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Dogmas and controversies in the handling of nitrogenous wastes: Is exogenous ammonia a growth stimulant in fish?

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Summary

Traditionally, waterborne ammonia is considered a toxicant that decreases productivity in aquaculture. However, several recent studies have suggested, but not proven, that growth of salmonids might actually be stimulated by chronic exposure to very low levels of ammonia. In the present study, two 70–71 day growth experiments were conducted under rigorously controlled experimental conditions with juvenile rainbow trout at total ammonia concentrations ($[T_{Amm}]$)=0, 70 and 225 μmol l^{-1}, pH 7.6. In the first series, a small-scale laboratory proof-of-principle study at 15°C, there was a significant stimulation of mass gain, gross food conversion efficiency, condition factor and protein production per fish at $[T_{Amm}]$=70 μmol l^{-1}, without an increase in voluntary food consumption or change in 'in-tank' O_2 consumption or ammonia excretion rates. These growth stimulatory effects were not seen at $[T_{Amm}]$=225 μmol l^{-1}, where the fish consumed more food, and excreted more ammonia, yet achieved the same mass and protein content as the controls. In the second series, a larger study conducted in an aquaculture facility at 6.5°C, growth rate, conversion efficiency and protein production per fish over 71 days were all significantly stimulated at $[T_{Amm}]$=225 μmol l^{-1}, but not at 70 μmol l^{-1}, without any change in voluntary food consumption. These effects occurred despite an early inhibition of growth at both $[T_{Amm}]$ levels. When ration was restricted, growth was reduced and there were no longer any differential effects attributable to $[T_{Amm}]$. While the effective levels of $[T_{Amm}]$ differed between the two series, in both, the P_{NH_3} level stimulating growth was ~23 μtorr. The results are interpreted as reflecting either a stimulation of ammonia incorporation into amino acids and protein synthesis and/or a reduction in metabolic costs. The finding that low levels of exogenous ammonia can serve as a growth stimulant without altering food consumption may be important for aquacultural practice, and challenges traditional dogma that the effects of ammonia are detrimental to growth.

Key words: sublethal ammonia, salmonids, protein, specific growth rate, conversion efficiency.

Introduction

Traditionally, ammonia has been considered as a toxicant in aquaculture with negative effects on the general health of the fish, their susceptibility to disease and other stressors, and most importantly on growth (e.g. Robinette, 1976; Beamish and Tandler, 1990). Indeed, there has been much debate on the relative roles of NH_3 and NH_4^+ in causing toxicity, on the acceptable or 'safe' levels for total ammonia and these components, and on the water flow requirements, water chemistry, feed formulation and feeding rations needed to ensure that such levels are not exceeded. Excellent reviews of the earlier literature are provided by Alabaster and Lloyd (1980), Haywood (1983), Meade (1985) and Tomasso (1994), and critical analysis of more recent findings by Ip et al. (2001) and Randall and Tsui (2002). However, almost all of the research performed has been directed at determining the thresholds for acute or chronic toxic effects, and therefore has looked for the 'no effect' level, i.e. the concentration below which toxicity does not occur. Overlooked until recently was the possibility that ammonia at even lower levels may actually have positive effects on productivity in aquaculture.

In theory, it seems possible that exogenous ammonia could stimulate protein synthesis and therefore growth if it could be taken up by the fish and incorporated into amino acids, or if it caused endogenous ammonia to 'back up' inside the fish and be similarly incorporated. Certainly metabolic pathways exist in fish whereby ammonia incorporation could occur – for example, glutamate dehydrogenase will 'fix' ammonia onto alpha-ketoglutarate to form glutamate, and in turn glutamine synthetase will 'fix' ammonia onto glutamate to form glutamine. While this is a well known pathway for detoxifying high ammonia (Randall and Tsui, 2002), it is often overlooked that the amino groups can be transferred from glutamate by transamination (e.g. alanine amino transferase, aspartate amino transferase) to form other amino acids if an excess of carbon skeletons (e.g. pyruvate, oxaloacetate) is available (Wood, 1993). The evidence that this actually occurs in fish is rather

limited; however, Iwata et al. (2000) reported that ^{15}N from exogenous (waterborne) ammonia is incorporated into amino-N and amide-N in the liver and muscle of the goby, and Ip et al. (2001) summarize a number of studies showing that various tropical species accumulate amino acids in their tissues during situations associated with internal ammonia retention. Perhaps most convincing is the report of Hayashi et al. (1993) that eel hepatocytes in culture grew better and produced more protein when ammonium chloride was added to the culture medium.

Further anecdotal support for this concept comes from a series of studies simulating the effect of global warming on the physiology of rainbow trout, in which +2°C was superimposed on the natural thermal cycle, in the presence and absence of sublethal pollutants (reviewed by Morgan et al., 2001). One of these pollutants was ammonia, at the very low level of 70 μmol l^{-1}. In general, there appeared to be a stimulatory effect of the ammonia treatment on growth that was statistically significant in some studies (Linton et al., 1997, 1999), and below significance in others (Linton et al., 1998a,b), though interpretation was somewhat confounded by the fluctuating thermal regime, and by variations in ration and season.

With this background in mind, the goal of the present study was to rigorously test whether low levels of ammonia would stimulate growth and protein production of juvenile rainbow trout under chronic exposure conditions where food consumption was monitored, and temperature and environmental ammonia concentrations were carefully controlled. Two experimental series were performed, each ten weeks in length, at the same two low ammonia levels (70 and 225 μmol l^{-1}) in each. The first, a small scale 'proof-of-principle' experiment, was conducted in our laboratory, using a relatively small number of fish. The second, a more ambitious experiment with many more fish and two different feeding regimes, was conducted at an aquaculture facility. Differences in temperature between the two sites provided an opportunity to examine whether temperature influenced the response. The results clearly demonstrate that elevated environmental ammonia can stimulate growth and protein production without an increase in food consumption.

Materials and methods

Series 1

Approximately 200 juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum, mass 2–5 g) from Humber Springs Trout Farm (Orangeville, ON, Canada) were held indoors at McMaster University (Hamilton, ON, Canada) in a 270 liter polyethylene tank continuously supplied with aerated, dechlorinated Hamilton tapwater (from Lake Ontario; ion concentrations in mmol l^{-1}: Ca^{2+} 1.0, Mg^{2+} 0.30, Na^{+} 0.6, Cl^{-} 0.7, K^{+} 0.05, pH 7.6) at a temperature of 12±1°C. Light conditions were controlled to mimic the natural autumn photoperiod for Hamilton. Fish were held under these conditions for 31 days, and were hand-fed to satiation every 2–3 days with Zeigler's Trout Starter #3 (protein >50%, lipid >15%, moisture 12%; Zeigler Brothers, Inc., PA, USA).

The experiment was run over a 70-day period starting in mid-November. Following acclimation, groups of 12 fish were randomly selected and transferred to each of 12 experimental 17.7 l polyethylene tanks on day –2, and allowed to settle for 48 h. Half of the fish (6) in each tank were lightly anaesthetized (MS-222 0.1 g l^{-1}, adjusted to pH 7.5 with NaOH) and freeze-branded for individual identification. To calculate growth rates of these individually marked fish, their wet mass (0.01 g accuracy using a Sartorius bench scale; Gottingen, Germany) and total length (to 1 mm accuracy) were measured on days 0, 31, 52 and 70 following light anaesthesia by MS-222. In addition, the bulk mass (total biomass) of all fish in each tank was measured weekly as part of the protocol for the measurement of O_2 consumption and N-waste excretion rates (see below). This allowed us to monitor whether the periodic handling of the marked fish impacted growth. Following completion of the rate determinations, all the fish from each tank were transferred by sieve-net to a tared container of water, weighed in bulk on a GSE Scale Systems macro-balance (Detroit, MI, USA; 5 g accuracy) and thereafter returned to their individual tanks.

Each experimental tank received a 0.5 l min^{-1} flow-through of dechlorinated Hamilton tapwater heated to 15±1°C by a heat exchanger. There were three treatment groups: control, low ammonia and high ammonia (nominal total ammonia [T_{Amm}]=0, 70 and 225 μmol l^{-1}, respectively), each replicated in four different tanks. Starting on Day 0, the desired concentration was achieved by delivering the required amount of $(NH_4)_2SO_4$ stock solution *via* Mariotte bottles (Mount and Brungs, 1967) into common mixing header tanks, ensuring that each of the four replicates received identical [T_{Amm}]. No exogenous ammonia was added to the control treatment tanks. Ammonia concentrations, pH, temperature and oxygen levels were monitored at least once a week, ensuring that the latter remained >80% saturation throughout the 70-day experiment.

Fish were hand-fed to satiation once daily at 10:00 h from pre-weighed containers of Zeigler trout starter #3 following the methods of Wilson et al. (1994). In brief, separate weighed bags of food were used for each tank. Food was offered in small portions at 1-min intervals, and feeding was stopped when uneaten pellets still remained after a period of 2 min. Individual tank food bags were weighed before and after each feeding session. This allowed a determination of daily appetite (voluntary food consumption), as well as cumulative food consumption in each tank. Tanks were siphoned every morning before feeding to remove faeces, and also before the metabolic rate measurements.

Every 7 days, from weeks 1–9, measurements of 'in-tank' rates of oxygen consumption ($\dot{M}_{O_2}$) and ammonia excretion ($\dot{M}_{Amm}$) were performed in the afternoon, starting approximately 5 h after feeding. Aeration and water flow were suspended, and each of the tanks was fitted with a sealing lid. Water samples for O_2 measurements were withdrawn at 15-min intervals for 1 h (i.e. 5–6 h post-feeding), after which the lid was removed and aeration restarted but the water flow remained closed. Samples for total ammonia measurements

were made at 0 h and 3 h (i.e. 5–8 h post-feeding), after which water flow was resumed and the daily feeding was performed. Measurement blanks demonstrated that microbial $\dot{M}_{O_2}$ and $\dot{M}_{Amm}$ were negligible, so no corrections were applied.

In order to determine changes in protein content, 23 fish from the stock tank were killed on Day 0 to yield a mean initial value, and three marked fish from each experimental tank on Day 70 to yield individual final values, using an overdose of neutralized MS-222 (1 g l^{-1}). The bodies were blotted dry, weighed, frozen immediately in liquid N_2, and stored at –20°C for later protein analysis.

Series 2

Approximately 1400 rainbow trout (3–8 g) from Spring Valley Trout Hatchery (Langley, BC, Canada) were held in the aquaculture test facility, Fisheries and Oceans Canada (West Vancouver, BC, Canada) housed in an outdoor shed. Fish were acclimated for 2 weeks in two 500 liter polyethylene tanks to the facility-supplied well water (ion concentrations in mmol l^{-1}: Ca^{2+} 1.0, Mg^{2+} 0.26, Na^{+} 1.0, Cl^{-} 2.0, K^{+} 0.09, pH 6.3) at a temperature of 7–8°C. Light conditions reflected natural autumn photoperiod for West Vancouver. During the acclimation period, fish were fed to satiation every second day with Aquamax Grower 500 #2 (St Louis, MO, USA; protein ≥40%, lipid ≥10%, moisture 12%, fibre ≤5%, ash ≤10.5%, minerals ≤1.5%).

The experiment was run over a 70-day period starting at the end of November. Experimental temperature was 6.5±0.5°C. The exposure system comprised 12 exposure tanks (150 liter each), each receiving a flow-through of 4 l min^{-1}. Each tank was divided into two sections by a mesh barrier, thereby creating 24 sections in total. The barrier was designed to allow free flow of water between both sides, but to prevent mixing of fish and food particles from side to side. Thus the fish (2×54 fish) on the two sides could receive identical [T_{Amm}] but different feeding regimes. On Day –2, 54 fish were individually weighed and transferred to each section, and 16 of these fish were individually marked by inserting a passive integrated transponder (PIT) tag into their peritoneal cavities, and allowed to settle for 48 h. We employed 12 mm identity tags (TX1400L, 125 kHz) together with the portable transceiver system (FS2001) from the same manufacturer (Destron Fearing, MN, USA).

Weights were then measured for the 16 marked fish in each tank on Days 1, 8, 15, 22, 29, 36, 43, 50, 57, 64 and 71, using an Ohaus bench scale (Pine Brook, NJ, USA; 0.1 g accuracy). The procedure was performed quickly, without anaesthesia. The weights of all fish in every tank were also measured on Day –2 and Day 70 by the same procedure, which allowed us to evaluate whether the weekly handling of the marked fish impacted growth.

Each of the 12 tanks was randomly chosen for exposure to one of three ammonia concentrations (nominally 0, 70 and 225 μmol l^{-1}), with four replicates for each. Starting on Day 0, the desired concentrations were achieved by delivering the required amount of NH_4Cl stock solution *via* separate Mariotte bottles into each tank. No exogenous ammonia was added to the four control tanks, but every tank received an addition of NaOH from the separate Mariotte bottles so as to maintain pH at ~7.6, the same level as in Series 1. Exposures lasted 70 days. Water pH, temperature and total ammonia concentration were monitored on a daily basis in each experimental tank, and oxygen level on a weekly basis to ensure that saturation remained >80%.

Fish in the front halves of each tank were fed to satiation (with Aquamax Grower 500 #2) every morning as described for Series 1, thereby allowing measurement of appetite and daily food consumption. In contrast, those in the back halves were fed a fixed ration. Rationed diets were calculated as 2% of body mass per day based on the mean mass of the fish in control tanks that were measured every 7 days. This food was delivered as a single bolus to the tank. While this was initially intended to standardize food consumption in all of the fixed ration tanks, in practice, the 2% ration delivered in this manner turned out to be more than the fish would voluntarily consume at this experimental temperature, because this brand is a slowly sinking food. Thus, a significant but unquantified portion was lost by sinking, and was removed by daily siphoning, performed at 17:00 h each day.

In order to determine differences in protein content, 20 fish from the stock tanks were killed and stored as in Series 1 on Day 0 for initial values, plus five unmarked fish from each of the experimental tanks on Days 35 and 70, to yield intermediate and final values.

Analytical techniques

In both Series 1 and 2, water ammonia concentrations were measured by the colorimetric assay of Verdouw et al. (1978). Water pH was monitored using a Radiometer GK2401C (Copenhagen, Denmark) glass combination electrode and pHM 82 meter in Series 1 and a Corning (NY, USA) sealed body combination electrode and portable meter in Series 2. Water O_2 levels were measured in Series 1 (as P_{O_2}) using a Cameron Instruments O_2 electrode (Port Avansis, TX, USA) connected to a Cameron OM 200 oxygen meter, and in Series 2 (as % saturation) using a YSI 85 O_2 combination probe and meter (Ohio, USA). In Series 1, whole-body protein concentration was determined using the Lowry method as modified by Miller (1959). In Series 2, the Bradford (1976) method was employed using Sigma reagents (St Louis, MO, USA; Kit B6916). In both series, Sigma protein standard solution (2 mg ml^{-1} bovine serum albumin) was used to construct standard curves. In addition, an internal control of the same powdered trout whole-body pool was run with every assay to adjust for any day-to-day variability. Frozen carcasses were initially ground into a fine powder using a grinding mill (IKA–M10/M20; Wilmington, NC, USA) cooled to approximately –40°C by a methanol/dry ice mixture. In Series 1, a small portion of this powder was dried in an oven at 65°C for 56 h to obtain the water content, and the remainder was lyophilized for 72 h at –55°C for the measurement of other parameters (not reported) in addition to protein. In Series 2,

protein assays were conducted directly on frozen, non-lyophilized powder as no other parameters were to be measured. Protein concentrations were calculated on a wet body mass basis in both series.

Calculations and statistical analysis

Daily food intake was calculated by dividing the total amount fed per tank (Series 1) or per section (Series 2) by the total mass of fish present to express it on a % ration basis [(g food g^{-1} fish)×100)] or by the total number of fish present to express it on an appetite basis (g food $fish^{-1}$). Daily addition of the latter values yielded cumulative food consumption per fish.

Gross food conversion efficiency (CE) was estimated for each tank (Series 1) or section (Series 2) by dividing the total mass gained per fish (mean value per fish for the tank/section) by the cumulative food consumption per fish in that tank/section, and adjusting to a % basis.

Specific growth rate (SGR, % day^{-1}) over an interval (T, days) of time 1 to time 2 were calculated from the measurements of body mass (M_b, g) for each individual marked fish as:

$$\mathrm{SGR} = (\ln M_{b2} - \ln M_{b1}) / T \times 100 . \quad (1)$$

Condition factors (CF) were determined for each individual marked fish (Series 1 only) based on the measurements of M_b and length (L, cm):

$$\mathrm{CF} = (M_b / L^3) \times 100 . \quad (2)$$

Protein production per fish was calculated assuming that each fish started with the mean % protein measured in fish sampled from the pool on Day 0. In Series 1, as % protein was measured in individually marked fish on Day 70, the mean % protein value on Day 0 was applied to the individual Day 0 body mass of the marked fish, and the measured individual % protein value to the individual Day 70 body mass of the same fish to yield the protein production per fish. In Series 2, as % protein was measured in unmarked fish, the mean protein content per fish on Day 0 (% protein × M_b) was subtracted from the final measured protein content per individual fish (% protein × M_b) on Day 35 or 70 to yield the protein production per fish.

In Series 1, O_2 consumption rates ($\dot{M}_{O_2}$) of fish in each tank were calculated from measurements of the average rate of change of $\dot{P}_{O_2}$ values ($\Delta\dot{P}_{O_2}/T$, torr h^{-1}; 1 torr=133.3 Pa) over the 1 h sampling period:

$$\dot{M}_{O_2} = [(\Delta\dot{P}_{O_2}/T)\alpha_{O_2}V] / B , \quad (3)$$

where α_{O_2} (μmol l^{-1} $torr^{-1}$) is the solubility constant for O_2 in water at the experimental temperature (from Boutilier et al., 1984), V is the volume of water in each tank (17.7 l), and B (g) is the measured total biomass of fish in the tank.

Similarly, total ammonia excretion rates ($\dot{M}_{Amm}$) of fish in each tank were calculated from measurements of the rate of change in total ammonia concentration (T_{amm}/T, μmol l^{-1} h^{-1}) over the 3 h sampling period:

$$\dot{M}_{Amm} = (\Delta T_{Amm}/T)V / B . \quad (4)$$

To allow for valid comparisons between fish of different size, all the $\dot{M}_{O_2}$ and $\dot{M}_{Amm}$ data were mass-corrected using the mass exponent 0.824 determined for rainbow trout by Cho (1990).

Data are expressed as means ± S.E.M. (N) where N = number of fish or samples for individual measurements or number of tanks/sections for group measurements. Percentage data were transformed to normalize variances prior to analysis. Differences in feeding and growth rates were tested using an analysis of covariance (ANCOVA). For comparison of ammonia responses, if one-way analysis of variance (ANOVA) indicated significance, a multiple comparisons test was then performed to determine which treatment means were different. For comparison of measures at discrete time points, the Tukey–Kramer HSD test was used, whereas for measures integrated across time (e.g. SGR, protein production rate), the Bonferroni test was employed. Student's t-test was used to compare the same treatment means between satiation-fed and rationed fish in Series 2. In all tests, differences were evaluated at the two-tailed significance level of $P<0.05$, unless otherwise noted.

Results

Series 1

This experiment was conducted at 15±1°C. Two of the four treatment tanks in the nominal [T_{Amm}]=70 μmol l^{-1} exposure were lost early in the experiment due to water flow failure, and their data were discarded. There were no treatment-related mortalities. The average measured [T_{Amm}] was 6.6±0.6 μmol l^{-1} (N=27) for the control tanks, 88.7±2.4 μmol l^{-1} (N=14) for the low ammonia treatment group, and 198.5±6.2 μmol l^{-1} (N=28) for the high ammonia treatment group, respectively, which were reasonably close to the nominal values of 0, 70 and 225 μmol l^{-1}.

Approximately 40% of the marked fish could no longer be individually identified by Day 70 because of blurring of the freeze-brands, so only data from those fish that were positively identified at every weighing time are included in the growth plots of Fig. 1. While there were no significant differences in individual mass between treatments at any time, ANCOVA indicated a higher overall growth in the [T_{Amm}]=70 μmol l^{-1} exposure ($P<0.055$, two-tail; $P<0.028$, one-tail). This trend was also confirmed by a significantly higher mass gain per individual fish over 70 days in the low ammonia treatment group (Fig. 2A), though the SGR increase was not significant (Fig. 2B). Relative to the controls, the extra mass gain amounted to about 7 g per fish by the end of the experiment. Fish in the low ammonia treatment group also became plumper, with a significantly higher CF on Days 31, 52 (data not shown) and 70 (Fig. 2C). There was no difference between the control and high ammonia treatment groups in any of these parameters (Figs 1, 2). The weekly bulk measurements of biomass showed a very similar pattern, indicating that the data sets on the smaller numbers of individual fish were representative and not impacted by the periodic handling of the

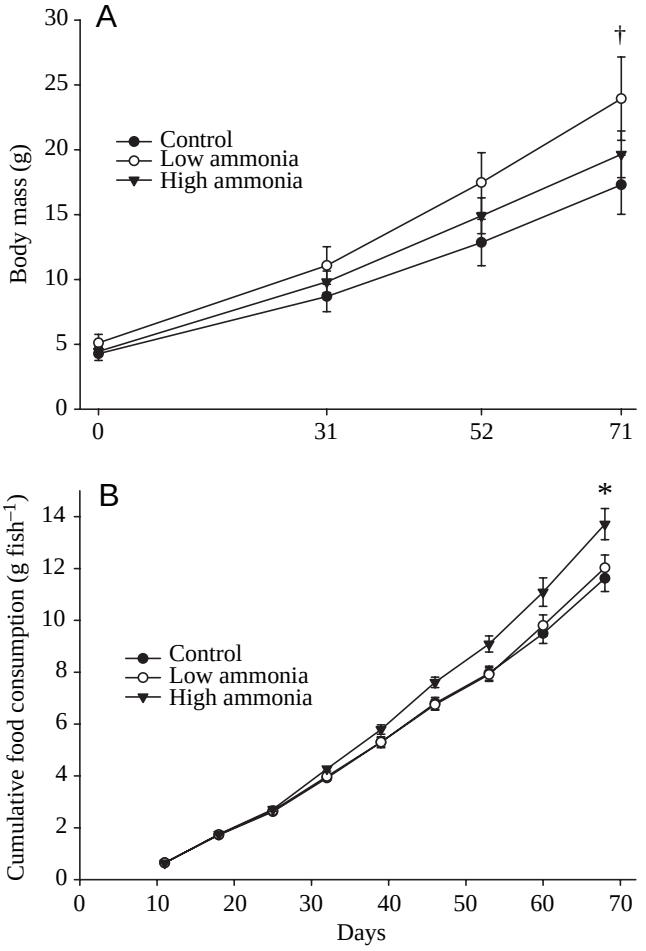

Fig. 1. (A) Body mass of individually marked fish over 70 days in the three experimental treatments of Series 1: nominal, [T_{Amm}]=0 μmol l^{-1} (control, N=13), [T_{Amm}]=70 μmol l^{-1} (low ammonia, N=7) and [T_{Amm}]=225 μmol l^{-1} (high ammonia, N=16). Values are means ±1 S.E.M. There were no significant differences at any time, but ANCOVA indicated higher overall growth ($^{\dagger}P$<0.055, two-tail; P<0.028, one-tail) in the low ammonia treatment relative to the control. (B) Cumulative food consumption per fish, measured on a tank basis (N=4 for control and high ammonia, N=2 for low ammonia) in this same series. By Day 70, cumulative food consumption in the high ammonia treatment group was significantly greater (asterisk) than in the other two treatment groups.

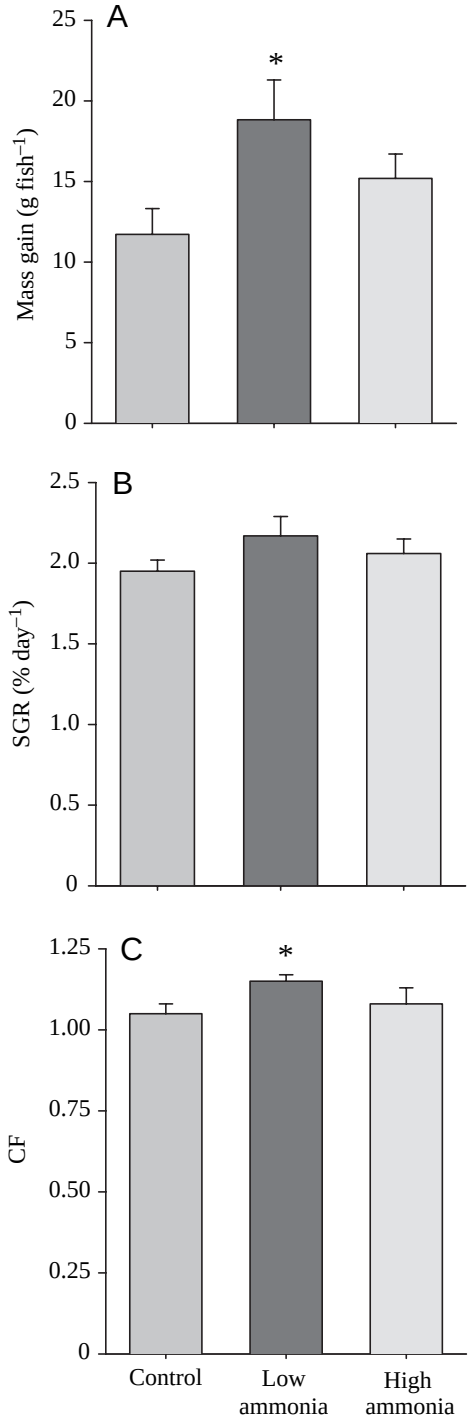

Fig. 2. Growth indices of individually marked fish over 70 days in the three experimental treatments of Series 1: nominal [T_{Amm}]=0 μmol l^{-1} (control, N=13), 70 μmol l^{-1} (low ammonia, N=7), and 225 μmol l^{-1} (high ammonia, N=16). (A) Mass gain per fish, (B) specific growth rate (SGR) and (C) condition factor (CF). Values are means ±1 S.E.M. *Significant difference from control.

fish for individual weighing. For example, mean bulk mass per fish at the end of the experiment was: control, 18.4±0.8 g (N=4), low ammonia, 23.0±0.7 g (N=2) and high ammonia, 19.6±0.5 g (N=4).

Initially, appetite was the same in all three treatment groups, i.e. all voluntarily consumed the same amount of food per fish. However, by Day 42, fish in the high ammonia treatment group started consuming more food than those in the two other treatment groups, and by Day 70, the difference in cumulative food consumption was significant, an excess of about 2 g per fish or 20% (Fig. 1B), even though these fish did not grow more (Figs 1A, 2A,B). In contrast, there was no elevation in cumulative food consumption in the low ammonia treatment group (Fig. 1B), even though these fish tended to exhibit better growth (Figs 1A, 2A,B).

This stimulatory effect of the low ammonia exposure on growth, without an increase in food consumption, was reflected

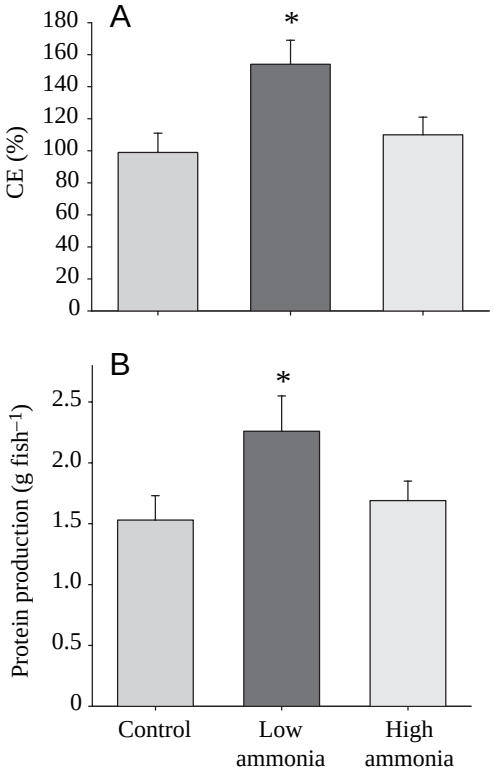

Fig. 3. (A) Gross food conversion efficiency (CE) measured on a tank basis (N=4 for control and high ammonia, N=2 for low ammonia) and (B) protein production per individual fish (N=12 for control and high ammonia, N=6 for low ammonia) over 70 days in the three experimental treatments of Series 1: nominal [T_{Amm}]=0 μmol l^{-1} (control), 70 μmol l^{-1} (low ammonia), and 225 μmol l^{-1} (high ammonia). Values are means ±1 S.E.M. *Significant difference from control.

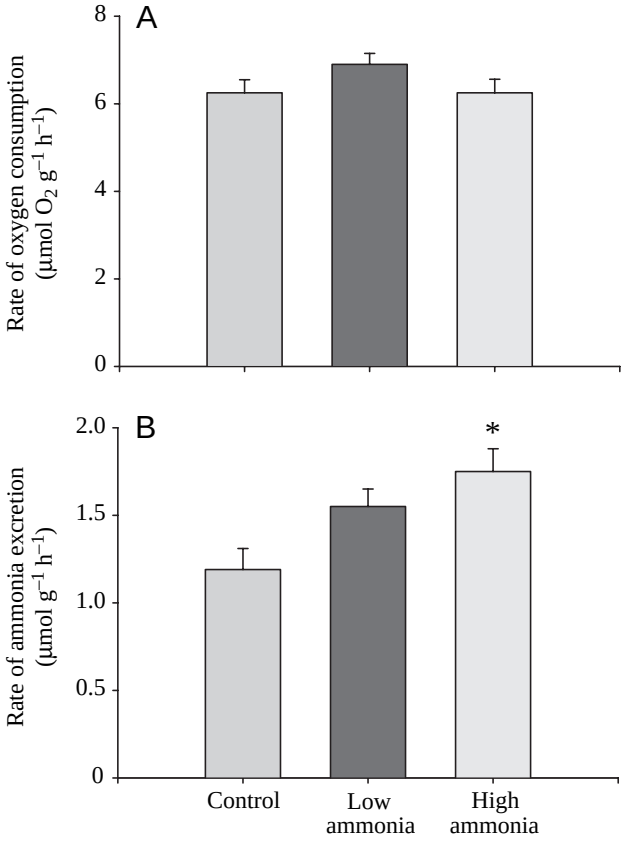

Fig. 4. Mean rates of (A) 'in-tank' O_2 consumption ($\dot{M}_{O_2}$, 5–6 h post-feeding) and (B) 'in-tank' ammonia excretion ($\dot{M}_{Amm}$, 5–8 h post-feeding) measured once per week over 9 weeks in each of the treatment tanks of Series 1 (N=36 for the control and high ammonia treatments, N=22 for the low ammonia treatment). The data were mass-corrected using the mass exponent 0.824 determined for rainbow trout by Cho (1990). Values are means ±1 S.E.M. *Significant difference from control.

in a significantly higher gross conversion efficiency (CE, measured on a per tank basis) over 70 days (Fig. 3A). Furthermore, protein production per individual fish was also significantly higher in this treatment group (Fig. 3B). All fish significantly increased their % whole-body protein from 8.70±0.21% (N=23) on Day 0 to 11.00±0.30% (control, N=12), 11.29±0.25% (low ammonia, N=6), and 10.50±0.29% (high ammonia, N=12) on Day 70. Differences among the latter were not significant.

Measured rates of 'in-tank' O_2 consumption ($\dot{M}_{O_2}$, 5–6 h post-feeding) and total ammonia excretion ($\dot{M}_{Amm}$, 5–8 h post-feeding) showed no consistent patterns over time, so all measurements were averaged for each of the treatment groups (Fig. 4). There were no significant differences in $\dot{M}_{O_2}$ related to treatment (Fig. 4A), but $\dot{M}_{Amm}$ was significantly greater in the high ammonia treatment group than in the controls (Fig. 4B). $\dot{M}_{Amm}$ in the low ammonia treatment group did not differ from the control values.

Series 2

This experiment was conducted at 6.5°C. Both sections (i.e. satiation-fed and rationed) of one of the four treatment tanks in the nominal [T_{Amm}]=225 μmol l^{-1} exposure were lost early in the experiment due to a Mariotte bottle failure (i.e. pH and T_{Amm} surge), and their data were discarded. Mortalities in all of the other sections were less than 5%, and all of the marked fish survived and retained their PIT tags throughout the 71-day experiment. The average measured [T_{Amm}] was 1.8±0.1 μmol l^{-1} (N=280) for the control tanks, 77.0±1.1 μmol l^{-1} (N=280) for the low ammonia treatment, and 238.8±4.2 μmol l^{-1} (N=210) for the high ammonia treatment, respectively (Fig. 5). These values were relatively constant over time, again close to the nominal values of 0, 70 and 225 μmol l^{-1}.

Body mass of 16 individually tagged fish per section was measured weekly (×3–4 sections per treatment), allowing a very accurate assessment of growth, as well as of all fish at the beginning and end of the experiment. Fish in all treatment groups started at the same body mass.

Within the satiation-fed treatment groups, ammonia exposure exerted complex effects on growth. Growth in the control group was virtually linear over time, whereas growth

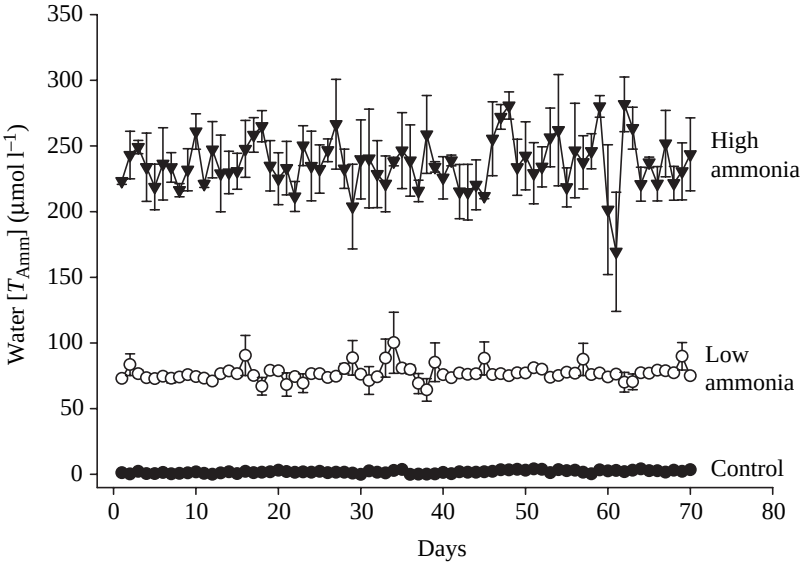

Fig. 5. Measured levels of total ammonia on each day in the nominal [T_{Amm}]=0 μmol l^{-1}, (control, N=4), 70 μmol l^{-1} (low ammonia, N=4) and 225 μmol l^{-1} (high ammonia, N=3) treatment tanks of Series 2. Values are means ±1 S.E.M.

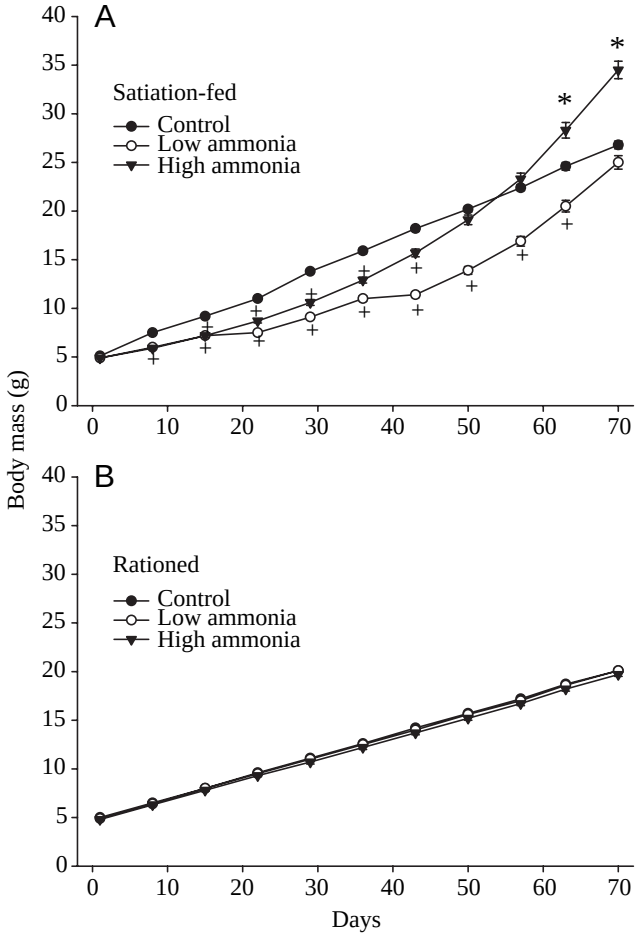

Fig. 6. Body mass of individually marked fish at weekly intervals over 71 days in the three experimental treatment groups of Series 2: nominal [T_{Amm}]=0 μmol l^{-1} (control, N=64), 70 μmol l^{-1} (low ammonia, N=64), and 225 μmol l^{-1} (high ammonia, N=48). Values are means ±1 S.E.M. (A) Satiation-fed fish, (B) rationed fish. Within A, asterisks indicate mean body mass significantly higher than the corresponding control mean, and crosses indicate mean body mass significantly lower than the corresponding control mean. ANCOVA indicated significantly higher overall growth over 71 days in the high ammonia treatment, and no significant difference in overall growth over 71 days in the low ammonia treatment relative to controls. Within B, there were no significant differences.

in the ammonia treatment groups was closer to an exponential pattern (Fig. 6A). Fish in the low ammonia treatment group exhibited significantly lower mass than controls from Day 8 through Day 64, but had caught up to the controls by Day 71. ANCOVA demonstrated that growth rate in this treatment group was depressed relative to the controls only until Day 43, but thereafter was accelerated, so that there was no overall difference. Fish in the high ammonia treatment group exhibited significantly lower body mass until Day 43, but thereafter caught up with the controls (Days 50 and 57) and then significantly surpassed the controls on Days 64 and 71. ANCOVA demonstrated that growth rate in this treatment group was depressed only until Day 29, and was thereafter accelerated so as to significantly exceed the control rate over 71 days. Relative to the controls, the extra mass gain amounted to about 8 g per fish by the end of the experiment.

These data on individually marked fish were confirmed by the measurements made on every fish at the end of the experiment, demonstrating that the weekly handling of the marked fish did not impact their growth. These measurements, where N was approximately threefold higher than in Fig. 6 [control, 26.8±0.4 g (N=191); low T_{Amm}, 25.0±0.7 g (N=189); high T_{Amm}, 34.5±0.7 g (N=147)] essentially superimpose on the final means in Fig. 6, and exhibit the same statistical pattern, i.e. significantly higher mean mass in the high ammonia treatment group, but no difference from control in the low ammonia treatment group.

Within the rationed treatment groups, there were no effects of ammonia exposure on growth. Growth in all groups was virtually linear over time (Fig. 6B), and over 71 days, lower than in any of the satiation-fed treatments. Compared to the comparable treatments in the satiation-fed fish, body mass was lower in the rationed controls from Day 29 onwards, in the rationed high ammonia treatment group from Day 50 onwards, and in the rationed low ammonia treatment group only on Day 71. The mass measurements of all fish at the end of the experiment were in accord with these results.

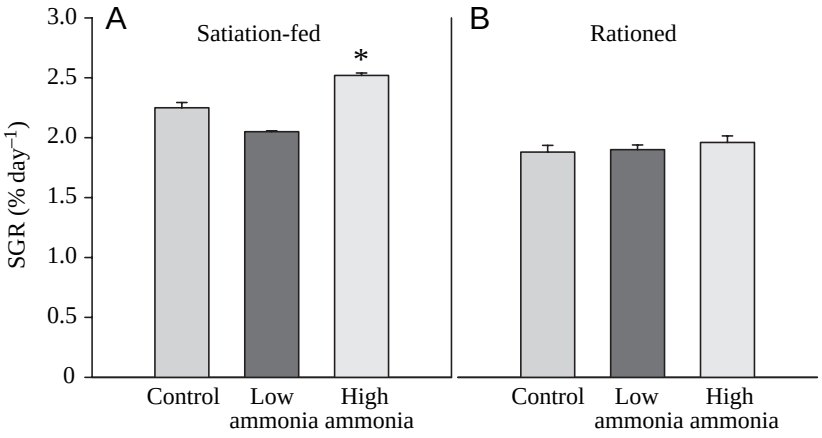

Fig. 7. Specific growth rates (SGR) of individually marked fish over 71 days in the three experimental treatment groups of Series 2: nominal [T_{Amm}]=0 μmol l^{-1} (control, N=64), 70 μmol l^{-1} (low ammonia, N=64), and 225 μmol l^{-1} (high ammonia, N=48). (A) Satiation-fed fish, (B) rationed fish. Values are means ±1 S.E.M. *Significant difference from control within the same panel. SGR values for the same treatments were all significantly lower in the rationed fish than in the satiation-fed fish.

These trends were all captured in the SGR calculation (Fig. 7). Over 71 days, in the satiation-fed fish, SGR was significantly higher in the high ammonia treatment group than in the controls or low ammonia treatment group, which did not differ (Fig. 7A). In the rationed fish, SGR values were virtually identical in the three treatment groups, and all were significantly lower than in the comparable treatments for satiation-fed fish (Fig. 7B).

Within the satiation-fed treatments, appetite was essentially identical throughout the 71 days in the control, low ammonia, and high ammonia exposure groups, such that cumulative food consumption per fish was the same in the three groups. These data are shown as daily ration in Fig. 8, which started around 1.3% body mass per day but fell to about 0.8% body mass per day by Day 71. Expressed on this basis, the ration was slightly lower late in the exposure in the high ammonia fish (which grew the best), but overall there was no significant difference. Thus, the improved growth was independent of food consumption. This also illustrates that the rationed fish were given the intended 2% body mass ration per day, but it was clearly much more food than they could voluntarily consume under these conditions.

Gross conversion efficiencies (CE) were remarkably high at these low temperatures, with a stimulatory effect of high ammonia clearly evident. CE, calculated over the entire 71 days, surpassed 300% in the high ammonia treatment group, significantly greater than values around 250% in the control and low ammonia treatment groups (Fig. 9A). CE values were much lower, around 100%, in the rationed fish and independent of treatment, but not all of the food was consumed with this feeding regime.

Within satiation-fed fish, % whole-body protein was significantly elevated in the high ammonia treatment group on both Days 34 and 71, and in the low ammonia treatment group on Day 34 (Table 1). This effect was also seen in the rationed fish, but only on Day 71 for the high ammonia treatment. Calculated over 71 days, net protein production in the individual satiation-fed fish was greatly elevated in the high ammonia treatment group, and depressed by a small but significant amount in the low ammonia treatment group (Fig. 10A). There was also

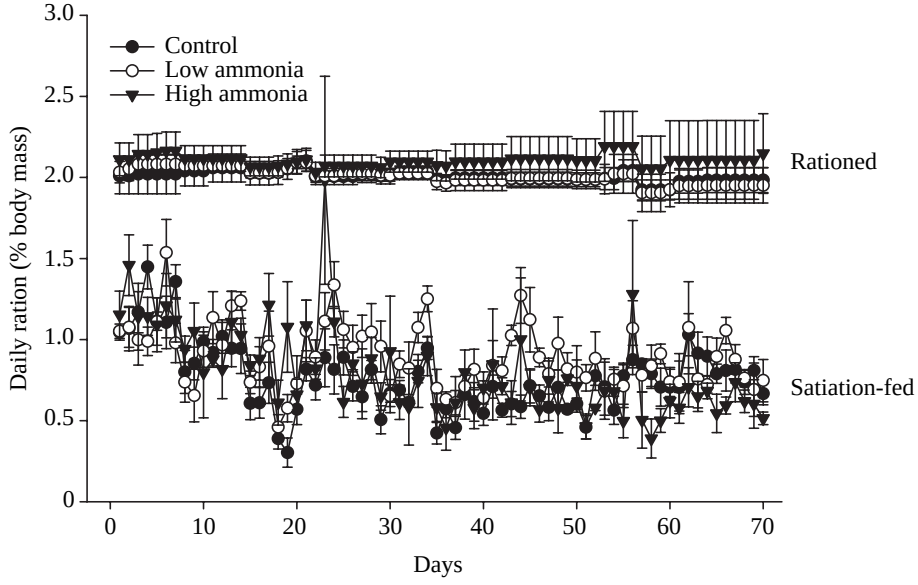

Fig. 8. Daily ration, measured on a per tank basis and expressed on a % body mass per day basis [(g food g^{-1} fish)×100] over 71 days in three experimental treatments of Series 2: nominal [T_{Amm}]=0 μmol l^{-1} (control, N=4 tanks), 70 μmol l^{-1} (low ammonia, N=4 tanks) and 225 μmol l^{-1} (high ammonia, N=3 tanks). Values are means ±1 S.E.M. Data for satiation-fed fish represent voluntary food consumption. Values for the high ammonia treatment group tended to be lower than the controls late in the exposure, but overall there were no statistically significant differences. Data for rationed fish reflect the fact that these fish were fed a fixed ration of 2% of their body mass per day, not all of which was consumed. Again, there were no significant differences overall.

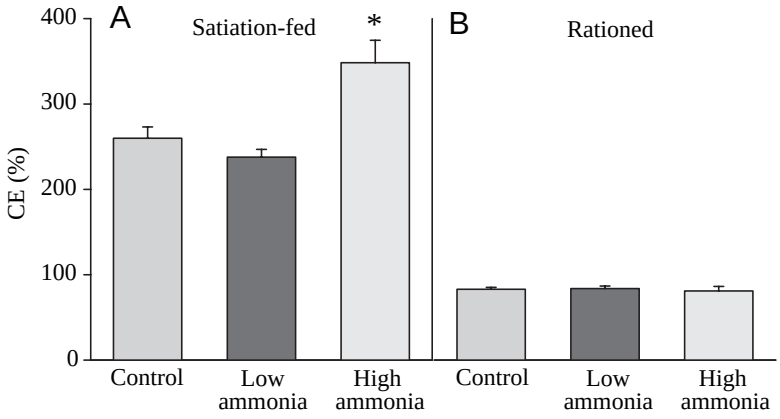

Fig. 9. Gross food conversion efficiency (CE) measured on a tank basis (N=4 for control and low ammonia groups, N=3 for high ammonia group) over 71 days in the three experimental treatments of Series 2: nominal [T_{Amm}]=0 μmol l^{-1} (control), 70 μmol l^{-1} (low ammonia) and 225 μmol l^{-1} (high ammonia). (A) Satiation-fed fish, (B) rationed fish. Values are means ±1 S.E.M. *Significant difference from control within the same panel. CE values for the same treatments were all significantly lower in the rationed fish than in the satiation-fed fish.

a small, significant depression in the rationed fish caused by low ammonia exposure (Fig. 10B). Protein production was consistently lower in the rationed fish than in the comparable treatment groups for the satiation-fed fish.

Discussion

The results of both Series 1 and Series 2 clearly demonstrate that chronic exposure to moderately elevated environmental ammonia can significantly stimulate growth and protein production in juvenile salmonids (by up to 40% over 10 weeks), without an increase in food consumption. These data therefore corroborate anecdotal evidence of earlier studies from our laboratory, performed under more variable temperature and ration conditions, where stimulation of growth was significant in some studies (Linton et al., 1997, 1999) but not in others (Linton et al., 1998a,b). The value for [T_{Amm}] used by Linton et al., was ~70 μmol l^{-1}, the same as the low ammonia treatment in the present study, which was effective in Series 1 performed at 15°C. It is interesting to note that in those studies where there was no significant growth stimulation (Linton et al., 1998a,b), there was also an extended period at cold temperatures (4–10°C). Similarly, in the present study, [T_{Amm}]=70 μmol l^{-1} was ineffective in stimulating growth in Series 2, performed at 6.5°C, at least over the 71-day period of the exposure, whereas the high ammonia treatment (~225 μmol l^{-1}) did cause significant stimulation of growth and protein accretion at this temperature.

Table 1. *Whole-body protein (%) on Days 34 and 71 in the control, low ammonia and high ammonia treatment groups for the satiation-fed and rationed fish in Series 2*

	[Ammonia] (μmol l^{-1})		
	Control (0)	Low (70)	High (225)
Day 34			
Satiation-fed fish	7.85±0.39‡	9.62±0.62*	9.95±0.52*
Rationed fish	9.13±0.32	8.40±0.68	9.18±0.72
Day 71			
Satiation-fed fish	9.31±0.46	8.58±0.45	11.29±0.73*,†
Rationed fish	9.30±0.46	8.04±0.39	10.71±0.75†

Values are means ± 1 S.E.M., N=19–20 (control), N=20 (low), N=14–15 (high).
*Significantly different from comparable control value.
†Significantly different from comparable low ammonia value.
‡Significantly different from comparable value for rationed fish.

At least two explanations may be offered. Firstly, it may be the P_{NH_3} level, rather than the [T_{Amm}], which is critical in initiating the growth response. Taking into account the effects of temperature on ammonia solubility and the NH_4^+–NH_3 dissociation reaction in water (Cameron and Heisler, 1983), together with minor differences in pH between the two series, we calculate that mean P_{NH_3} was 22.7 μtorr in low ammonia treatments and 50.7 μtorr in high ammonia in Series 1, and 7.9 μtorr in low ammonia treatments and 23.0 μtorr in high ammonia in Series 2. Thus in both, the effective P_{NH_3} levels were the same, around 23 μtorr. Approximately the same mean P_{NH_3} can be calculated from the Linton et al. (1997, 1999) studies performed close to a mean temperature of 15°C, where significant growth stimulation was observed. This explanation suggests that the critical range of effectiveness is fairly small, with beneficial effects being lost when P_{NH_3} rises too high or falls too low.

Alternatively or additionally, the time of exposure needed to initiate the growth stimulation may be critical, and may be longer at a lower temperature. In this regard, had Series 2 ended at 43 days, we would have concluded that both of the ammonia treatments were inhibitory to growth (Fig. 6A). Alternatively, by extrapolating the growth curves beyond Day 71, we might have concluded that both ammonia levels were stimulatory had the experiment been continued for a longer period. The reason why this pattern of early inhibition and later stimulation occurred in Series 2 is unclear; it was not seen in Series 1.

The observations that growth stimulation occurs at one low ammonia level and not another (Series 1), and that inhibition can change to stimulation with time of exposure (Series 2), both suggest that the 'window' for ammonia's effectiveness as a growth stimulant might be quite small. This is perhaps not surprising, because the levels of ammonia that cause chronic

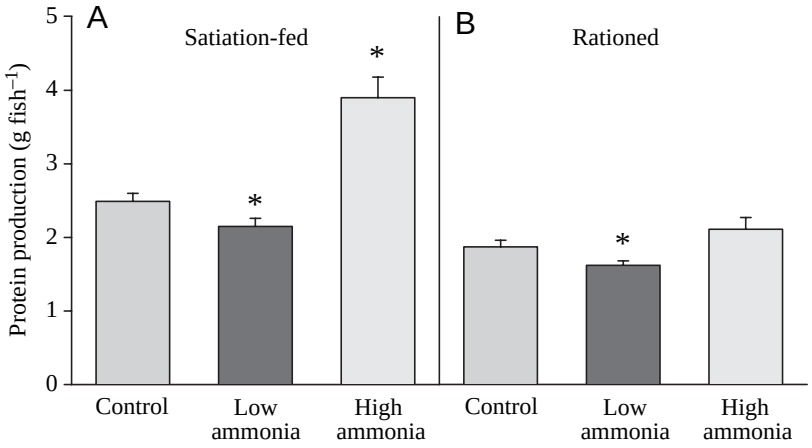

Fig. 10. Protein production per individual fish (N=20 for control and low ammonia, N=15 for high ammonia) over 71 days in the three experimental treatments of Series 2: nominal [T_{Amm}]=0 μmol l^{-1} (control), 70 μmol l^{-1} (low ammonia) and 225 μmol l^{-1} (high ammonia). (A) Satiation-fed fish, (B) rationed fish. Values are means ±1 S.E.M. *Significant difference from control within the same panel. Means for the same treatments were all significantly lower in the rationed fish than in the satiation-fed fish.

sublethal negative effects in fish are quite low, and according to recent US EPA (1999) guidelines, even lower in salmonids than in other teleosts. To put the levels used here in toxicological perspective [T_{Amm}]=70 μmol l^{-1} at pH 7.6 is approximately 50% of the US EPA criteria chronic concentration (CCC) and about 9% of the criteria maximum (i.e. acute) concentration (CMC) for salmonids. Therefore [T_{Amm}]=225 μmol l^{-1} is just above the threshold for chronic toxicity, probably explaining its ineffectiveness as a growth stimulant at 15°C in Series 1, and the need for these fish to eat more food to sustain the same growth (Figs 1, 2). Temperature has only a modest effect on ammonia toxicity when concentration is expressed in units of T_{Amm} (US EPA, 1999; Ip et al., 2001; Randall and Tsui, 2002); nevertheless, toxicity is slightly reduced at lower temperatures, perhaps explaining why [T_{Amm}]=225 μmol l^{-1} was able to serve as a growth stimulant in Series 2 at 6.5°C (Fig. 6A).

The stimulatory effect of ammonia on growth was seen under satiation feeding conditions in both Series 1 and 2. Satiation feeding must be considered a relative term, as the absolute amount eaten will depend on the nature of the food, on the number of feeding bouts offered per day, and on the exact criteria applied (Jobling, 1994). Nevertheless, the protocol provides an experimental situation where the fish are able to eat more food if they so wish. In neither series did those fish that grew more (Figs 1A, 2A, 6A, 7A) and produced more protein (Figs 3B, 10A) actually eat more food under ammonia stimulation than their respective controls (Figs 1B, 8). Thus, gross food conversion efficiency clearly increased in ammonia exposure (Figs 3A, 9A). Linton et al. (1997) reported the same phenomena – better growth and protein production without an increase in voluntary food consumption in a study performed at a mean temperature close to 15°C. Furthermore, in a similar study at this same temperature range but performed under restricted rations (nominally 1% body mass per day, where it was impossible for the fish to increase their food consumption), Linton et al. (1999) again found the stimulatory effect of [T_{Amm}]=70 μmol l^{-1} on protein accretion.

The SGR and CE values (Figs 2B, 3A) achieved by the satiation-fed trout of Series 1 were well within typical ranges reported in the literature for rainbow trout of comparable size at 15°C, but in Series 2 (Figs 7A, 9A) were above typical values reported close to 6°C (e.g. Wurtsbaugh and Davis, 1978; Brett, 1979; Elliott, 1982; Jurss et al., 1987; Cho, 1990, 1992; Azevedo et al., 1998). CE generally increases at lower temperatures, though exceptions have been reported (e.g. Azevedo et al., 1998). However, the CE values achieved in the satiation-fed fish of Series 2 were remarkable, especially in the high ammonia treatment group. Taking relative water contents into account (food, 12%; fish, 85%), the true conversion efficiencies on a dry matter basis were about 44% for the control and low ammonia treatments, and 59% for the high ammonia treatment, whereas literature values are typically less than 30%.

The mechanism by which exogenous ammonia might stimulate growth was proposed in the Introduction as an incorporation of ammonia into amino acids, resulting in increased protein synthesis, and the limited evidence that this occurred in fish was summarized (Hayashi et al., 1993; Iwata et al., 2000; Ip et al., 2001). Certainly, in both series, net protein production per fish was increased (Figs 3B, 10A), and in Series 2, % whole-body protein was also significantly increased (Table 1), so net protein synthesis was clearly stimulated. It is well established in salmonids that instantaneous protein synthesis rates rise quickly after a meal, as do plasma T_{Amm} and P_{NH_3} levels (e.g. Wicks and Randall, 2002a,b; for a review, see Wood, 2001). Furthermore, the tolerance of the fish to formerly lethal levels of exogenous T_{Amm} also increases (Wicks and Randall, 2002a). Therefore, one may speculate that the rise in plasma ammonia levels may serve as a signal both to activate ammonia detoxifying pathways (i.e. amino acid synthesis) and also to activate increased protein synthesis. If plasma ammonia levels are already modestly elevated as a result of moderately elevated external T_{Amm} (as was shown for trout at ~70 μmol l^{-1} by Linton et al., 1997), then the stimulation of these pathways may be greater. In this regard it is interesting that Reid et al. (1998), using the radio-labeled phenylalanine technique (Garlick et al., 1980; Houlihan et al., 1986), reported increased instantaneous rates of protein synthesis in the liver and white muscle of the

faster-growing, ammonia-exposed trout of Linton et al. (1997), which had been fed to satiation at temperatures close to 15°C. However, this effect was not seen by Morgan et al. (1999) when assaying the faster-growing trout exposed by Linton et al. (1999) to the same conditions on limited rations, so this may not be the complete explanation of the phenomenon.

An alternative or additional mechanism by which ammonia might stimulate growth without altering food consumption is by reducing metabolic costs. It is now well established that sublethal levels of external T_{Amm}, albeit considerably higher than those used here, will reduce critical swimming speed in salmonids, probably by depolarizing effects on the muscle membrane potential (Shingles et al., 2001; Wicks et al., 2002). It therefore seems possible that lower levels of T_{Amm} might reduce spontaneous activity and therefore metabolic rate. However, there is no clear picture in the literature on this point. Linton et al. (1997) reported lower 'in-tank' $\dot{M}_{O_2}$ values (integrated over the daily feeding cycle) in satiation-fed trout exposed to $[T_{Amm}]$=70 μmol l^{-1}, though again this was not seen in rationed fish (Linton et al., 1999) under otherwise similar conditions. Shingles et al. (2001) reported that $\dot{M}_{O_2}$ values at slow swimming speeds as well as at zero activity ('maintenance' metabolic rate) were actually raised by sublethal ammonia exposure (T_{Amm}=288 μmol l^{-1} at pH 8.4) in trout at 16°C.

In the present study (Series 1), 'in-tank' $\dot{M}_{O_2}$ was measured for only a 1 h period, approximately 5 h post-feeding, on nine successive weeks. There was no change in $\dot{M}_{O_2}$ at either of the ammonia exposure levels (Fig. 4A), suggesting that metabolic costs were not altered. Furthermore, there was no reduction in $\dot{M}_{Amm}$ in the $[T_{Amm}]$=70 μmol l^{-1} treatment (Fig. 4B), which is counterintuitive in view of the fact that these trout grew more and produced more protein without consuming more food. However, it is now clear that many other N-compounds besides ammonia may be excreted by teleost fish, and that their importance increases with feeding (e.g. Kajimura et al., 2004; for a review, see Wood, 2001). Obviously there is room for alternate scenarios – i.e. the expected changes in $\dot{M}_{O_2}$ may have been missed by these short-term measurements, there may have been a reduction in the excretion of other unmeasured N-wastes, and of course, spontaneous activity levels were not quantified.

Regardless of the exact explanation, this study now provides clear evidence that low levels of exogenous ammonia can serve as a growth stimulant in fish. Given the intense economic pressures of salmonid aquaculture, a factor that can increase productivity by up to 40% while reducing water flow requirements and not changing food requirements has great potential significance. Ammonia, often viewed as the fish farmer's enemy, may actually be a friend if carefully managed. Clearly, it will be important to find the full physiological explanation for this stimulatory effect of low levels of ammonia on growth. In future experiments, continuous recording of 'in-tank' metabolic rate, total N-waste excretion, and spontaneous activity, on a 24 h basis, will be required, similar to the pioneering measurements of Brett and Zala (1975), together with detailed measurements of internal nutrient dynamics, such as amino acid and protein synthesis rates, blood ammonia, and amino acid levels and key enzyme activities.

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