very tolerant to ammonia compared to other fish.

4.1. Plasma NH_4^+

In African catfish plasma, T_{Amm} is predominantly present (84–98%) as NH_4^+ (Ip et al., 2004b). The capability to maintain a low plasma NH_4^+ concentration as seen in this study during exposure to millimolar water

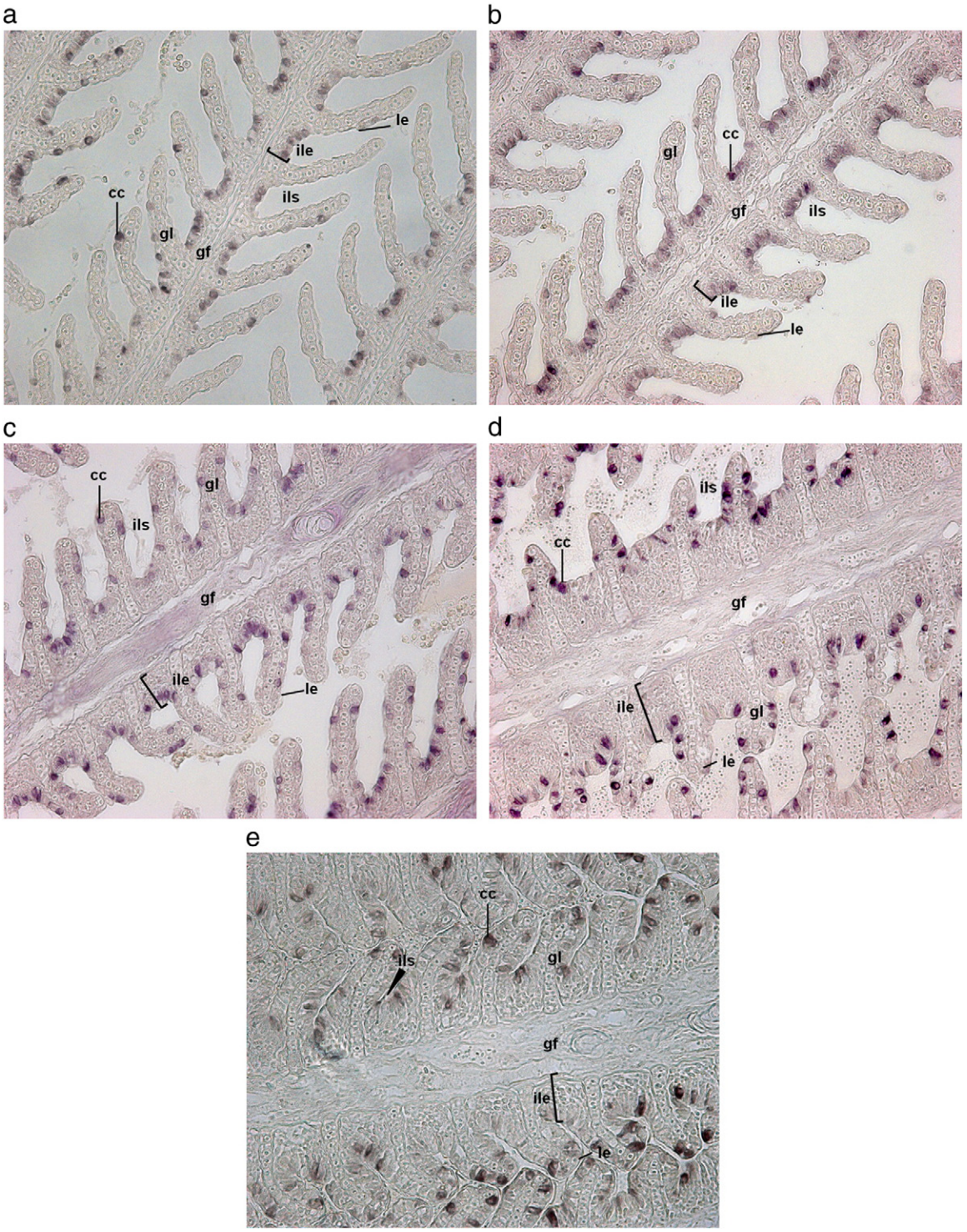

Fig. 1. Histology of gill epithelium immunohistochemically stained for Na^+/K^+-ATPase-rich cells (chloride cells) of the 4 μM (a) 14 μM (b) 38 μM (c) 176 μM (d) and 1084 μM (e) NH_3 treatment groups (400× magnification). Thickening of inter-lamellar and lamellar epithelium (inter-lamellar space reduction) increases gradually with increasing water ammonia level (c). The 1084 μM treatment (e) reveals distal and basal hyperplasia with lamellar fusion, epithelial hypertrophy accompanied with enhanced mucus secretion. Legend: ile = inter-lamellar epithelium; le = lamellar epithelium; ils = inter-lamellar space; cc = chloride cell; gf = gill filament; gl = gill lamellae.

NH_3 has been previously demonstrated for African catfish (Ip et al., 2004b): plasma NH_4^+ concentrations after 5 days exposure to 0.69 mM ambient NH_3 came to 2.12 mM, a value approximately ten times higher than reported here after 34 days exposure of the same species to up to 1084 μM NH_3. This suggests that in the African catfish exposure to high water NH_3 initially results in a plasma NH_4^+ peak due to an NH_3 influx, followed by the on-set of NH_3 defence mechanisms over time and a subsequent decline of plasma NH_4^+ concentrations to basal levels. Time-kinetic studies are needed to exactly define this pattern for African catfish, but our results do support a successful acclimation to rather extreme NH_3 concentrations.

African catfish actively excretes NH_4^+ against an inward concentration gradient as the major defence mechanism against ammonia toxicity (Ip et al., 2004a,b). The low plasma NH_4^+ levels observed in the present study can possibly be attributed to this mechanism. Indeed, as sodium is a counter ion in this NH_4^+ export, the 5 g/L salinity in our

Table 4
Mean (SD) values per treatment (N = 2) for final weight, specific growth rate (SGR), total feed intake and feed conversion rate (FCR). Mean values with different superscripts are significantly different (one-way ANOVA, P values as shown).

Treatment	Ammonia (μM)	Final weight (g)	Total feed intake (g)	SGR (%/BW/d)	FCR
1	4	412 (1.2)^{ab}	2339 (2)^{a}	3.25 (0.01)^{ab}	0.72 (0.004)^{a}
2	14	468 (10.4)^{a}	2705 (104)^{b}	3.64 (0.07)^{a}	0.69 (0.005)^{a}
3	38	466 (44.8)^{a}	2668 (170)^{b}	3.61 (0.29)^{a}	0.69 (0.004)^{a}
4	176	370 (17.1)^{b}	2017 (181)^{c}	2.92 (0.14)^{b}	0.73 (0.011)^{a}
5	1084	224 (9.6)^{c}	986 (32)^{d}	1.40 (0.13)^{c}	1.00 (0.15)^{b}
P-value		<0.01	<0.01	<0.01	0.03

experiments may have facilitated NH_4^+ excretion. We do not exclude contribution of other defence mechanisms (see Introduction), but we have no data to directly support such mechanisms.

The maintenance of low plasma NH_4^+ at a chronically high water ammonia concentration is exceptional among farmed fish species. Plasma ammonia concentrations in Atlantic salmon (*Salmo salar*) and European seabass (*Dicentrarchus labrax*) were found to increase linearly up to 1 mM with chronic exposure to high water ammonia with no signs of rebound or acclimation (Knoph and Thorud, 1996; Lemarié et al., 2004).

4.2. Plasma cortisol, glucose and lactate

Plasma cortisol concentrations were (typically) rather variable among individual fish and no significant differences were found among treatments (Table 3). In fish, acute stress results in rapid 10 to 100 fold increase of the plasma cortisol concentration, followed by a return to basal concentrations within hours. Basal concentrations are generally low but variation among life stages, sexes, individuals within a population, and species exist (Wendelaar Bonga, 1997). In case of chronic stress, such as high water ammonia, plasma cortisol concentrations may remain elevated above basal concentrations, although at lower concentrations than the concentrations associated with acute stress (Wendelaar Bonga, 1997). In the present study, mean plasma cortisol concentrations ranged from 30.0 to 72.9 nM, well below a basal concentration of 122.8 nM (45.5 ng/mL) reported earlier for the same species (Martins et al., 2006a; Martins et al., 2006b). We conclude that the current experimental design did not induce chronic stress in the fish, except maybe for the highest NH_3 group. The lower plasma cortisol values of the 1084 μM NH_3 treatment group could be interpreted as an exhaustion of the pituitary–inter-renal-axis as a prolonged hyperactivity of this system (Hontela et al., 1992). This is supported by the observation of significantly elevated plasma glucose in the 1084 μM NH_3 treatment; the effects are still mild, corroborated by no more than a tendency for plasma lactate concentrations to go up in this treatment. High plasma glucose and lactate concentrations are both common to stressed fish (Wendelaar Bonga, 1997). The elevated plasma glucose concentration in 1084 μM NH_3 treatment can possibly be explained by the energy demand required to fuel active NH_4^+ excretion. The elevated plasma lactate concentrations in this treatment suggest the use of lactate as substrate for gluconeogenesis (Wendelaar Bonga, 1997). This adaptation of the energy metabolism may be related to the energy demand of active NH_4^+ excretion while energy (food) intake is reduced (Table 4).

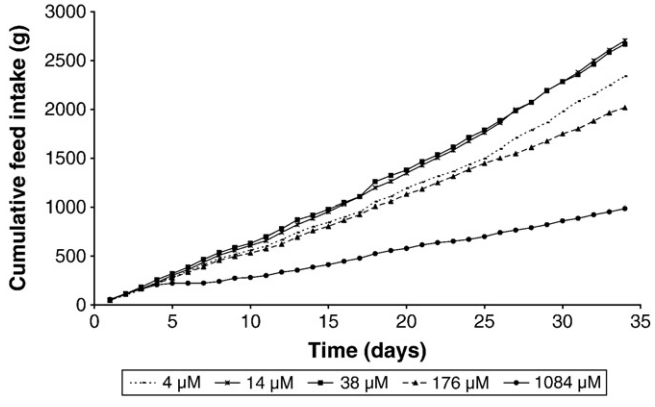

Fig. 2. NH_3 exposure and mean (N = 2) cumulative feed intake.

4.3. Plasma osmolality

Plasma osmolality was very similar among the 4 to 176 μM NH_3 treatments and ranged from 267.3 to 278.3 mOsmol/kg. Compared to these treatments the plasma osmolality was significantly elevated in the 1084 μM NH_3 treatment to 347 mOsmol/kg (Table 3). Teleostean fishes are strong regulators, tightly regulating their plasma osmolarity in a species-dependent range of salinities (Varsamos et al., 2005). The water

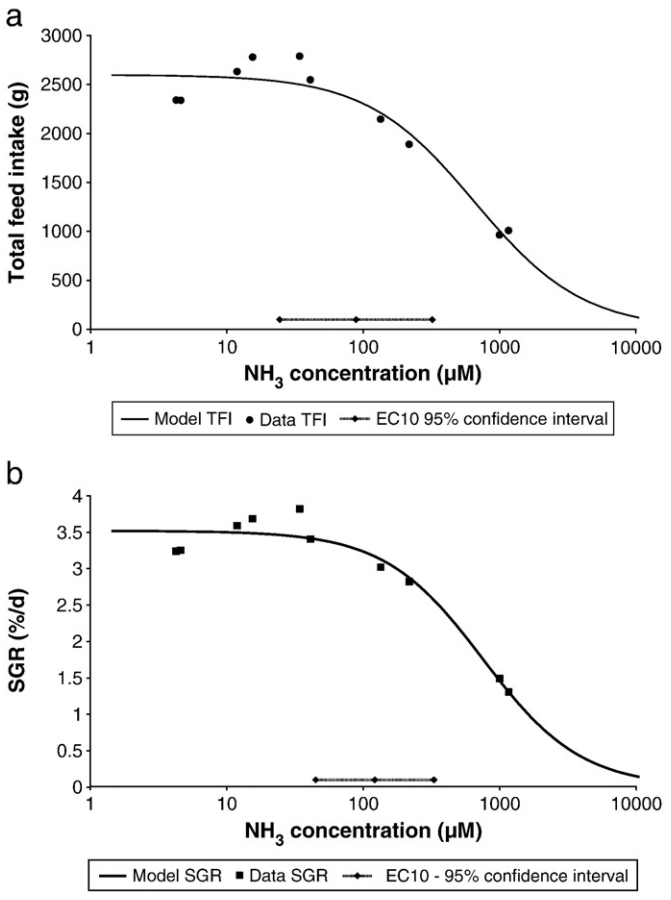

Fig. 3. Concentration–effect curves for total feed intake (TFI) (a) and specific growth rate (b) in relation to the water NH_3 concentration. $TFI = 2559/(1+10^{(2.819-\log[NH3])})^{*} - 1.095$ ($r^2 = 0.93$) and $SGR = 3.519/(1+10^{(2.877-\log[NH3])})^{*} - 1.205$ ($r^2 = 0.95$), where $[NH_3]$ is the NH_3 concentration (μM).

osmolality among treatments in this study was essentially constant (see Section 2.1 and Table 1) and no significant differences in conductivity of the water were observed among treatments (Table 2). The differences in plasma osmolality must therefore be attributed to the experimental treatments. Unfortunately, we ran out of plasma (the samples compromised the economical PhOx-equipment we normally use for plasma mineral analysis) and therefore we cannot support plasma osmolality data with sodium and chloride values. However, the elevated plasma osmolality in the 1084 μM NH_3 treatment group could very well be a consequence of facilitated influx of NaCl to the blood in exchange for NH_4^+ excretion as the higher (compared to normal fresh water) ambient Na (1.6 g/L) and Cl (2.5 g/L) levels in the tanks would favour this. On the other hand, reduced ion exchange with the environment may decrease plasma volume and increase of plasma osmolality (Wendelaar Bonga, 1997), and this could also explain this observation.

4.4. Gill morphology/Na^+/K^+-ATPase activity

The branchial epithelium is where gas exchange, ion regulation, acid–base balance and nitrogenous-waste excretion occur (Evans et al., 2005). The direct contact with the medium and delicate structure make gills vulnerable for water pollutants and a sensitive site to develop anomalies (Erkmen and Kolankaya, 2000), such as epithelial hypertrophy, epithelial lifting, necrosis and hyperplasia with lamellar fusion (Evans, 1987; Erkmen and Kolankaya, 2000).

The gradual changes in gill morphology observed with increasing water ammonia concentrations culminated in drastic lesions in the 1084 μM NH_3 treatment group. Similar morphological impairments were observed in an Anatolian khramulya (*Capoeta tinca*) population living in a stream with high concentrations of water pollutants, including NH_3 (58 μM; Erkmen and Kolankaya, 2000). These histopathological changes may underlie a disrupted ion transport (Ip et al., 2004a). The epithelium from the filaments is known to be more permeable than the lamellar epithelium, due to the presence of 'tight junctions' that require strict regulation to guarantee epithelial permeability to water and ions (Evans, 1987). One should further consider that the filamental epithelium harbours the chloride cells that facilitate the major part of ion transports in the gills, and that the inherent cellular make-up involves specific junctional complexes to seal the epithelium as required for the transports taking place; in control lamellar epithelium chloride cells are normally absent and thus one may predict a differential permeability in filamental and lamellar epithelium. Hypertrophy of the filament epithelium may represent an adaptive response to increase a barrier to reduce the inflow of NH_3. Hypertrophy of the epithelium associated with lamellar fusions, as well as mucous cells proliferation can be interpreted as adaptations to increase the distance between the water and the blood flow, reducing the permeability of the gills.

Whereas chloride cell migration towards the lamellae expands the transport capacity to facilitate ion exchange, it may simultaneously increase branchial permeability, as it potentially extends the leakiness of the branchial epithelium as a whole. Clearly a balance needs to be made up to weigh contributions of extra ion transport capacity (more chloride cells) and an enhanced branchial surface with increased permeability (and thus passive movement of water and ions) to the adaptive response seen.

Gills are the predominant place for NH_3 excretion in freshwater fish (Evans et al., 2005; Wilkie, 2002). Chloride cells in the branchial epithelium are the site of active excretion of NH_4^+ against an inwardly directed electrochemical gradient of ammonia, through the Na^+/K^+-ATPase (1 ATP: $2K^+$ or NH_4^+: 3 Na^+) (reviewed by Heisler, 1984; Evans, 1987; Evans et al., 2005; Ip et al., 2004a) at the basolateral plasma membrane of the chloride cells; NH_4^+ may be exchanged at the apical membrane for waterborne chloride. As described before, African catfish are able to maintain a relatively low plasma NH_4^+ concentration despite the high external ammonia concentration (Ip et al., 2004b). In this condition, it is assumed that as the external ammonia concentration increases, the active excretion of NH_4^+ is enhanced. This is supported by an increased, energized Na^+/K^+-ATPase mediated export in the 1084 μM treatment group.

4.5. Feed intake, specific growth rate, feed conversion rate and mortality

The absence of mortality in this experiment shows that the lethal concentration for NH_3 for chronic exposure lies above 1084 μM. The 96 h LC_{50} for African catfish is reported to be as high as 380 mM for T_{Amm} (Britz, 1988 in Ip *et al.*, 2004b). However the pH at which the acute toxicity was tested is not reported, and this hinders comparison just based on NH_3 of our study with these studies.

In the first week of the experiment feed intake was similar among treatments (Fig. 2). This we attribute to the experimental design where we have chosen to gradually built-up the water ammonia during the first four days of the experiment.

Surprisingly, a higher total feed intake was observed in the 14 and 38 μM NH_3 treatments compared to the control treatment. We hypothesize that the fish overcompensated for the extra energy demand associated to the active excretion of NH_4^+ by increasing their feed intake, a phenomenon described as hormesis (Calabrese, 2005).

The cumulative feed intake in the 176 μM NH_3 treatment and the control were equal until approximately day 25 of the experiment and after day 25, cumulative feed intake was lower in the 176 μM NH_3 treatment (Fig. 2). This suggests that the fish exposed to 176 μM NH_3 were aiming to maintain normal feed intake, but were unable to sustain this during prolonged ammonia exposure. Overall this resulted in a lower total feed intake in the 176 μM NH_3 treatment compared to the control (Table 3).

Total feed intake and SGR were lower in the 176 μM NH_3 treatment compared to the 14 and 38 μM NH_3 treatments. Since the feed conversion rate was equal among these treatments, the lower SGR in the 176 μM NH_3 treatment must be attributed to reduced feed intake rather than reduced feed utilization. This notion is corroborated by studies on turbot, where it was found that NH_3 reduces growth as a result of reduced feed intake (Person-Le Ruyet et al., 1997).

Total feed intake and SGR were negatively affected in the fish exposed to the highest NH_3 concentration: exposure to 1084 μM NH_3 resulted in a 58% lower total cumulative feed intake and a 57% lower SGR compared to the control treatment. Fish exposed to 1084 μM NH_3 showed a higher FCR compared to the fish in all other treatments. This suggests a high energy demand for the maintenance of low plasma NH_4^+ concentrations at high water ammonia at the cost of growth.

4.6. NH_3 threshold concentrations

African catfish chronically exposed to NH_3 concentrations as high as 176 μM (2.5 mg NH_3-N/L) did not show major physiological disturbances, except for gill morphology. This suggests that the threshold NH_3 concentration for physiological disturbance is at least 176 μM. However, both feed intake and specific growth rates were found to be reduced at much lower NH_3 concentrations: the EC_{10} for NH_3 was found to be 89 μM for feed intake and 122 μM for SGR.

The physiological parameters measured in this experiment include both primary (plasma cortisol) and secondary (plasma glucose and plasma lactate) stress responses. Feed intake and growth rate are tertiary stress responses (Wendelaar Bonga, 1997). The observed effects on feed intake and growth in the absence of major physiological disturbances are therefore surprising, but indicate that the priority for the fish apparently lies in proper stress regulation over regulation of growth. Feed intake and growth are thus good indicators for negative effects of high water ammonia on African catfish, in particular when evaluating chronic suboptimal conditions. The threshold concentration for chronic NH_3 exposure of African catfish is in our view best based on NH_3 effects on feed intake and growth. Considering that the lowest EC_{10}

value was obtained for feed intake and taking into account the lower limit of its 95% confidence interval, the NH_3 threshold concentration should preferably be set at 24 μM (0.34 mg NH_3-N/L). At this NH_3 concentration the risk of a 10% reduction in feed intake is 5% maximally and growth will not be compromised.

5. Conclusions

This study clearly demonstrates that plasma levels of NH_4^+, cortisol, glucose and lactate, as well as plasma osmolality are not indicators of first choice for chronic toxicity of high water ammonia in African catfish. We advise for African catfish not to exceed a water NH_3 concentration of 24 μM (0.34 mg NH_3-N/L) to reduce the risk of reduced growth and feed intake. Below this NH_3 threshold concentration less obvious, but potentially dangerous disturbances such as deteriorated gill fine structures are avoided.

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