Aquatic Toxicology 195 (2018) 77–87

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

The effects of high environmental ammonia on the structure of rainbow trout hierarchies and the physiology of the individuals therein

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ARTICLE INFO

Keywords:
Aggression
Dominance
Growth
Feeding
O_2 consumption
Ammonia excretion
Plasma cortisol

ABSTRACT

Our goals were: (i) to determine whether sublethal concentrations of water-borne ammonia would prevent the formation of a dominance hierarchy, or alter its structure, in groups of 4 juvenile trout; (ii) to investigate the behavioral and physiological responses of individuals of different social rank exposed to a concentration of ammonia that still allowed hierarchy formation. Social hierarchies were created by using a technique in which a food delivery system that created competition also served to isolate individual fish for respirometry. Groups of 4 fish were exposed to elevated ammonia (NH_4HCO_3) 12 h before first feeding; aggression was recorded by video camera during morning feedings. Experimental ammonia concentrations were 700, 1200 and 1500 μmol L^{-1} at pH 7.3, 12 °C (9.8, 16.8, and 21.0 mg L^{-1} as total ammonia-N, or 0.0515, 0.0884, and 0.1105 mg L^{-1} as NH_3-N). Aggression was severely reduced by 1200 and abolished by 1500 μmol L^{-1} total ammonia, such that hierarchies did not form. However, groups exposed to 700 μmol L^{-1} total ammonia still formed stable hierarchies but displayed lower levels of aggression in comparison to control hierarchies. Exposure continued for 11 days. Physiological parameters were recorded on day 5 (end of period 1) and day 10 (end of period 2), while feeding and plasma cortisol were measured on day 11. In control hierarchies, dominant (rank 1) trout generally exhibited higher growth rates, greater increases in condition factor, higher food consumption, and lower cortisol levels than did fish of ranks 2, 3, and 4. In comparison to controls, the 700 μmol L^{-1} total ammonia hierarchies generally displayed lower growth, lower condition factor increases, lower O_2 consumption, lower cortisol levels, but similar feeding patterns, with smaller physiological differences amongst ranks during period 1. Effects attenuated during period 2, as aggression and physiological measures returned towards control levels, indicating both behavioral and physiological acclimation to ammonia. These disturbances in social behavior and associated physiology occurred at an ammonia concentration in the range of regulatory significance and relevance to aquaculture.

1. Introduction

In fish, stable dominance hierarchies may be beneficial to both subordinate and dominant individuals by reducing aggressive behavior. An additional benefit is that stable hierarchies may entrain different feeding strategies for fish of different rank (Gurney and Nisbet, 1979; Sneddon et al., 2006). There are also clear physiological differences in fish of different social rank, such as higher growth rates and lower cortisol levels in dominant individuals (Sloman et al., 2001a; Gilmour et al., 2005; Grobler and Wood, 2013). However, there is now increasing recognition that the social behavior of aquatic animals, especially the formation of dominance hierarchies, is a sensitive target for aquatic toxicants (for reviews, see Atchison et al., 1996; Scott and Sloman, 2004; Sloman and Wilson, 2006).

Ammonia (the sum of NH_3 and NH_4^+) is highly toxic to fish (for reviews, see Randall and Tsui, 2002; Eddy, 2005; Ip and Chew, 2010). Ammonia is also the most manufactured molecule in the world (N-fixation by the Haber-Bosch process), most of it being used for fertilizer manufacture, thereby supporting over 45% of the world's population (Erisman et al., 2008; Fowler et al., 2013). However, most of this is ultimately lost to the environment, resulting in increasing ammonification of natural waters by non-point source pollution. Ammonia is also generated by a wide variety of other anthropogenic processes. Indeed Ankley et al. (2011) identified ammonia as the key toxicant of concern in 37% of municipal discharges evaluated by Toxicity Identity Evaluation (TIE) tests in the US. Environmental Protection Agency's effluent testing program. Additionally, teleost fish produce ammonia as their major N-waste product (Wright, 1995), so ammonia build-up in

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https://doi.org/10.1016/j.aquatox.2017.12.006
Received 14 April 2017; Received in revised form 9 December 2017; Accepted 17 December 2017
Available online 19 December 2017
0166-445X/

aquaculture is of great concern (Handy and Poxton, 1993; Tomasso, 1994; Colt, 2006; Crab et al., 2007). Given this background, it is curious that, to our knowledge, there has been only one study to date on ammonia's effect on social interactions in fish (Tudorache et al., 2008), and none on the possible effects of ammonia on the physiology of fish within dominance hierarchies.

Other toxicants (e.g. organics, metals, complex effluents) at sublethal levels, as well as general environmental disturbances (e.g. lowered water levels, increased water turbulence, hypoxia) have been shown to disrupt or alter the formation of dominance hierarchies. Commonly reported effects include decreased stability of hierarchies or a failure to form hierarchies, either decreased or increased rates of aggression, and reductions in physiological differences among members of different rank (reviewed by Atchison et al., 1996; Scott and Sloman, 2004; Sloman and Wilson, 2006). Sublethal ammonia is well known to affect many of the processes thought to be important in hierarchy dynamics such as brain function (Walsh et al., 2007), swimming activity and muscle function (Shingles et al., 2001; Wicks et al., 2002; Tudorache et al., 2010), growth (Foss et al., 2003, 2004, 2009, Wood, 2004; Madison et al., 2009), appetite (Wicks and Randall, 2002a; Ortega et al., 2005), and cortisol regulation (Knoph and Olsen, 1994; Wicks and Randall, 2002b; Tsui et al., 2009).

Therefore in the present study, we tested the overall hypotheses that sublethal exposure to high environmental ammonia would (i) disrupt the formation of dominance hierarchies in juvenile rainbow trout, (ii) alter aggression rates, and (iii) reduce physiological differences among members of different rank. We used a method developed by Grobler and Wood (2013) to establish dominance hierarchies in groups of four rainbow trout and to measure respirometric parameters (O_2 consumption, and ammonia excretion rates) of each member over an 11-day period. Social rank was assessed by measurements of aggression; additional determinations included growth, condition factor, feeding, and terminal plasma cortisol concentrations. Several levels of total ammonia were tested initially to assess concentration-dependency, and then detailed measurements were made at the lowest concentration.

2. Methods and materials

Procedures were approved by the McMaster University Animal Research Ethics Board (AUP 09–04–10), and complied with the regulations of the Canada Council for Animal Care.

2.1. Experimental animals and holding conditions

Juvenile rainbow trout (6–10 g) were obtained from Humber Spring Trout Hatchery in Orangeville, Ontario. At McMaster University, the fish were held for 3 weeks prior to experimentation under a 12.5 h light: 11.5 h dark photoperiod in batches of 50 individuals per 200-L aerated aquaria. The tanks were supplied with flowing ($\sim 1\,L\,min^{-1}$) dechlorinated Hamilton tap water (12 °C, pH ~ 7.3, $Na^+ = 0.5$, $Cl^- = 0.7$, $Ca = 1.0\,mmol\,L^{-1}$, hardness ~140 ppm as $CaCO_3$). A 1% total tank weight ration of commercial dried pellet feed (1 point, Martin Mills Inc., Elmira, Ontario) was fed to the fish, three times per week. Water composition was: (in $mmol\,L^{-1}$) $Na^+ = 0.5$, $Cl^- = 0.7$, $Ca = 1.0$, hardness ~140 $mg\,L^{-1}$ as $CaCO_3$. Access to food and space was sufficient during holding that aggression was minimal and social hierarchies did not form.

2.2. Control and experimental group preparation

After anaesthesia in neutralized MS – 222 (0.08 g tricaine methanesulfate L^{-1}), fish were weighed (0.01 g), measured for fork length (0.1 cm), uniquely freeze branded to allow for visual identification, as described by Grobler and Wood (2013), and assigned to groups, as described below. Normal behavior was re-established by 24 h, and food then was then provided on day 2, when the experiments were started.

Seven control groups, ten ammonium bicarbonate (NH_4HCO_3) groups, and four sodium bicarbonate ($NaHCO_3$) groups were formed, each containing 4 fish, using methods similar to those of Grobler and Wood (2013). The 4 fish in each group were size-matched in terms of both length and mass, and were sourced from the same batch of 50-fish in the holding aquaria. Each group was housed in a 30-L aerated tank (53 × 26.7 × 30 cm) fitted with a clear lid to facilitate observations and 5 pieces (1 floating) of PVC pipe (7 × 2.5 cm) for shelter. Tanks were supplied with aeration and flowing ($\sim 0.5\,L\,min^{-1}$), dechlorinated Hamilton tap water. Water quality was the same as in the holding conditions, and water pH was measured daily using a combination glass electrode (GK24O1C) and pHM 84 meter (Radiometer, Copenhagen, Denmark).

For the ammonia treatments, three different total ammonia concentrations (700, 1200 and 1500 $\mu mol\,L^{-1}$) were created by adding solutions of analytical grade ammonium bicarbonate (NH_4HCO_3, Sigma-Aldrich, St. Louis, Missouri), dispensed through drip bottles, to the tanks 12 h before first feeding. These correspond to 9.8, 16.8, and 21.0 $mg\,L^{-1}$ as total ammonia-N, or 0.0515, 0.0884, and 0.1105 $mg\,L^{-1}$ as NH_3-N (unionized ammonia-N) at a mean pH = 7.3and temperature = 12 °C, calculated using the online calculator accompanying the USEPA (2013) ammonia criteria document. These experimental groups were set up in the exact same way as the control groups. Two groups were exposed to 1200 $\mu mol\,L^{-1}$ total ammonia for 5 days, two groups to 1500 $\mu mol\,L^{-1}$ for 5 days, and six groups to 700 $\mu mol\,L^{-1}$ for 11 days. Total ammonia concentrations were verified daily using a modified Verdouw et al. (1978) procedure with concentrations varying by no more than ± 10 $\mu mol\,L^{-1}$ from the nominal concentration for each concentration. In tank measurements of total ammonia in control tanks were routinely 0–6 $\mu mol\,L^{-1}$, and pH was 7.2–7.4 in both control and elevated ammonia tanks. Ammonium bicarbonate (NH_4HCO_3) was chosen for the ammonia treatments as it did not appreciably alter the water pH.

To account for any effect that the elevated bicarbonate in the ammonia-exposed groups (dissociation of NH_4HCO_3) might have on hierarchy structure or individual physiology, four groups were exposed to 700 $\mu mol\,L^{-1}$ $NaHCO_3$, (Sigma-Aldrich, St. Louis, Missouri) for 11 days using the exact same protocol as outlined for the 700 $\mu mol\,L^{-1}$ NH_4HCO_3 exposures. All parameters except ammonia excretion rate, % feeding, and plasma cortisol were measured in the 700 $\mu mol\,L^{-1}$ $NaHCO_3$ treatments.

2.3. Hierarchy creation and feeding regime

The experimental design comprised an 11-day exposure, divided into two 4-day periods (days 1–4, and 6–9) in which social interactions were observed, 2 days (days 5 and 10) in which physiological measurements (respirometry for oxygen consumption and ammonia excretion rate, growth indices) were made, and a final day 11 when feeding and plasma cortisol measurements were made. As days 5 and 10 involved disturbance to the fish (handling, anaesthesia), the two periods were treated separately for analysis.

The method of Grobler and Wood (2013) was used to create social hierarchies and record physiological differences among fish with a minimum of disturbance. The basic principle is that food delivery was restricted to a chamber, thereby creating competition. This chamber also served to isolate individual fish for respirometry. The technique

utilized a darkened container (17.8 × 14 × 12 cm, volume = ~ 2.8 L) that would serve as a respirometer when closed; see Fig. 1 of Grobler and Wood (2013) who provide a more detailed description of the method. During feeding, a darkened plastic container (identical size and dimensions as the respirometer) that had a feeding tube attached was inserted into the tank. Food pellets were dropped into the feeding tube so that food was highly concentrated within the container. Fish attempted to monopolize the container as they associated the darkened feeding area with food.

Fish were placed on a strict feeding regime using this method. A 1% total tank biomass ration was fed twice daily (morning between 7:30–9:00 AM and evening between 6:30–8:00 PM), with food being delivered into the feeding tube pellet by pellet. Food delivery took less than 1 min and the feeding container was left inside the tank for 15 min. A video camera (Sanyo VPC-WH1, Osaka, Japan) set up on scaffolding surrounding the tanks recorded group behavior during this 15-min period. Video recordings were made on days 1–4 in all treatments, and on days 1–4 and 6–9 in the control, 700 μmol L^{-1} NH_4CO_3, and 700 μmol L^{-1} $NaHCO_3$ treatments. Respirometry was performed on days 5 and 10, and direct measurement of individual feeding by the Ballotini beads X-radiographic method of McCarthy et al. (1992) as described in Section 2.5, followed by terminal sampling for cortisol, was performed on day 11. Days 1–5 are termed period 1, and days 6–10 are termed period 2.

2.4. Behavioral measurements

Preliminary video analysis showed that aggression was higher during the morning feedings than during the evening feedings, and therefore the former were selected for detailed analysis. Each chase, approach, and nip over the 15-min morning observation period was given a point of 1, allowing each fish to have a total aggression score from which a social hierarchy could be deduced. The most aggressive fish, and thus most dominant individual, was labeled 'Rank 1′ while the least aggressive fish was 'Rank 4′.

2.5. Physiological measurements

On days 5 and 10, physiological parameters were recorded in fasted fish (24 h since last meal) by confining individual fish in the 'dummy' feeding apparatus which served as a thermostatted (12 °C) respirometer (see Grobler and Wood, 2013 for details). Fish would enter the respirometer voluntarily in search of food. All fish in a hierarchy were sampled at the same time, using four respirometers. Therefore individual rates of oxygen consumption (MO_2) and ammonia excretion (M_{Amm}) were measured and compared simultaneously under identical conditions, on each of the four fish in a hierarchy, and capture disturbance was avoided. Fish were held in the respirometers for 6 h, with MO_2 measured within the first h, and ammonia excretion rate (M_{Amm}) measured over 6 h by spot sampling; the respirometer was sealed during the MO_2 determination, then aerated for the remaining period. Water P_{O2} was measured with an oxygen electrode (Cameron Instruments, Port Aransas, TX, USA) thermostatted to 12 °C and connected to a model 1900 A-M Systems polarographic amplifier (Carlsborg, WA, USA). Water total ammonia concentration was measured by the colorimetric method of Verdouw et al. (1978), using calibration curves in the 0–250 μM range. Where dilution of samples was required, both initial and final samples were diluted identically. At the end, fish were anaesthetized in neutralized MS-222 (0.08 g L^{-1}), weighed, and measured for fork length as at the start of the experiment to allow calculation of growth rate and condition factor. Thereafter, the four fish were simultaneously returned to their hierarchy for overnight recovery. Thus, fish had their physiological profile measured at the end of period 1 (day 5) and period 2 (day 10).

On the morning of day 11, the method of McCarthy et al. (1992) was used to measure the individual food consumption in a single meal by each member of the hierarchy. A standard ration of 1% total tank weight of the regular dried pellet feed was given, but the feed had been repelleted to contain 6% (by mass of dried powdered food) Ballotini lead glass beads (0.400–0.455-mm, 8.5- grade; Jencons USA Inc., Bridgeport, PA, USA). In preliminary trials, we found that the trout consumed the labeled pellets as readily as normal pellets. One hour after feeding, the fish were terminally anaesthetized by netting them

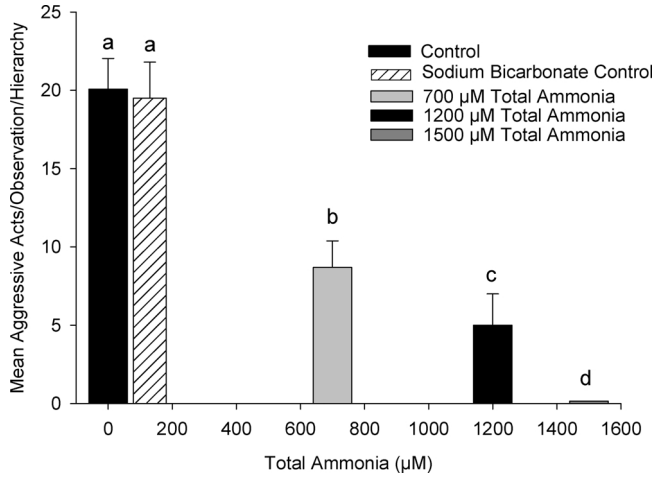

Fig. 1. Mean aggressive acts per 15-min observation for whole hierarchy in groups of 4 trout exposed to different concentrations of total ammonia. Control, 700 μmol L^{-1} sodium bicarbonate control, and 700 μmol L^{-1} total ammonia groups were exposed for 10 days, while 1200 μmol L^{-1} total ammonia and 1500 μmol L^{-1} total ammonia groups were exposed for 5 days. Values are means of groups ± S.E.M. (P = 0.035, one-way ANOVA; post-hoc Fisher LSD test; different letters denote significant differences (P < .05) between exposure groups). Control, N = 7; 700 μmol L^{-1} $NaHCO_3$ control, N = 4; 700 μmol L^{-1} ammonia, N = 6; 1200 μmol L^{-1}, N = 2; 1500 μmol L^{-1}, N = 2.

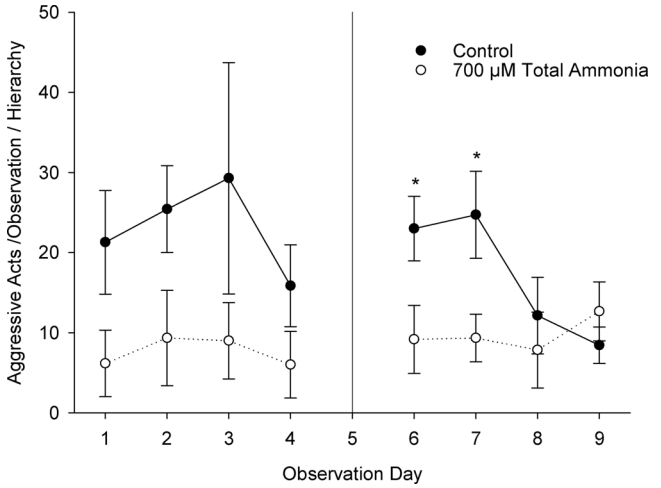

Fig. 2. Daily aggressive acts per 15-min observation for control and 700 μmol L^{-1} total ammonia hierarchies in period 1 (days 1–4) and period 2 (days 6–9). Values are daily means of hierarchical groups ± S.E.M. There are no significant differences within each treatment over time (Ammonia P = 0.590; Control P = 0.237; Friedman's one way repeated measures ANOVAs on ranks) Significant differences (P < .05; Mann-Whitney *U* tests) between aggressive acts per day for control and 700 μmol L^{-1} total ammonia on specific days denoted by asterisk. (Control N = 7; 700 μmol L^{-1} total ammonia N = 6).

simultaneously into a high concentration (5 g L^{-1}) of neutralized MS-222 to cause quick death without struggling. Blood was collected immediately for cortisol determination (see below), and then the carcasses were frozen at −20 °C until X-rayed (Faxitron 805 portable X-ray machine, Wheeling, Illinois; 1 s exposure at 70 kVP) to determine the number of glass beads ingested.

For cortisol determinations in the control and 700 μmol L^{-1} NH_4CO_3 treatment groups, blood was collected from the euthanized trout via tail severance into heparinized capillary tubes, and centrifuged immediately in order to obtain plasma. Plasma was stored at −20 °C until assayed with a Cayman Chemical EIA Kit (Ann Arbor, MI, USA), as described by Grobler and Wood (2013). In particular, following the manufacturer's instructions, each of the multiple plates used contained two blanks, two non-specific binding wells, two maximum binding wells, and an eight point standard curve run in duplicate. All plates were run within a 24-h period. Note that the plasma cortisol data for the control group (only 6 of 7 hierarchies were sampled) have been previously reported by Grobler and Wood (2013).

2.6. Calculations

During each morning feeding (days 1 through 4 for period 1, days 6 through 9 for period 2, 8 days in total), all fish were scored for aggressive behavior. Aggression was not monitored on days 5 and 10 as physiological measurements were taken on these days. At the end of the experiment, each fish would have a daily aggression score for each 15-min observation period as well as a total score for aggressive acts for each 4-day interval, which was divided by 4 to yield mean aggressive acts per observation period.

Specific growth rate (SGR) was calculated as:

$$\ln(W_2) - \ln(W_1)/(t_2 - t_1) \times 100 \quad (1)$$

where W_1 and W_2 are body weights (g) at times t_1 and t_2 (d) respectively.

Fulton's condition factor (CF) was calculated as:

$$(W/L^3) \times 100 \quad (2)$$

where W is the weight (g) and L is the fork length (cm) of the fish.

Percent change in condition factor (CF) was calculated as:

$$([CF_i - CF_f]/CF_i) \times 100\% \quad (3)$$

where CFi and CFf are initial and final condition factors for a period.

SGR and percent change in condition factor were calculated for each fish for periods 1 and 2 separately.

Oxygen consumption rate (MO_2) was calculated as:

$$(\Delta PO_2 \times \alpha O_2 \times v)/(W \times t) \quad (4)$$

where ΔPO_2 (mmHg) is the measured change in water P_{O2}, αO_2 (μmol L^{-1} $mmHg^{-1}$) is the solubility constant for O_2 in water at 12 °C (Boutilier et al., 1984), v is the volume (L) of the 'dummy' feeding container, W(g) is the weight of the fish, and t is the time (h). Total ammonia excretion rate (M_{Amm}) was calculated similarly, substituting total ammonia for ΔPO_2 x αO_2.

The feeding measurement was based on food pellets uniformly labeled with 6% glass beads (see McCarthy et al., 1992 for details). The number of beads in each fish on the X-rays was counted, and expressed as a percentage of the total number of beads recovered. This yielded the percent of the total 1% ration meal consumed by each member of the hierarchy.

2.7. Statistical analyses

SigmaStat 3.5 (Systat Software, Inc. 2006) and Statistica 7.0 (StatSoft Inc. 2004) softwares were used for statistical analyses. One-way ANOVA followed by the Fisher's Least Significant Difference (LSD) post-hoc test were used to test for differences in aggressive acts per observation among control, 700, 1200 and 1500 μmol L^{-1} NH_4HCO_3-exposed groups. Two-way ANOVA (with ammonia and social rank as factors) followed by the Fisher's LSD post-hoc test were performed to test for differences in mean aggressive acts, SGR, percent change in condition factor, MO_2, M_{Amm}, % feeding, and plasma cortisol. Data were checked for normality and homogeneity of variance prior to ANOVA tests, and when criteria were not satisfied, the distribution of the data was plotted for inspection, and then appropriate transformation was applied (log, square root, inverse, or arc-sin). Aggressive acts, MO_2, M_{Amm}, % feeding, and plasma cortisol data were satisfactorily transformed. However, this was not possible with the daily aggressive acts data of Fig. 2, where Friedman's one way repeated measures ANOVAs on ranks were conducted to test for time-dependent differences in aggressive acts per observation within each treatment group. Mann-Whitney *U* tests were used to compare aggressive acts per observation within specific days between control and 700 μmol L^{-1} NH_4HCO_3 treatments. Data have been expressed as means ± 1 S.E.M., and significance was accepted at $P < 0.05$.

3. Results

3.1. Behavioural measurements

3.1.1. Effects of ammonia on overall aggression and dominance hierarchy formation

Control groups exhibited significantly higher rates of aggression than any of the ammonia treatments. Absolute controls and sodium bicarbonate controls displayed very similar aggression levels of 20.0 and 19.5 aggressive acts per observation respectively, both significantly greater than the 8.7 and 5.0 acts per observation in the 700 and 1200 μmol L^{-1} total ammonia groups respectively (Fig. 1). Aggression was essentially abolished in the 1500 μmol L^{-1} treatment.

Clear dominance hierarchies were formed in both control and 700 μmol L^{-1} total ammonia treatments. However, due to the reduced occurrence of aggressive acts, dominance hierarchies could not be determined in 1200 and 1500 μmol L^{-1} total ammonia treatments. Therefore, physiological parameters were not recorded in these groups, and detailed analyses focused only on the control and 700 μmol L^{-1} treatments. The 700 μmol L^{-1} total ammonia groups will be referred to as 'ammonia hierarchies' for the remainder of this paper.

The four hierarchies treated with 700 μmol L^{-1} $NaHCO_3$ (data shown in Fig. 1, and Supplementary Information Tables S1 and S2) displayed very similar patterns to the control hierarchies. Thus the behavioral and physiological effects seen in the ammonia hierarchies were attributable to elevated ammonia, and not to elevated bicarbonate.

3.2. Daily levels of aggression over time in control and ammonia hierarchies

Control hierarchies tended to display higher aggressive acts per observation than ammonia hierarchies in both periods 1 and 2, an effect which was significant on days 6 and 7 when daily comparisons were made (Fig. 2). The differences seemed to disappear on days 8 and 9, but this was due to reduced aggression in the controls rather than to increased aggression in the ammonia treatments. Within control or ammonia hierarchies, there were no significant differences amongst days. There was only one observed switch in social status from period 1 to period 2, occurring in one control group where one fish switched from rank 1 to rank 2 and *vice versa*. This did not occur in the ammonia hierarchies.

3.3. Aggression as a function of social status and time in control and ammonia hierarchies

During period 1, there was a significant overall effect of ammonia in

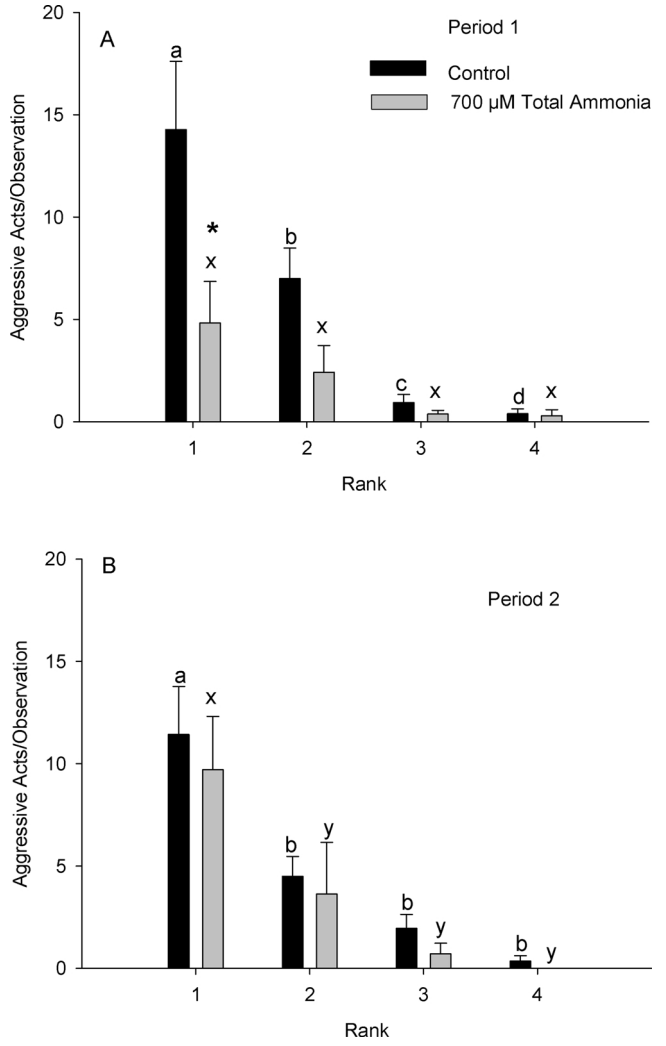

Fig. 3. Mean aggressive acts per 15-min observation as a function of social status for control and 700 µmol L^{-1} total ammonia hierarchies during period 1 and period 2. Values are means ± S.E.M. Period 1: there is a significant overall influence of treatment between control and 700 µmol L^{-1} total ammonia hierarchies (P = .007). There is also a significant overall influence of social rank (P < .001). There is a significant interaction effect (P = .021). Period 2: there is no significant overall influence of treatment between control and 700 µmol L^{-1} total ammonia hierarchies (P = .701). There is a significant overall influence of social rank (P < .001). There is no significant interaction effect (P = .976). (Control N = 7; 700 µmol L^{-1} total ammonia N = 6; two-way ANOVA; post-hoc Fisher LSD test). Different letters denote specific significant differences (P < .05) among ranks within a treatment. Asterisks denote significant differences (P < .05) between treatments within a social rank.

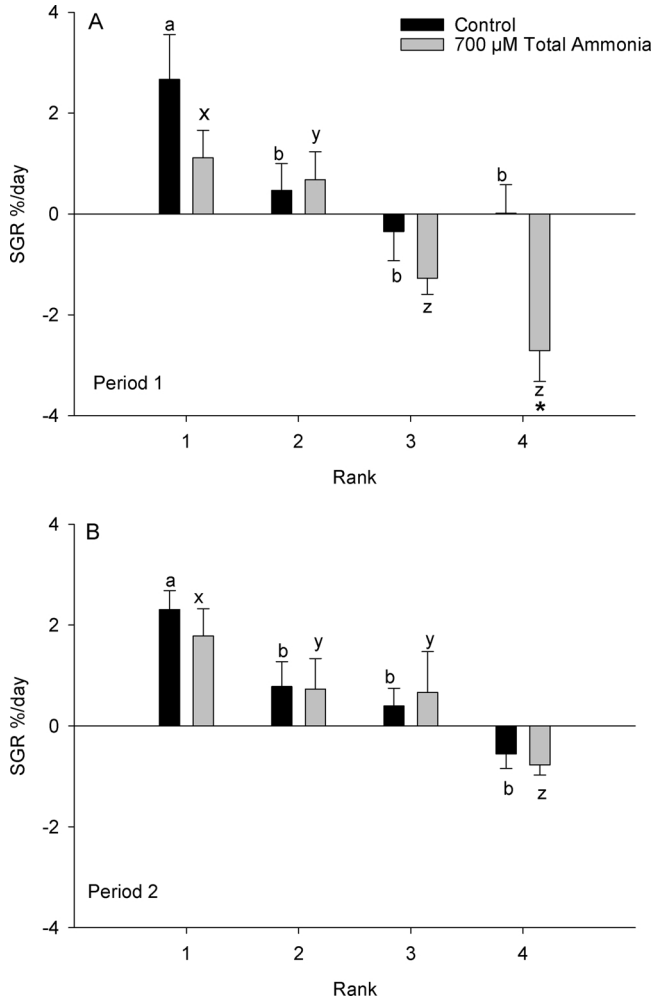

Fig. 4. Specific growth rate as a function of social status for control and 700 µmol L^{-1} total ammonia hierarchies during period 1 and period 2. Values are means ± S.E.M. Period 1: there a significant overall influence of treatment between control and 700 µmol L^{-1} total ammonia hierarchies (P = .004). There is also a significant overall influence of social rank (P < .001). There is no significant interaction effect (P = .177). Period 2: there is no significant overall influence of treatment between control and 700 µmol L^{-1} total ammonia hierarchies (P = .700). There is a significant overall influence of social rank (P < .001). There is no significant interaction effect (P = 0.866). (Control N = 7; 700 µmol L^{-1} total ammonia N = 6; two-way ANOVA; post-hoc Fisher LSD test). Different letters denote specific significant differences (P < .05) among ranks within a treatment. Asterisks denote significant differences (P < .05) between treatments within a social rank.

reducing aggression; however rank-specific differences were significant only for rank 1 fish (Fig. 3A). Among ranks, there were significant overall differences in aggression regardless of treatment but differences amongst specific ranks were significant only in the control hierarchy. There was a significant interaction effect.

In period 2, there were again significant differences in aggression among ranks in both control and ammonia hierarchies, but there was no overall difference between ammonia hierarchies and control hierarchies, and no significant rank-specific differences, unlike the situation in period 1 (Fig. 3B). There was no significant interaction effect.

3.4. Physiological parameters as a function of social status and time in control and ammonia hierarchies

3.4.1. Specific growth rate

Initial weight averaged 9.56 ± 0.55 g (N = 52). During period 1, there was a lower SGR in the ammonia treatment which was significant overall, as well as a significant overall effect of rank in both treatments (Fig. 4A). There was no significant interaction effect. In the controls, SGR was about 2.6% per day for rank 1 fish, and this was significantly higher than in ranks 2, 3, and 4 where SGR was close to 0% per day. In the ammonia groups, SGR was only about 1.1% per day in the rank 1 trout, and this dropped significantly to highly negative values (i.e. weight loss) of −1.3 and −2.7% per day in ranks 3 and 4 respectively. Rank-specific differences between the treatments were significant only for rank 4 fish.

In period 2, there was no longer a significant overall difference between control and ammonia groups in SGR, which had largely caught up to the control group in the ammonia treatment (Fig. 4B). However, a significant effect of rank persisted regardless of the exposure. There was no significant interaction effect. Control rank 1 fish still displayed the highest SGR (2.3% per day), but rank 1 ammonia fish now exhibited a

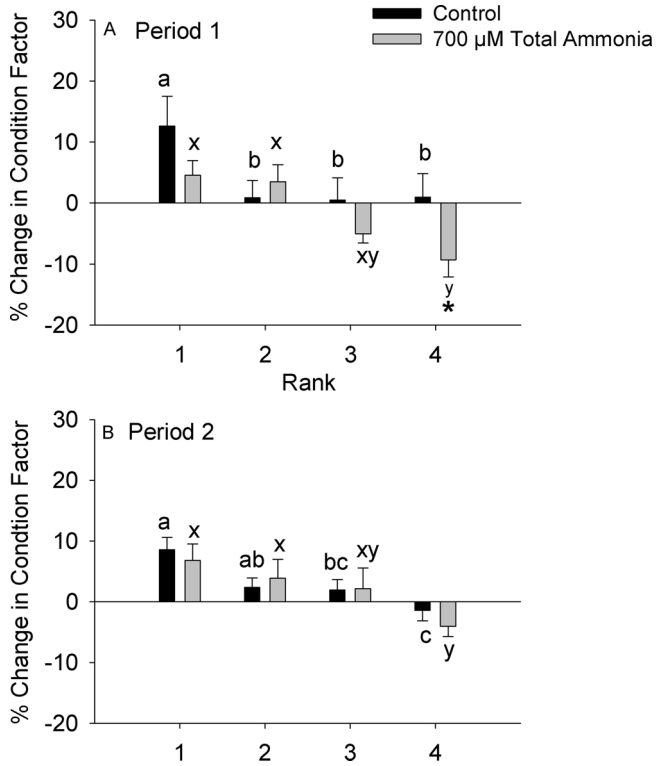

Fig. 5. Percent change in condition factor as a function of social status for control and 700 µmol L^{-1} total ammonia hierarchies during period 1 and period 2. Values are means ± S.E.M. Period 1: there is a significant overall influence of treatment between control and 700 µmol L^{-1} total ammonia hierarchies (P = .036). There is also a significant overall influence of social rank (P = .003). There is no significant interaction effect (P = .253). Period 2: there is no significant overall influence of treatment between control and 700 µmol L^{-1} total ammonia hierarchies (P = .693). There is a significant overall influence of social rank (P < .001). There is no significant interaction effect (P = .817). (Control N = 7; 700 µmol L^{-1} total ammonia N = 6; two-way ANOVA; post-hoc Fisher LSD test). Different letters denote specific significant differences (P < .05) among ranks within a treatment. Asterisks denote significant differences (P < .05) between treatments within a social rank.

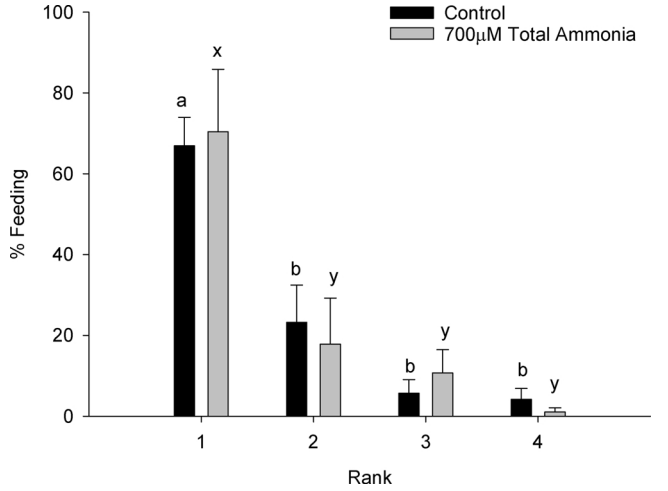

Fig. 6. Percent feeding for one meal (on day 11) as a function of social status for control and 700 µmol L^{-1} total ammonia hierarchies. Values are means ± S.E.M. There is no significant overall influence of treatment between control and 700 µmol L^{-1} total ammonia hierarchies (P = .871). There is a significant overall influence of social rank (P < .001). There is no significant interaction effect (P = .899). (Control N = 7; 700 µmol L^{-1} total ammonia N = 6; two-way ANOVA; post-hoc Fisher LSD test). Different letters denote specific significant differences (P < .05) among ranks within a treatment. Asterisks denote significant differences (P < .05) between treatments within a social rank.

more similar SGR (1.7% per day), and within the same ranks, values were almost identical between treatments in rank 2, 3, and 4 animals. Notably, negative growth (-0.6 to −0.7% per day) was now seen only in rank 4 fish of both treatments.

3.4.2. Percent change in condition factor

Initial condition factor averaged 0.9699 ± 0.0380 (N = 52). Changes in condition factor (Fig. 5) with respect to time and treatment more or less paralleled those in SGR (Fig. 4). In period 1, there was a significant overall difference in percent change in condition factor between control and ammonia hierarchies, as well as a significant overall effect of rank (Fig. 5A). There was no significant interaction effect. In the controls, condition factor increased by 12.6% over 5 days, and this was significantly higher than in ranks 2, 3, and 4 where the changes in condition factor were close to zero. In the ammonia groups, the increase in condition factor was only about 4% per day in rank 1 and 2 trout, and this dropped significantly to −5.1% and −9.3% in ranks 3 and 4. Similar to the pattern for SGR, rank-specific differences between the treatments were significant only for rank 4 fish.

In period 2, there was no longer a significant overall difference between control and ammonia groups in percent change in condition factor, which had essentially caught up to the control level in the ammonia treatment (Fig. 5B). However, a significant overall effect of rank persisted regardless of the exposure. There was no significant interaction effect. Now only rank 4 fish exhibited a negative change in condition factor (−1.4 to − 4.1%) which was significantly lower than in rank 1 trout (6.6–8.6%) Fish of ranks 2 and 3 exhibited small increases in condition factor (2.4–4.8%) in both treatments.

3.4.3. Percent feeding

Feeding was quantified on day 11, the final day of the experiment. Ammonia exposure did not affect the amount of food consumed on this day, as the entire meal was eaten in both treatments. However, there was a significant effect of rank, together with a similar pattern in the two treatments (Fig. 6). There was no significant interaction effect. Rank 1 fish consumed by far the largest proportion of the single meal (~70%), and this was significantly greater than ranks 2 (~20%), 3 (~8%) and 4 (~2%). The percentages of the meal consumed by the latter three ranks were not significantly different from one another in either treatment.

3.4.4. Oxygen consumption

Overall, at the end of period 1 (day 5), MO_2 tended to be lower in the ammonia hierarchies relative to the control treatments, a difference which just escaped significance (P = .056) (Fig. 7A). There was no significant overall influence of social rank. Control rank 1 fish exhibited an MO_2 of 13.0 µmol $g^{-1}h^{-1}$, which was not significantly different than rates of 20–25 µmol $g^{-1}h^{-1}$ in control ranks 2, 3, and 4. In the ammonia treatment, MO_2 was similar in the four ranks at 11–15 µmol $g^{-1}h^{-1}$. There was no significant interaction effect.

At the end of period 2 (day 10), there were no significant overall effects of either ammonia exposure or social rank on MO_2, and there was no significant interaction effect (Fig. 7B). Rates tended to be lower in rank 1 fish, and slightly lower in the ammonia treatment, with values ranging from 13.7 to 24.0 µmol $g^{-1}h^{-1}$ in control hierarchies and 13.6–19.5 µmol $g^{-1}h^{-1}$ in ammonia hierarchies, but there were no significant differences.

3.4.5. Ammonia excretion

At the end of period 1 (day 5), M_{Amm} tended to be higher and more variable in the ammonia hierarchies relative to the control treatments, but there was no significant overall effect of ammonia exposure (P = .160) (Fig. 8A). There were also no significant differences among ranks regardless of exposure, and no significant interaction effect.

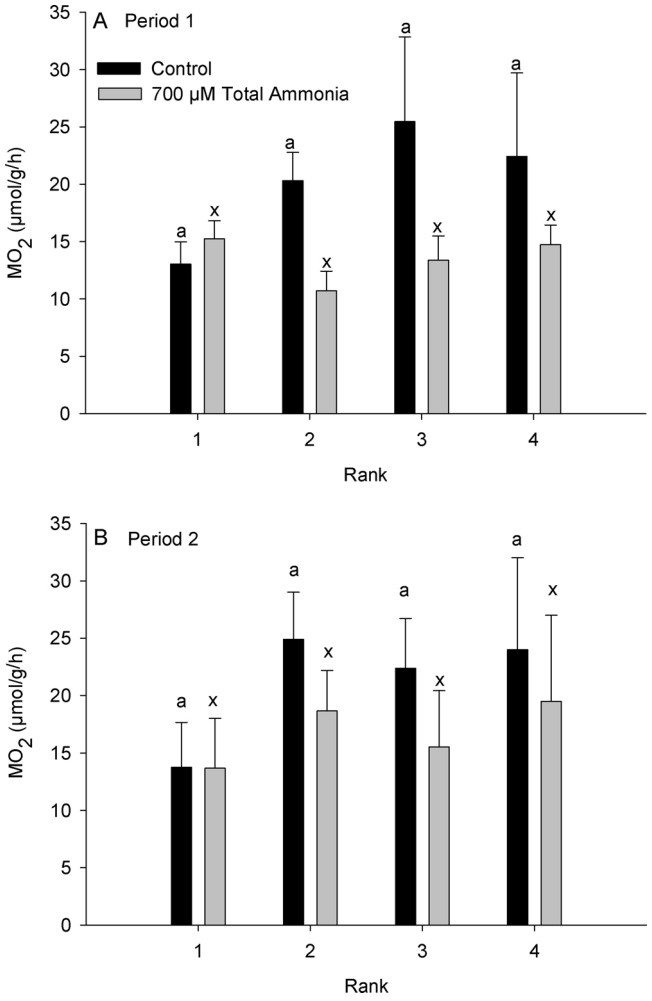

Fig. 7. Oxygen consumption rates (MO_2) as a function of social status for control and 700 µmol L^{-1} total ammonia hierarchies during period 1 and period 2. Values are means ± S.E.M. Period 1: there is no significant overall influence of treatment between control and 700 µmol L^{-1} total ammonia hierarchies (P = .056). There is also no significant overall influence of social rank (P = .798). There is no significant interaction effect (P = .199). Period 2: there is no significant overall influence of treatment between control and 700 µmol L^{-1} total ammonia hierarchies (P = .222). There is also no significant overall influence of social rank (P = .349). There is no significant interaction effect (P = .903). (Control N = 7; 700 µmol L^{-1} total ammonia N = 6; two-way ANOVA; post-hoc Fisher LSD test). Different letters denote specific significant differences (P < .05) among ranks within a treatment. Asterisks denote significant differences (P < .05) between treatments within a social rank.

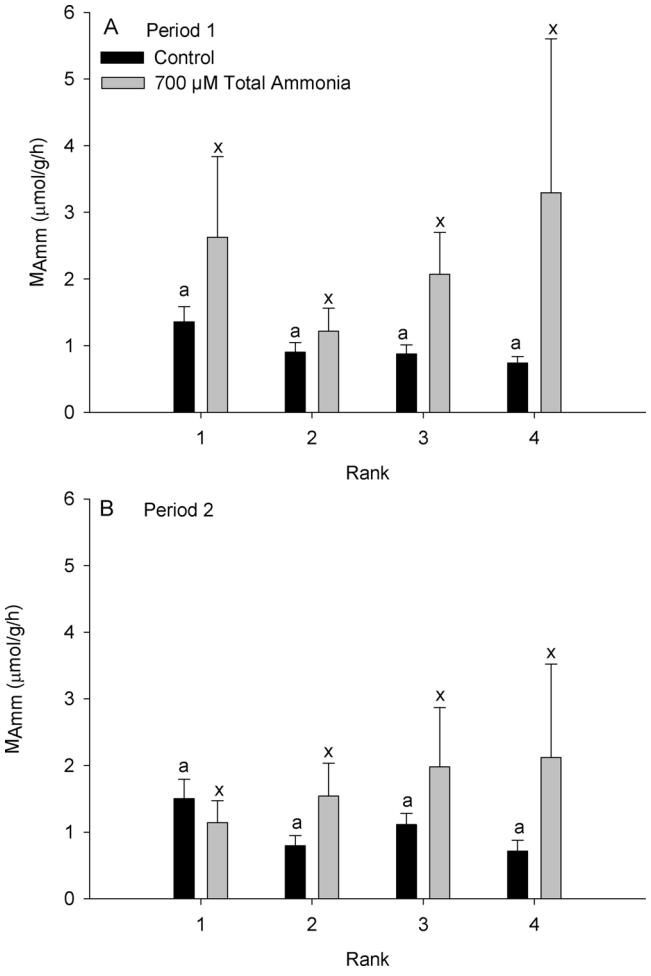

Fig. 8. Ammonia excretion rates (M_{Amm}) as a function of social status for control and 700 µmol L^{-1} total ammonia hierarchies during period 1 and period 2. Values are means ± S.E.M. Period 1: there is no significant overall influence of treatment between control and 700 µmol L^{-1} total ammonia hierarchies (P = .160). There is also no significant overall influence of social rank (P = .671). There is no significant interaction effect (P = .755). Period 2: there is no significant overall influence of treatment between control and 700 µmol L^{-1} total ammonia hierarchies (P = .691). There is also no significant overall influence of social rank (P = .420). There is no significant interaction effect (P = .538). (Control N = 7; 700 µmol L^{-1} total ammonia N = 6; two-way ANOVA; post-hoc Fisher LSD test). Different letters denote specific significant differences (P < .05) among ranks within a treatment. Asterisks denote significant differences (P < .05) between treatments within a social rank.

M_{Amm} values ranged from 0.7 to 1.4 µmol $g^{-1}h^{-1}$ in control hierarchies and 1.2–3.3 µmol $g^{-1}h^{-1}$ in ammonia hierarchies.

At the end of period 2 (day 10), there was again no significant overall effects of either ammonia exposure or social rank on M_{Amm}, and no significant interaction effect. However in ranks 2, 3, and 4, M_{Amm} again tended to be higher and more variable in ammonia hierarchies than in the controls (Fig. 8B). Control rates ranged from 0.8–1.5 µmol $g^{-1}h^{-1}$, while rates in the ammonia-exposed fish ranged from 1.1 to 2.1 µmol $g^{-1}h^{-1}$.

3.4.6. Plasma cortisol

Plasma cortisol was quantified 1 h after feeding on day 11, the final day of the experiment. Overall, there was a significant effect of ammonia exposure on plasma cortisol, with ammonia hierarchies having lower concentrations compared to control hierarchies (Fig. 9). There was no overall effect of social rank, but cortisol concentrations tended to be higher and more variable in the less dominant social ranks of both treatments. There was no significant interaction effect. Up to 4-fold rank-specific differences between treatments were significant for ranks 1, 2, and 3, with cortisol concentrations ranging from 59 to 130 ng ml^{-1} in the control hierarchies, and only 17–36 ng ml^{-1} in the ammonia hierarchies. Rank 4 fish in the two treatments had similar cortisol concentrations (~ 100 ng ml^{-1}) which were high and variable.

4. Discussion

4.1. Overview

This is the first study, to the authors' knowledge, to investigate the effect of high external ammonia on complex social hierarchies in salmonids, and the physiology of the fish therein. The results clearly confirm the original hypotheses. (i) Sublethal ammonia reduced or abolished the formation of dominance hierarchies in juvenile rainbow trout in a concentration-dependent fashion (Fig. 1). (ii) The overall intensity of aggression was reduced (Figs. 2, 3A), even at an ammonia level (700 µmol L^{-1}) which allowed the hierarchies to still be

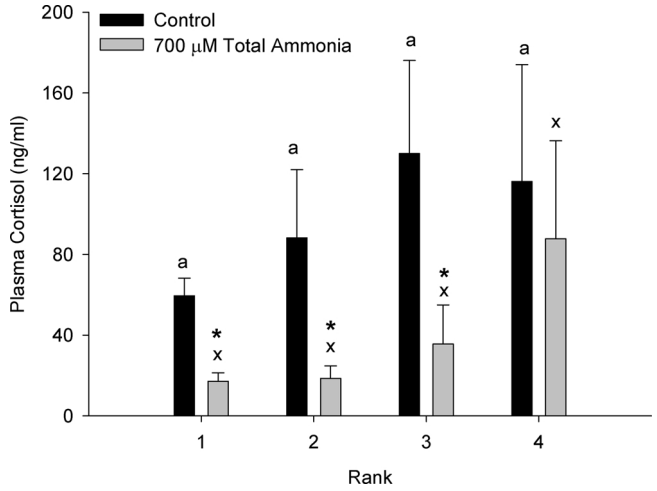

Fig. 9. Plasma cortisol based as a function of social status for control and 700 μmol L^{-1} total ammonia hierarchies. Values are means ± S.E.M. Different letters denote significant differences ($P < .05$). There is a significant overall influence of treatment between control and ammonia hierarchies ($p < .001$). There is no significant overall influence of social rank ($p = .287$). There is no significant interaction effect ($P = .769$). (Control N = 6; 700 μmol L^{-1} total ammonia N = 6; two-way ANOVA; post-hoc Fisher LSD test). Different letters denote specific significant differences ($P < .05$) among ranks within a treatment. Asterisks denote significant differences ($P < .05$) between treatments within a social rank. Control cortisol data have been previously reported by Grobler and Wood (2013).
Aggression was severely reduced

established. Nevertheless, the hierarchies remained stable. (iii) For most physiological parameters, ammonia exposure (700 μmol L^{-1}) reduced differences among members of different rank – i.e. there was an overall leveling effect. These included SGR (Fig. 4A), percent increase in condition factor (Fig. 5A), MO_2 (Fig. 7A) and plasma cortisol (Fig. 9). An additional finding of interest was that many of these effects of ammonia (decreased aggression, decreased growth and condition factor, depressed MO_2) attenuated over time, such that differences from the control patterns in period 2 were less than in period 1. These results suggest that acclimation to high ammonia occurred, in terms of both behavior and physiology.

4.2. Environmental relevance

Significant hierarchy disturbance (reduced aggression, altered physiology of members of different rank) was seen at a total ammonia concentration of 700 μmol L^{-1}at a mean pH = 7.3 and temperature = 12 °C. This represents 9.80 mg L^{-1} as total ammonia-N, or 0.0515 mg L^{-1} as NH_3-N (unionized ammonia-N). The most recent ammonia criterion of the United States Environmental Protection Agency (USEPA) at this pH and temperature, calculated using the online calculator accompanying the USEPA (2013) document, is 17.5 mg L^{-1} total ammonia–N for acute toxicity in waters where salmonids are present, and between 2.21 and 10.72 mg L^{-1} total ammonia -N for chronic toxicity depending on the presence or absence of freshwater mussels and fish early life stages. The criterion at this pH and temperature of the Canadian Council of Ministers of the Environment (CCME, 2000), which is for protection against chronic toxicity, is 6.33 mg L^{-1} as total ammonia-N. In estuaries subject to anthropogenic inputs, levels exceeding 10 mg L^{-1} total ammonia -N have been observed (Eddy, 2005), and in salmonid aquaculture, peak values of 4–9 mg L^{-1} total ammonia -N (or NH_3-N equivalences) are common occurrences (Soderberg, 1985; Colt, 2006; Hjeltnes et al., 2012). Clearly, disruption of hierarchies and associated physiological disturbances will occur at ammonia concentrations within the range of environmental relevance and regulatory concern. In practical terms, when fish are periodically size-sorted in aquaculture, they are routinely introduced into a new tank with conspecifics with whom they have no prior experience, and this new tank often has elevated ammonia concentrations. In the wild, when fish get trapped in pools during times of low water flow, ammonia levels are elevated and new hierarchies must again form. At least from an aquacultural perspective, the lower aggression and cortisol levels observed in ammonia-exposed fish might be considered an advantage, especially given that growth rates in these fish rebounded to control levels after just a few days of ammonia exposure.

4.3. Physiology as a function of social rank

The physiological differences seen among different social ranks in the control treatment were typical of those previously reported in salmonid hierarchies, with dominant individuals displaying higher growth rates, higher food consumption, lower routine MO_2, and lower cortisol levels, all indicative of better physiological performance and lower stress levels (Abbot and Dill, 1985; Peters et al., 1988; Pottinger and Pickering, 1992; McCarthy et al., 1992; Gregory and Wood, 1999; Sloman et al., 2001b, Gilmour et al., 2005). In general, in the present study, the differences between ranks 1 and 2 were greatest, while ranks 2, 3, and 4 exhibited fairly similar physiological profiles, even though their ranking based on aggression scores remained very stable over time. This same similarity in ranks 2, 3, and 4 was seen in a previous study from our lab (Grobler and Wood, 2013) using similar methods.

4.4. The nature of ammonia's effects on dominance hierarchies relative to those of other environmental stressors

Ammonia exposure (700 μmol L^{-1}) significantly reduced the overall level of aggression (Figs. 1, 2, 3) but did not alter the stability of dominance hierarchies. In general, both increases and decreases in aggression levels have been reported with different toxicants and environmental perturbations (reviewed by Atchison et al., 1996; Scott and Sloman, 2004; Sloman and Wilson, 2006). Often aggression increases in dominant individuals and decreases in subordinate ones, but this was not observed with ammonia in the present study, where aggression decreased in all social ranks. Tudorache et al. (2008) studied pairs of brown trout, and reported that after exposure to a 10-fold lower concentration of total ammonia at comparable pH for 96 h, the dominant individual exhibited less aggressive behavior but remained dominant, whereas the subordinate individual never exhibited any aggressive behavior, a somewhat similar pattern to that of the present study. In other investigations, moderate to complete reorganization of social status has been seen, caused by physical disturbances such as lowered water levels (Sloman et al., 2001b; Sneddon et al., 2006), increased water turbulence (Sneddon et al., 2006), and hypoxia (Sneddon and Yerbury, 2004), as well as by insecticide (fenitrothion) exposure (Symons, 1973). On the other hand, disruption of hierarchy structure did not occur with sublethal metal exposures (copper, zinc, nickel, lead: Sloman et al., 2003a; cadmium, lead: Sloman et al., 2005), similar to the situation in the present study. One important difference of the present study from some (but not all) of these previous investigations is that it evaluated the effect of the toxicant on the original formation and initial aggression levels of the hierarchy, rather than the toxicant's effect on previously formed hierarchies. The latter is of interest for future study. Clearly, different types of stressors differ in whether they increase or decrease aggression levels, and in their specific effects on hierarchy structure, and these may relate to their different physiological effects. As discussed subsequently (Section 4.5), the overall decrease in aggression levels yet stable hierarchy structure caused by ammonia may be explained by some of this toxicant's known effects on physiology.

4.5. The physiological effects of ammonia in dominance hierarchies

Decreased swimming ability was likely an important cause of the changes in social interactions seen during ammonia exposure. Notably, Tudorache et al. (2008) noted a decrease in fast start performance in ammonia-exposed brown trout. This would decrease the capacity of the fish to perform aggressive acts, without affecting their motivation, thereby explaining decreased overall levels of aggression without affecting hierarchy stability, as well as the trend for decreased MO_2 (Fig. 7). It is well established that ammonia exposure and/or internal ammonia accumulation decreases both spontaneous swimming activity and overall swimming performance in fish (e.g. Beaumont et al., 1995; Israeli-Weinstein and Kimmel, 1998; Shingles et al., 2001; Wicks et al., 2002; Tudorache et al., 2008; Madison et al., 2009; Tudorache et al., 2010). High total ammonia in the water leads to elevated ammonia in the blood plasma. From here, ammonia also accumulates in the muscle tissue (Tudorache et al., 2010), which can disrupt muscle function by multiple mechanisms. High intracellular NH_4^+ can cause muscle depolarization (Beaumont et al., 1995; Beaumont et al., 2000a,b; Wicks et al., 2002). Ammonia can also affect neurological function by substituting NH_4^+ for K^+ (Cooper and Plum, 1987). At a biochemical level, ammonia can stimulate phosphofructokinase which will increase the rate of glycolytic flux, reducing glycogen reserves, and affecting the potential for anaerobic work (Beaumont et al., 2000b; McKenzie et al., 2003). Ammonia can also limit aerobic metabolism by inhibiting various steps of the citric acid cycle (Lai and Cooper, 1991).

Lower growth rate and lower percent increases in condition factor were observed in the ammonia hierarchies (Figs. 4 and 5) during period 1, though overall metabolic costs were if anything lower, as indicated by the tendency for lower MO_2 (Fig. 7). Madison et al. (2009) similarly recorded lower routine MO_2 in ammonia-exposed walleye. Other investigations have demonstrated that a reduction in food consumption may also contribute to lower growth rates in ammonia-exposed fish (Person-Le Ruyet et al., 1997; Foss et al., 2003; Foss et al., 2004; Wicks and Randall, 2002a; Ortega et al., 2005). This was not apparent in the single feeding measurement made on day 11 of the present study where the entire meal was consumed (Fig. 6), but anecdotally, we observed decreased food consumption in the ammonia treatments in period 1, an effect that disappeared in period 2.

Changes in percent increases in condition factor (Fig. 5) paralleled those in SGR (Fig. 4), in contrast to the possibility of reduced SGR without alteration in percent increases in condition factor. This indicates that the deleterious effects of ammonia affected not only weight gain, but the allometry of the relationship between growth and body length such that weight impacts were greater than length impacts. Reduced condition factor is thought to reflect reduced nutritional or energy status of the fish (Barton et al., 2002) and may also suggest that maturation is being delayed by ammonia (Thorpe et al., 1998).

The marked depression in plasma cortisol concentrations in ammonia-exposed trout of all social ranks (Fig. 9) was surprising, in light of the well-known effects of ammonia in elevating plasma cortisol in salmonids (Knoph and Olsen, 1994; Wicks and Randall, 2002b; Ortega et al., 2005; Tsui et al., 2009). One possible interpretation is that effects of reduced aggression in lowering plasma cortisol are stronger than the effects of ammonia in raising it. However, Sloman et al. (2001b) reported that a chronic physical stressor (lowered water levels) tended to reduce plasma cortisol concentrations in hierarchies of brown trout, while Madison et al. (2009) noted reduced cortisol levels in walleye chronically exposed to low concentrations of ammonia. Whether this reflects the reduced aggression noted above, a “calming” effect associated with reduced activity (Madison et al., 2009), an impaired ability to mobilize cortisol after prolonged exposure to a sublethal stressor (e.g. Pickering and Pottinger, 1987; Hontela et al., 1992; Tellis et al., 2012), or true acclimation to ammonia (Section 4.6) remains to be determined. Alterations in brain monoaminergic activity play an important role in modulating social behavior in salmonids (e.g. Winberg and Nilsson, 1993; Gilmour et al., 2005), so in future studies it would be of interest to monitor this in individuals of different social status during chronic ammonia exposure.

In freshwater fish, it is now generally accepted that ammonia movement through the branchial epithelium is facilitated by Rh glycoproteins (Nakada et al., 2007; Nawata et al., 2007; Wright and Wood, 2009), and there is evidence that cortisol increases the expression levels of the gill Rh proteins (Nawata and Wood, 2009; Tsui et al., 2009). These Rh proteins transport ammonia bidirectionally, as a function of prevailing gradients (Nawata et al., 2010; Lim et al., 2015), so greater ammonia loading could be associated with higher levels of cortisol. In future studies, simultaneous measurements of plasma total ammonia and cortisol concentrations in dominant and subordinate individuals should cast light on this issue. Increased toxicant uptake has been seen previously in subordinates exposed to other toxicants, for example both copper and silver, which are analogues of sodium (Sloman et al., 2002; Sloman et al., 2003b). The authors attributed this effect to increased rates of branchial Na^+ uptake associated with increased diffusive losses of this ion in subordinates.

4.6. Behavioral and physiological acclimation to ammonia

It appears that ammonia hierarchies ‘rebounded’ in period 2, as aggression levels (Fig. 3), as well many physiological parameters, including growth (Fig. 4), condition factor (Fig. 5), and MO_2 (Fig. 7), were partially or completely restored to control levels. Previous studies have shown that during prolonged ammonia exposure, fish make physiological adjustments over time such as up-regulated ammonia detoxification mechanisms (Wicks and Randall, 2002b; Wright et al., 2007; Zhang et al., 2013), up-regulated excretory processes (Nawata et al., 2007; Tsui et al., 2009), decreased ventilatory disturbance (Zhang et al., 2011; Zhang et al., 2013), reductions in cortisol levels (Ortega et al., 2005; Madison et al., 2009), and restoration of feeding and growth (Dosdat et al., 2003). The present results suggest that acclimation to chronic ammonia exposure occurs not only in physiology, but also in social behavior.

Acknowledgements

Supported by an NSERC (Canada) Discovery grant to CMW. We thank Drs. Sigal Balshine, Kath Sloman, Jim Quinn, and Kevin Stiller for advice, Sunita Nadella and Linda Diao for technical support, and two anonymous reviewers for constructive comments that improved the paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aquatox.2017.12.006.

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