THE EFFECTS OF FEEDING AND STRESS ON AMMONIA TOXICITY IN RAINBOW TROUT

by

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Abstract

The effect of ammonia on aquatic organisms is an area that has been well studied. Results from various types of toxicity tests, including chronic and acute tests, have been used to establish the water ammonia standards for protection of aquatic life. Tests often follow standard protocols and are conducted under static conditions on unfed and unstressed animals. These conditions are in fact ones when fish are least susceptible to ammonia toxicity since feeding and stress cause increased internal ammonia production. This thesis had two goals. The first determined whether feeding or stress exacerbated ammonia toxicity to a level where standards promulgated by the US EPA were no longer protective for rainbow trout. In this regard, it was determined, using 96-h LC_{50} tests that present standards did protect rainbow trout that were either feeding, or stressed by high-density conditions. Feeding decreased ammonia toxicity during the first 24-h of ammonia exposure, increasing the LC_{50} concentration by 30 mg N/L. In contrast, both exposure to stress and cortisol injections exacerbated ammonia toxicity in fish. The second purpose of this research was to determine the physiological consequences of feeding and stress in rainbow trout exposed to sub-lethal ammonia concentrations. Results from experiments where fish were exposed to 10 mg N/L for 48-h and then terminally sampled for measurement of plasma ammonia, metabolites (glutamate and glutamine) and enzyme activities (glutaminase, glutamine synthetase-GSase, glutamate dehydrogenase-GDH, alanine aminotransferase and aspartate aminotransferase) in brain, liver and muscle tissue, indicated that protection from ammonia toxicity is evoked in feeding fish because glutamine synthetase is upregulated and α-ketoglutarate is likely not limiting the formation of glutamate and glutamine. The non-toxic glutamine is accumulated in the brain, liver and muscle tissue, thus removing ammonia from circulation. The same experiments conducted on fasted fish exposed to stressors (high-density, air-exposure and 3 ppm ambient cortisol) and then exposed to ammonia showed a different response. These fish were not able to regulate internal ammonia when ammonia exposure commenced and the down-regulation of GSase and glutaminase and up-regulation of GDH indicated that this was likely due to a limitation in α-ketoglutarate available for glutamate formation. Based on two models formulated from the present studies, glutamate regulation is critical in preventing ammonia toxicity.

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Several of the chapters contained in this thesis have either been published in or are submitted for consideration to referred journals. Chapter 2. The effect of feeding and fasting on ammonia toxicity in juvenile rainbow trout, *Oncorhynchus mykiss* is currently "In press" in Aquatic Toxicology. The authors are B.J. Wicks and D.J. Randall. The manuscript will be in print in 2001 and is 12 pages in length. Chapter 3. The effect of sub-lethal ammonia exposure on fed and unfed rainbow trout: the role of glutamine in regulation of ammonia is being reviewed by Comparative Biochemistry and Physiology and is authored by B.J. Wicks and D.J. Randall. Chapter 4. The effects of dietary protein on ammonia metabolism in rainbow trout under control and elevated ammonia conditions has been submitted for publication to Aquaculture and is authored by B.J. Wicks, I. Kakuta and D.J. Randall. The contributions to these papers by David Randall were through supervision and guidance of my thesis research and provision of funding from his N.S.E.R.C. grant. Izuru Kakuta's contribution to Chapter 4 was assisting in the formation of the custom-made diets and with sampling procedures. These Chapters have been written so that they may be read without significant reference to other parts of the thesis. The thesis as a whole provides new insight into how natural factors in the environment of rainbow trout, whether it is in its wild state or under intensive aquaculture, affects ammonia toxicity in these animals.

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Chapter 1. Introduction and review of ammonia toxicity in fish.

Ammonia is the end product of the food chain, formed as a metabolic waste when amino acids are catabolized. It is an interesting natural substance in that it can become internally toxic if an organism is unable to excrete or convert ammonia to a non-toxic form. The toxic nature of ammonia is sufficient that all organisms have some means of eliminating it from the body. There are three main excretory mechanisms known through which organisms deal with excess ammonia. Ureotelic animals, for example most mammals, excrete nitrogenous wastes as urea. Urea is less toxic than ammonia and being soluble in water is excreted in urine. Uricotelic animals excrete nitrogenous wastes predominantly as uric acid, a very concentrated form of nitrogenous waste. The last mechanism used for ammonia excretion is ammonotely, which is the excretion of ammonia waste directly. This type of excretion typically requires a large volume of water for dilution, and thus is predominant in aquatic animals. It is ammonotelic animals, more specifically the rainbow trout that will be discussed in this thesis.

There has been an enormous quantity of research conducted on ammonia as it relates to the aquatic environment. The research covers a range of topics including: methods of excretion, metabolic adaptation of organisms to extreme environments, the mode of action of ammonia toxicity and acceptable environmental standards for the protection of aquatic organisms. Of particular interest is how the water ammonia criteria have been established. Aquatic toxicological tests are conducted in order to establish the effect of toxicants on any given organism. In order for results to be comparable a standard set of protocols have been recommended and are generally used (ASTM, 1993). Results from these standardized tests are then used to develop criteria for allowable limits of anthropogenic toxicants in the environment. These limits should ensure that the health of aquatic organisms is protected.

Although much of the toxicity testing follows standard protocols the types of tests used can take many forms. Tests are done to determine either acute or short-term effects or chronic responses, which consider effects over a longer period. Typically in an acute toxicity test only one species is considered at a time. Test species are usually those that have economic importance or are easily available and maintainable under laboratory conditions. Chronic studies are used to investigate the toxic effects of a substance on an individual species over longer time periods and often consider reproductive costs and generation effects. Whole ecosystem studies

using systems such as mesocosms, which imitate the natural environment, have been used to study the effect of toxicants on many organisms at once and therefore consider interaction between species. The compilation and analysis of data collected in these various types of studies are used to set water quality criteria for the protection of all aquatic organisms.

An extensive literature base is available on water ammonia, however information can be confusing due to the variety of units used to express concentration. Haywood (1983) suggests maximum permissible ammonia levels in fresh water for teleosts in Canada and the US Environmental Protection Agency (EPA) provides the American criteria (1998, 1999). Both of the North American standards use mg N/L to express concentrations of total ammonia nitrogen in ambient water ammonia. Hence, for the purpose of the following studies ambient ammonia will be expressed in these units. Although Canadian ammonia standards exist they are likely based on the more comprehensive data from the US EPA and for that reason the US EPA criteria will be referred to throughout this document. The US EPA water quality criteria fall into two categories, the Criterion Maximum Concentration (CMC) and the Criterion Continuous Concentration (CCC). The CMC value is based on one half the final acute value from compiled studies. This level is not to be exceeded for more than one hour more than once every three years and is expected to protect 95% of all organisms exposed. The CCC value is based on the compilation of values from chronic, animal, plant and residue studies, with the lowest value being selected for the criterion. The CCC average concentration should not be exceeded for more than four days once every three years. Many considerations regarding the nature of ammonia have been made when establishing these ambient water criteria, including temperature, pH, and ionic strength of the solution and sensitivity of different life stages to ammonia exposure. The impact of ammonia produced as natural internal metabolic waste on the toxicity of ambient ammonia in aquatic organisms remains unknown.

As previously mentioned, ammonia is an unusual toxicant in that it is both a naturally produced waste product as well being toxic if accumulation occurs internally. Ammonia excretion in ammonotelic organisms occurs mainly by diffusion and in fish this occurs across the gills. Accumulation can occur by two means, inability to excrete nitrogenous wastes or through a net influx of NH_3. In aqueous solution total ammonia has two species, NH_3 and the protonated NH_4^+. Externally, the concentration of NH_3 is of concern as it is lipophilic and therefore readily moves across membranes. As a result, if ammonia levels are high in the

environment, ammonia enters the fish as NH_3 but at body pH it is rapidly protonated to NH_4^+, which is highly toxic.

The mechanism of acute ammonia toxicity in fish is probably similar to that of mammals. Astrocytes are large cells associated with nervous tissue. They create an enzymatic barrier between the blood, the central nervous system and brain, which protects the brain from ammonia by converting it to glutamine (Cooper and Plum, 1987). When ammonia levels in the blood become elevated the "barrier" cannot detoxify all ammonia and brain ammonia levels become elevated. The elevated ammonia interferes with several aspects of brain function including, energy metabolism, electro physical properties, morphology of capillaries and reduced numbers of neurotransmitters (Cooper and Plum, 1987). Convulsions and hyperventilation occur before death in organisms exposed to acute ammonia levels supporting the theory of interference in brain function. Non-lethal chronic ammonia exposure in fish can cause blood alkalization (Cameron and Heisler, 1983) and changes in ion balance (Sousa and Meade, 1977). This disruption in the acid-base balance can decrease efficiency of the oxygen delivery system by negatively affecting haemoglobin (Sousa and Meade, 1977). Physical damage has also been noted at low environmental ammonia levels with the gill epithelium showing extensive hyperplasia (Burrows, 1964)

Toxicity testing follows standardized protocols and is usually conducted under laboratory conditions with variables such as photoperiod, food availability, temperature, oxygen levels, pH and stress levels being carefully controlled. These conditions are rare in the natural environment or under aquaculture conditions. Interestingly, the natural variables that result in elevated internal ammonia in fish, active swimming, feeding and stress are all factors that are avoided during toxicity testing.

Feeding is a normal activity for fish. During the catabolism of ingested proteins fish produce nitrogenous waste products that must be excreted, with the main end product being ammonia (Handy and Poxton, 1993). The majority of ammonia is produced in the liver of fish during degradation of excess amino acids. High levels of dietary protein, providing excess amino acids beyond what is required for protein synthesis, will increase production of nitrogenous wastes including ammonia. The increase of ammonia excretion with increased dietary protein has been well documented (Cai et al., 1996; Medale et al., 1995; Ballestrazzi et al., 1998; Jayaram and Beamish, 1992). Ammonia excretion rates are elevated 2-h after feeding

and remain high for up to 11-h (Leung et al., 1999; Gelineua et al. 1998; Dosdat et al., 1996; van Weerd et al., 1995) depending on fish species.

Based on the literature, ammonia excretion undoubtedly increases during the postprandial period in fish suggesting that the internal ammonia levels have risen. Surprisingly, few studies have been conducted that consider the internal ammonia levels in fish after feeding. One study indicates that after feeding the plasma total ammonia level can more than double (Kaushik and Teles, 1985). A fundamental part of the acute toxicity testing is that test specimens are not fed before or during the toxicity test; however due to the relationship between feeding and ammonia production such protocols may be inappropriate if they are to be applied to ecologically relevant situations. Remarkably, little literature is available on ammonia toxicity in fed fish or how exogenous ammonia affects feeding. The one published study on ammonia toxicity in turbot indicates that elevated ambient ammonia reduces growth rate as a result of reduced food consumption (Person-Le Ruyet et al., 1997). The following estimations can be made using available literature on postprandial ammonia excretion in fish. Based on data collected by van Weerd et al. (1995) a 1-kg fish fed continuously to satiation will produce 1050 mg N/day. Thus, using a typical aquaculture water flow rate between 0.2 and 0.4 L/min/kg the fish will be exposed to water concentrations between 1.8 and 3.4 mg N/L on a daily basis as a result of net nitrogen excretion. The postprandial ammonia pulse in fish peaks between 3 and 6-h after feeding and can last for up to 3-h. During this high ammonia excretion period, fish can produce 230-295 mg N/kg/hour (Leung et al., 1999). Using the previously mentioned flow rates, fish would experience between 12 and 25 mg N/L. These calculations indicate ammonia levels experienced by fish on a daily basis and undoubtedly during the postprandial period exceed the CCC standard set by the EPA at 2.28mg N/L at pH 7.5 and may even come close to the CMC value at 13.3 mg N/L (USEPA, 1998). Based on knowledge that feeding increases the internal ammonia levels as well as ammonia excretion in fish it is hypothesized that elevated ambient ammonia will decrease feeding and/or increase ammonia toxicity in fed fish.

With respect to swimming, recent studies have indicated that salmon show a linear relationship between critical swimming velocity (*Ucrit*), a measure of swimming performance, and ambient ammonia concentration (Wicks et al., 2001). These results indicated a decrease in swimming performance occurs with exposure to increasing concentrations of exogenous ammonia. In the same study a significantly higher mortality rate was noted in actively

swimming fish than in fish resting in a respirometer under conditions of elevated ambient ammonia (Wicks et al., 2001). The calculated LC_{50} concentration was in fact lower in that study than the criterion set by the US EPA, suggesting that actively swimming fish may not be protected by the promulgated standards. The increased toxicity of ambient ammonia in swimming versus static fish is probably related to a swimming induced elevation in plasma ammonia. Swimming fish not only have a net influx of ammonia from the water, but also have to cope with ammonia accumulation in muscle due to deamination of adenylates during exercise (Mommsen and Hochachka, 1988). Accumulation of ammonia in muscle causes depolarization of the muscle cell membrane potential (Beaumont et al., 2000; Wang et al., 1996), which likely caused the reduction in swimming ability. Since, the increase in environmental ammonia resulted in increased plasma ammonia and decreased swimming ability, it is probable that elevated ammonia in the environment could decrease survival of migrating salmon.

Understanding ammonia production during swimming is complicated in that it potentially triggers two responses that increase internal ammonia, the deamination of adenylates (discussed above), and a stress response. Forced swimming is only one of many stresses fish experience. Stress can be physical, social or environmental factors such as handling, starvation, confinement, extreme changes in temperature or salinity, disease, high density or toxicant exposure, as well as forced swimming. When fish are exposed to stress, either an internal change or an external stimulus evokes a chain of physiological reactions to try and compensate for and resist the change. In teleost fish, stress typically activates the hypothalamus-pituitary-interrenal axis, resulting in an increase in circulating cortisol (Donaldson, 1981). In fish cortisol affects intermediary metabolism, stimulating gluconeogenesis (Vijayan et al., 1997) and this cortisol surge provides the fish with glucose to meet the energetic demands to cope with the stress. Cortisol also decreases protein synthesis and increases protein catabolism to provide amino acids as a source of energy (Gapp, 1987) and this catabolism could elevate internal ammonia (Mommsen et al., 1999). This elevation in internal ammonia could exacerbate ammonia toxicity when fish are exposed to exogenous ammonia due to a reduced ability to excrete wastes.

Given the fact that ammonia is a waste product that all organisms have to cope with, it is not surprising that various mechanisms have evolved in fish that live in environments where eliminating endogenous nitrogenous waste is difficult. A brief summary of some of these

mechanisms is provided as a basis for the following studies. Excretion of ammonia as urea is one strategy that is adopted by several species of fish including, *Oreochromis alcalicus grahami* (Randall et al., 1989) and *Heteropneustes fossilis* (Saha and Ratha, 1998). It is thought that these fish can remove urea utilizing transmembrane transporters (Ip et al., 2001b). Urea is not the only form of nitrogen that is transported out of fish. Mudskippers, *Periophthalmodon schlosseri* are extremely tolerant of elevated exogenous ammonia. It is thought that the unique structural components of their gills may allow them to actively transport ammonium ions across the gills against a concentration gradient (Randall et al., 1999). Amino acids are the key components for production and/or storage of nitrogen. As such, it is not surprising that many of the mechanisms involving ammonia regulation or detoxification involve amino acids. One strategy adopted by fish is incomplete protein catabolism that results in a non-toxic intermediate such as alanine or glutamine. Increased tissue levels of alanine have been noted in *P. schlosseri* (Ip et al., 2001a) and increased muscle concentrations of glutamine have been found in the marble goby, *O. marmoratus*, (Jow et al., 1999) living under terrestrial conditions where ammonia excretion is restricted. These mechanisms are highly specialized and do not occur in all fish.

Rainbow trout, *Oncorhynchus mykiss*, the lab rat of the fish world, lives in a well-oxygenated, low ionic strength environment, favouring ammonia loss by diffusion. In spite of its environment, several studies have demonstrated that rainbow trout do have some mechanisms to defend against ammonia toxicity. More specifically, when these fish are exposed to elevated ambient ammonia a significant increase in brain glutamine corresponding to a decrease in glutamate occurs (Vedel et al., 1998; Arillo et al., 1981). In addition, reduced ammonia production has been demonstrated in rainbow trout held in high pH water when ammonia excretion is impaired (Wilson et al., 1998). As previously mentioned the brain is the most sensitive tissue to ammonia and thus it is not surprising that a protective mechanism exists in this tissue. The formation of glutamine occurs by the following reactions:

1) α-ketoglutarate + $NH_4^+ \Rightarrow$ glutamate catalyzed by glutamate dehydrogenase (GDH)
2) glutamate + $NH_3 \Rightarrow$ glutamine catalyzed by glutamine synthetase (GSase)

Thus, the formation of one glutamine eliminates two NH_4^+ from solution These are the only known mechanisms reported in rainbow trout for coping with an ammonia insult.

The studies contained in this thesis have been conducted to provide insight into new areas with respect to ammonia toxicity in rainbow trout and include the effects of feeding and

stress on ammonia toxicity and ammonia metabolism. Chapters 2-5 cover specific topics and have been written so they can be read without reference to the rest of the thesis. It is anticipated that these chapters will be published as separate articles. The details of publication can be viewed in the forward.

Chapter 2. The effect of feeding and fasting on ammonia toxicity in juvenile rainbow trout, Oncorhynchus mykiss.

1.0 Introduction

Aquatic toxicological tests are conducted in order to establish the effect of ambient toxicants on aquatic organisms. In order for test results to be comparable between studies a standard set of guidelines are used (ASTM, 1993). Results from standardized tests are then used to develop criteria for allowable limits of anthropogenic toxicants in the environment. Acute toxicity tests follow standardized protocols and are conducted under laboratory conditions with variables such as photoperiod, food availability, temperature, oxygen levels, pH and stress levels being carefully controlled. Such controlled conditions are rare in the natural environment.

Ammonia is an unusual toxicant in that animals naturally produce it, yet it is toxic if accumulated in body tissues. The main internal source of ammonia in fish is through catabolism of proteins. Feeding is a normal activity for non-migrating fish. During the catabolism of ingested proteins, fish produce nitrogenous waste products that must be excreted, with the main end product being ammonia (Handy and Poxton, 1993). The majority of ammonia is produced in the liver of fish during degradation of excess amino acids. Increased dietary protein, providing excess amino acids beyond that which is required for protein synthesis will increase production of nitrogenous wastes including ammonia. The increase of ammonia excretion with increased dietary protein has been well documented (Jayaram and Beamish, 1992; Ballestrazzi et al., 1998; Medale et al., 1995; Cai et al., 1996). Ammonia excretion associated with feeding changes during the postprandial period. Elevated ammonia excretion rates have been documented between 2 and 11-h after feeding (van Weerd et al., 1995; Dosdat et al., 1996; Gelineua et al., 1998; Leung et al., 1999). The rate and pattern of ammonia excretion varies with temperature (Leung et al., 1999; Zakes and Karpinski, 1999), diet, and the species in question.

Ammonia excretion increases during the postprandial period in fish, suggesting that internal ammonia levels have risen. Surprisingly, few studies have been conducted that consider the internal ammonia levels in fish after feeding. One study indicated that, after feeding, the plasma total ammonia level could more than double in rainbow trout, *Oncorhynchus mykiss*, (Kaushik and Teles, 1985). A fundamental part of the toxicity testing that has been done for

ammonia is that test specimens are not fed before or during the toxicity test; however due to the relationship between feeding and ammonia production such protocols may be inappropriate for ammonia toxicity testing. Remarkably, little literature is available on ammonia toxicity in fed fish or how elevated ambient ammonia levels affect feeding. One published study on ammonia toxicity in turbot indicates that elevated ambient ammonia reduces growth rate because of reduced food consumption (Person-Le Ruyet et al., 1997).

The purpose of this study was twofold: to determine the postprandial internal ammonia levels in fish and to determine if increased internal ammonia after feeding changes ambient ammonia toxicity in rainbow trout.

2.0 Methods

2.1 Fish Husbandry and Experimental Set-up

For all sections of this study rainbow trout from a standard brood stock were obtained from Spring Valley Trout Farm, Langley, British Columbia. Fish weighed approximately 30 g at the time of delivery and were maintained in circular outdoor tanks supplied with dechlorinated Vancouver city water. Fish were left undisturbed and fed a maintenance ration of 1 % body weight daily for three weeks prior to commencement of experiments.

All experiments were conducted in series of eight 60 L flow through indoor tanks supplied with dechlorinated Vancouver city water. Light was controlled on a 12:12 light/dark cycle. Depending on the experiment between eight and twenty fish were placed in each of the eight tanks. During the two-week acclimation period, fish were fed daily at 0800 hr at a rate of 2 % body weight. Each tank of fish was allowed 15 minutes to consume food before excess was removed from food traps and uneaten pellets counted to establish feeding rates. Five days prior to the beginning of each experiment, water was buffered and pH maintained at 7.3 with the addition of sodium bicarbonate, at a rate of 0.27 mmol/L, by peristaltic pump (Masterflex) to the head tank. The bicarbonate solution increased the water Na^+ from 3.95 to 5.94 ppm, Ca^+ from 0.98 to 1.24 ppm and water pH from 6.9 to 7.2. The change in water parameters did not affect the feeding rate of the fish, however no sampling was performed to determine blood parameters. Water pH, temperature, dissolved oxygen and ammonia were monitored daily in each tank.

Ammonia levels were always tested 6-h following feeding to correspond to the peak ammonia excretion times (Kaushik and Teles, 1985) unless otherwise noted.

2.2 Experimental Protocols

2.21 Postprandial plasma ammonia and glucose levels

After the completion of the two-week acclimation period the 8 fish/tank used in this experiment were sampled at scheduled times following the morning feeding to determine postprandial plasma ammonia and glucose levels. Feeding on the sampling day commenced at 0800 hr but was staggered by 15 minutes between tanks to allow time for sampling. Sampling times were zero (unfed fish), 0.5, 1, 2, 4, 6, 8, and 12-h after feeding. Fish were rapidly netted from tanks, killed by concussion, weighed and blood collected using heparinized syringes. Total sampling time for each group of 8 fish was under 10 minutes. Blood was centrifuged for 5 minutes at 1000 rpm to separate the blood cells and plasma. Plasma was then divided into 2 aliquots and frozen at –80 °C for later determination of ammonia and glucose. Plasma ammonia was measured using the GLDH-method (Sigma, 171-UV) and plasma glucose was measured using the enzymatic hexokinase method (Sigma, 115).

2.22 The effect of feeding on acute ammonia toxicity

A series of 3 flow-through 96-h acute ammonia toxicity experiments were conducted with adherence to EPA guidelines for toxicity testing except that fed fish were compared with the unfed group. Fish morbidity was used as an endpoint rather than mortality in order to meet animal care requirements for the University of British Columbia. A pilot study indicated that morbid fish (loss of equilibrium and unresponsive to touch) would not recover if left in elevated ammonia for the duration of the experiment and thus could be counted as mortality. The death of morbid fish typically occurred between 2 and 4-h from the time the fish became unresponsive to touching.

Ten fish were placed in each tank for this set of experiments and were left to acclimate as described above. The 8 tanks were set up in pairs, each pair to receive an identical 96-h dose

of ammonia. Stock solutions of NH_4Cl were delivered to each tank by peristaltic pump (Masterflex) at a rate that produced the required ammonia concentration. All fish were fed daily until the day before the experiment. On the starting day of the experiment one tank of fish in each pair was hand fed at a rate of 2% body weight (based on total mass of fish in each tank) at 0800 hr while the unfed group had the tank cover lifted to simulate onset of feeding. After 15 minutes all tanks were cleaned and uneaten pellets from the fed tanks were counted and their weight calculated. Food consumption was estimated based on the average number of dry pellets per gram. Ammonia treatments were started 3-h after feeding and these corresponded to the elevation of plasma ammonia due to feeding (Figure 1). Water ammonia levels were tested on the first day of each experiment, 3-h after the start of treatment and each day thereafter, as described previously. Fish were observed on a regular basis for morbidity, with morbid fish being removed when they met the criteria. Once a fish was removed from a tank, it was weighed and its weight was subtracted from the tank's total fish mass. The quantity of food for that tank was recalculated such that fish were only offered a ration based on 2% of the total body weight per tank daily.

The first of the three 96-h exposure experiments covered a range of nominal ammonia concentrations including 0 (control), 4, 20 and 40 mg N/L at a pH of 7.2. These values were selected as they represented values close to those recommended by the U.S. EPA (1998) as the chronic standard (3.0 mg N/L) and the acute standard (30 mg N/L) as well as a value in the middle. When no morbidity was noted in the first experiment, it was run a second and third time.

The second experiment in this series used exposure levels of 0, 50, 65 and 80 mg N/L again at pH 7.2 on a new group of fish. The method was the same as described above with one modification. At the end of the experiment, all fish were killed by a sharp blow to the head and weighed. Blood samples collected from each fish were treated and analyzed for plasma ammonia as described above. Plasma was also aliquotted and frozen for measurement of calcium and sodium using an atomic absorption spectrophotometer.

A third experiment was required in this series using nominal ammonia levels of 0, 120, 150 and 180 mg N/L. During the experiment, morbidity was noted and morbid fish were removed from the tanks and sampled. At the end of this 96-h exposure, again all remaining fish were sampled.

2.23 The effect of fasting on acute ammonia toxicity

In a third set of experiments using the same experimental protocol, two 48-h LC_{50} tests were run to test the effect of fasting on ammonia toxicity in rainbow trout. In this set of experiments, 18 fish were placed in each of the 8 tanks and again left to acclimate as described previously. The first of the 2 toxicity tests was conducted on fish that were fasted for 5 days prior to the onset of treatment, paired with fish fed at 2% body weight daily up to and during treatment. The second toxicity test examined rainbow trout fasted for 10 days paired with fed fish. Fish remaining at the end of the tests were not sampled in this set of experiments. The nominal ammonia levels used for both toxicity tests were 0, 130, 170 and 210 mg N/L at pH 7.2.

All LC_{50} values and 95 % confidence intervals were calculated using U.S. EPA software utilizing the trimmed Spearman-Karber Method.

3.0 Results

3.1 Postprandial plasma ammonia and glucose levels

The data collected in this preliminary study revealed two trends. Both plasma ammonia and glucose showed a significant increase above control levels 30 minutes after feeding (Figure 1). Plasma glucose (±S.E.M.) increased significantly from 11.1 (± 1.2) mmol/L in unfed fish to 14.9 (±1.1) mmol/L in fish 30 minutes after feeding. Glucose levels returned to control level approximately 2-h after feeding. Plasma ammonia increased significantly from 11.1 (±1.5) in unfed fish to 15.5 (±1.5) μg/mL (Figure 1). Plasma ammonia also returned to control levels two hours after feeding, but showed a second significant peak 8-h after feeding at a level of 17.9 (±3.4) μg/mL.

3.2 The effect of feeding on acute ammonia toxicity

The actual measured mean ammonia concentrations over 96-h for each of the three experiments were 0.31 (control), 6.13, 35.8, 54.7, 85.7, 94.3, 113.5, 189.0, 250.0 and 272 mg

N/L. The water ammonia concentrations within a treatment varied by no more than 3%. The water pH and temperature remained constant at 7.2 and 10 °C respectively in all tanks during all acute experiments.

No morbidity was noted in the control tanks of fed or unfed fish in any of the 3 acute toxicity tests. In the first of the three 96-h feeding acute toxicity tests no morbidity was noted in either fed or unfed fish at any of the ammonia levels tested (6.13, 35.8 and 54.7 mg N/L). In the second 96-h test at ammonia levels ranging from 0, 85.7, 94.3 and 113.5mg N/L, loss of equilibrium was noted in both fed and unfed fish at the two highest ammonia levels in the first 12-h. These fish, however remained responsive to touch and subsequently returned to an upright swimming position over the course of the experiment. No morbid fish were noted in any treatment over the 96-h exposure. During the third toxicity test at ammonia levels ranging from 0, 189.0, 250.0 and 272 mg N/L, high morbidity rates were noted at all treatment levels for both fed and unfed fish (Table 1). At the two highest ammonia concentrations, 250 and 272 mg N/L, all unfed fish had been removed from the tanks due to morbidity within 24-h and fed fish in 48-h. At the lowest ammonia concentration, 189 mg/L, six of ten fish were morbid after 96-h in both the fed and the unfed tanks. Using the morbidity rate of rainbow trout at the ammonia concentrations tested in the three experiments the 24, 48 and 96-h LC_{50} for both fed and unfed fish exposed to these ammonia levels was calculated. No significant difference was noted between the 24 and 48-h LC_{50} in fed and unfed fish. The 96-h LC_{50} was calculated to be 174 mg N/L (with 95% confidence intervals of 159-191 mg N/L). Again, there was no significant difference between the mortality rates of fed and unfed fish.

Food consumption showed a definite decrease in those groups exposed to elevated ammonia levels with feeding suppression becoming more pronounced at higher ambient ammonia levels (Figure 2). A significant linear relationship existed between initial food consumption (<48-h) and ammonia concentration (Figure 3). After the initial decline in food consumption feeding rate increased after 48-h at all concentrations except, 189 mg N/L where feeding never resumed. The increase in feeding rate after 48-h of ammonia exposure was significant ($p<0.05$) as determined by ANCOVA. Feeding rate did not return to the pre-treatment level in any of the treated tanks of fish over the 96-h experiment.

Plasma ammonia levels measured in both the second and third acute toxicity tests showed a significant linear increase with increasing ambient ammonia in both fed and unfed fish

(Figure 4). Unfed fish showed significantly higher concentrations of plasma ammonia, 37.9 μg/mL (±2.5 S.E.M.) than fed fish at 32.6 μg/mL (±1.7 S.E.M.) at ambient ammonia levels above 187 mg N/L ($p<0.05$, Figure 4). At lower concentrations of ambient ammonia, the plasma ammonia levels were not significantly different. A two-way analysis of variance showed no interaction effect between feeding and ambient ammonia on plasma ammonia levels; however both feeding ($p<0.05$) and water ammonia ($p<0.001$) affected plasma ammonia after allowing for the effects of differences in diet and ambient ammonia, respectively. Plasma calcium levels were not significantly different between treatments; however, sodium levels decreased significantly with increasing ambient ammonia (Figure 5) with a significant difference between treatment levels (ANOVA, Bonferroni's multiple comparison test).

3.3 The effect of fasting on acute ammonia toxicity

The actual measured mean water ammonia levels in the toxicity tests using rainbow trout fasted for both five and ten days paired with fed fish were 0.1, 112, 150.5 and 183 mg N/L and 0.1, 107, 153 and 180.5 mg N/L respectively. The variation of the water ammonia concentration within a tank was no more than 2.5% over the course of an experiment. The water pH and temperature remained constant at 7.2 and 10 °C, respectively, in all tanks during both fasting experiments. Again, no morbidity or change in food consumption was noted in the control tanks for both the five and ten day fasting toxicity tests. Food consumption was significantly reduced at all ammonia concentrations in both tests. Fish maintained at the lowest ammonia concentrations still consumed food at a rate >1% body weight daily.

The 24-h morbidity rate in the fed treatment groups of the five-day fasting test was low and a LC_{50} value could not be calculated. Table 2 provides the 24 and 48-h LC_{50} values for both tests. The 24-h LC_{50} for fish fasted five days prior to ammonia exposure was 135 (130-140) mg N/L. The calculated 48-h LC_{50} for fed fish was not significantly different from that of the fasted fish with the values being, 139 (92-209) and 132 (124-139) mg N/L respectively. Although a LC_{50} could not be calculated for fed fish after 24-h of ammonia exposure, this treatment group had the lowest morbidity rate.

In the toxicity test using fish fasted 10 days the 24-h LC_{50} for fed fish was 177 (160-199) and 143 (127-157) mg N/L for fasted fish. The 24-h LC_{50} for fed fish was significantly higher

than for the fasted fish in this test. The 48-h LC_{50} values for fed and fasted fish were not significantly different with calculated values being 141 (124-160) and 138 (122-154) mg N/L respectively.

When comparing the 5 and 10-day fasting toxicity tests there was no significant difference between the 24 or 48-h LC_{50}'s for fasted fish. The fed fish in both tests had either a significantly higher or an incalculable 24-h LC_{50} but similar 48-h LC_{50} values.

4.0 Discussion

Feeding fish must increase swimming activity to compete for food. In addition, fish maintained in the small indoor tanks showed a fright response when the covers were removed from their tanks for feeding. Both the increased swimming behaviour (Mommsen and Hochachka, 1988) and the stress caused by disturbing the fish (McDonald et al., 1993) could, 30 minutes after feeding, have increased both plasma ammonia and glucose levels (Figure 1). This increase probably corresponds to the increased metabolism required to provide energy for both swimming and to cope with the initial fright because, both plasma ammonia and glucose returned to control levels in approximately 1-h and glucose remained constant thereafter. Ammonia, however, increased a second time 4-h after feeding, peaking again 8-h postprandial. The trend depicted in Figure 1 for plasma ammonia is similar to the postprandial ammonia excretion curve observed by Gelineau et al., (1998) after the initial peak at 30 minutes. The feeding hierarchies that develop in tanks of fish (McCarthy et al., 1992; Jobling and Koskela, 1996) could explain the large variation noted between plasma ammonia levels in individual fish at the various sampling times. Some fish will consume a larger portion of the available food during the feeding period. A difference in feeding rate would be reflected by internal ammonia levels that result from breakdown of ingested protein.

The initial reaction of the fish to elevated ambient ammonia may be a stress response that stops or reduces feeding (Wendelaar Bonga, 1997). Stress in fish is known to upregulate gluconeogensis to provide a rapid supply of glucose in order to meet an increase in energetic demands (Mommsen et al. 1999). An increase in metabolism due to stress has also been documented to cause increased ammonia production (Mommsen et al. 1999), thus it is possible that a stress response will also increase ammonia toxicity in fish. It was noted that in the first

24-h of ammonia exposure, fish would struggle and thrash in the tanks, indicating a high level of stress and at some ammonia levels, fish would lose balance yet returned to an upright swimming position after 48-h. The combination of high activity and stress may have an additive effect on the internal ammonia levels, due to both increased metabolism to cope with stress and the deamination of adenylates caused by the extreme swimming behaviour. Once fish calm down and reestablish the ammonia equilibrium they may then start feeding again. The suppression of feeding below a normal level may allow fish to maintain internal ammonia concentrations below toxic levels. If this is true then internal ammonia levels may mediate the feeding rate. Reduction in feeding rates may increase survival but will undoubtedly reduce growth as noted in turbot (Person-Le Ruyet et al., 1998).

Based on the present studies excess quantities of nitrogen are produced as a result of protein catabolism after feeding and results in increased plasma ammonia as noted in the postprandial feeding experiment. In some fish the plasma ammonia level following feeding was as high as 35 and 37 μg/mL. In fact in some individuals the plasma ammonia level exceeded that found in fish at the LC_{50} ambient ammonia levels (Figure 4). This extreme elevation in plasma ammonia after feeding could thus exacerbate ammonia toxicity in fish encountering elevated ambient ammonia.

Contrary to my hypothesis, results from the present toxicity tests indicate that fish fed up to the time of ammonia exposure are no more susceptible to elevated ambient ammonia than unfed fish prior to and during the exposure. In the two 96-h LC_{50} trials where plasma ammonia was measured in the surviving fish at the termination of the experiment, levels increased with increasing ambient ammonia in both the fed and unfed fish. Surprisingly, at high external ammonia levels plasma ammonia was on average 5 μg/mL lower in fed fish than in unfed fish (Figure 4). It is possible that since plasma ammonia levels became naturally elevated in feeding fish in normal ammonia environments that fish have a mechanism that is upregulated during feeding and protein catabolism to deal with excess ammonia. It has been demonstrated that under conditions of high ambient ammonia that brain glutamine levels increase in fish (Arillo et al., 1981; Vedel et al., 1998) most likely as a means to remove excess ammonia by combining with glutamate via glutamine synthetase. Such a mechanism might also serve to protect sensitive areas like the brain from the elevation of ammonia during feeding and thus may also protect the fish from elevations in ambient ammonia.

The fasting toxicity experiments support the theory that feeding ameliorates ammonia toxicity during early stages of ammonia exposure. Fed fish were less sensitive than the fasted fish to the elevation of ambient ammonia in the first 24-h, regardless of the duration of fasting. The 24-hour LC_{50} level was approximately 30 mg N/L higher in fed fish than in the fasted fish; however a protective effect of feeding was not evident at 48-h. It is worth mentioning that the use of morbidity as opposed to mortality as an end point for the LC_{50} tests is unlikely to impose significant changes to the calculated values due to the rapid death of fish after morbidity is noted. The ability of the feeding fish to cope with the higher ambient ammonia levels was likely related to the lower plasma ammonia concentrations in these fish (Figure 4). It is evident that the protection that is manifested by feeding reaches a threshold sometime after 24-h of ammonia exposure when the fish is no longer able to cope with the high ambient ammonia concentrations resulting in higher mortality rates. The threshold may be affected by the fact that feeding was initially suppressed after 24-h of ammonia exposure (Figure 2) or it is possible that the mechanism may only work until a threshold is reached.

One possible mechanism that may protect feeding fish from increased ambient ammonia could be up-regulation of pathways that utilize ammonia directly in the production of amino acids. These could include alanine, aspartate, glutamate and glutamine. All four of these amino acids could act as temporary stores of toxic ammonia. Vedel et al. (1998) found that brain glutamine increased in unfed rainbow trout exposed to elevated ambient ammonia and brain glutamate decreased. Vedel et al., (1998) also noted that the concentration of glutamate in the brain reached a threshold at high ammonia exposures and was not depleted any further. It is possible that similar changes occur in feeding fish under normal ammonia conditions, but the threshold is never reached, as internal ammonia levels due to feeding are not high enough. Once this pathway is upregulated however, it is possible that it will help protect fish from both self-intoxification and/or ambient ammonia toxicity. The regulation of food consumption by individual fish may also contribute to its ability to regulate internal ammonia levels. It is clear based on the present studies that further ammonia toxicity testing is required under fed and fasting conditions to elucidate the mechanism(s) that rainbow trout are utilizing to avoid self-intoxification after feeding and how feeding ameliorates ammonia toxicity.

This study did reveal that the U.S. EPA's present acute criteria of 30 mg N/L at pH 7.2 would protect both feeding and fasting fish. It is however, important to note the significant

differences in mortality rates observed between fed and unfed fish exposed to ammonia during the first 24-h. Lemly, (1996) suggests that Winter Stress Syndrome, a condition experienced by stressed fish during winter months when water temperature and feeding is naturally reduced, should be considered when evaluating environmental toxicants. Extended periods of fasting is part of this proposed syndrome and is clearly important in ammonia toxicity.

In conclusion, this study determined that fish have internal ammonia pulses that correspond with feeding. The increase in internal ammonia does not exacerbate ammonia toxicity when fish are exposed to elevated ambient ammonia, but protects the fish during the first 24-h of exposure. In addition, a considerable decrease in food consumption, which never returns to control levels during a 96-h exposure period, prevents self-intoxification during ammonia exposure. Further experiments are needed to determine the mechanisms that protect feeding fish from ammonia toxicity.

Table 1. Number of morbid fed and unfed rainbow trout exposed to elevated ambient ammonia (0.4-272 mg N/L) for 96-h at pH=7.2 and 10 °C. Each tank contained 10 fish.

Total Ammonia mg N/L	0.4	0.4	189	189	250	250	272	272
Feeding	Yes	no	yes	no	yes	no	yes	no
Morbidity (24-h)	0	0	4	5	8	10	8	10
Morbidity (48-h)	0	0	1	0	2	-	2	-
Morbidity (72-h)	0	0	1	1	-	-	-	-
Morbidity (96-h)	0	0	0	0	-	-	-	
Total morbidity	0	0	6	6	10	10	10	10

Table 2.Calculated LC_{50} values for fed and fasted (5 and 10 days) rainbow trout (n=18 fish/treatment) exposed to a range of ammonia concentrations at pH 7.2 and 10°C. LC_{50} values were calculated using the trimmed Spearman-Karber method and are presented with 95% confidence intervals (CI). Significant differences between LC_{50} values are indicated by *.

	24-h LC_{50} in mg N/L (95% CI)		**48-h LC_{50} mg N/L (95% CI)**
Fed	No mortality	*	139 (92-209)
5 day fast	135 (130-140)		132 (124-139)
Fed	177 (160-199)	*	141 (124-160)
10 day fast	143 (127-157)		138 (122-154)

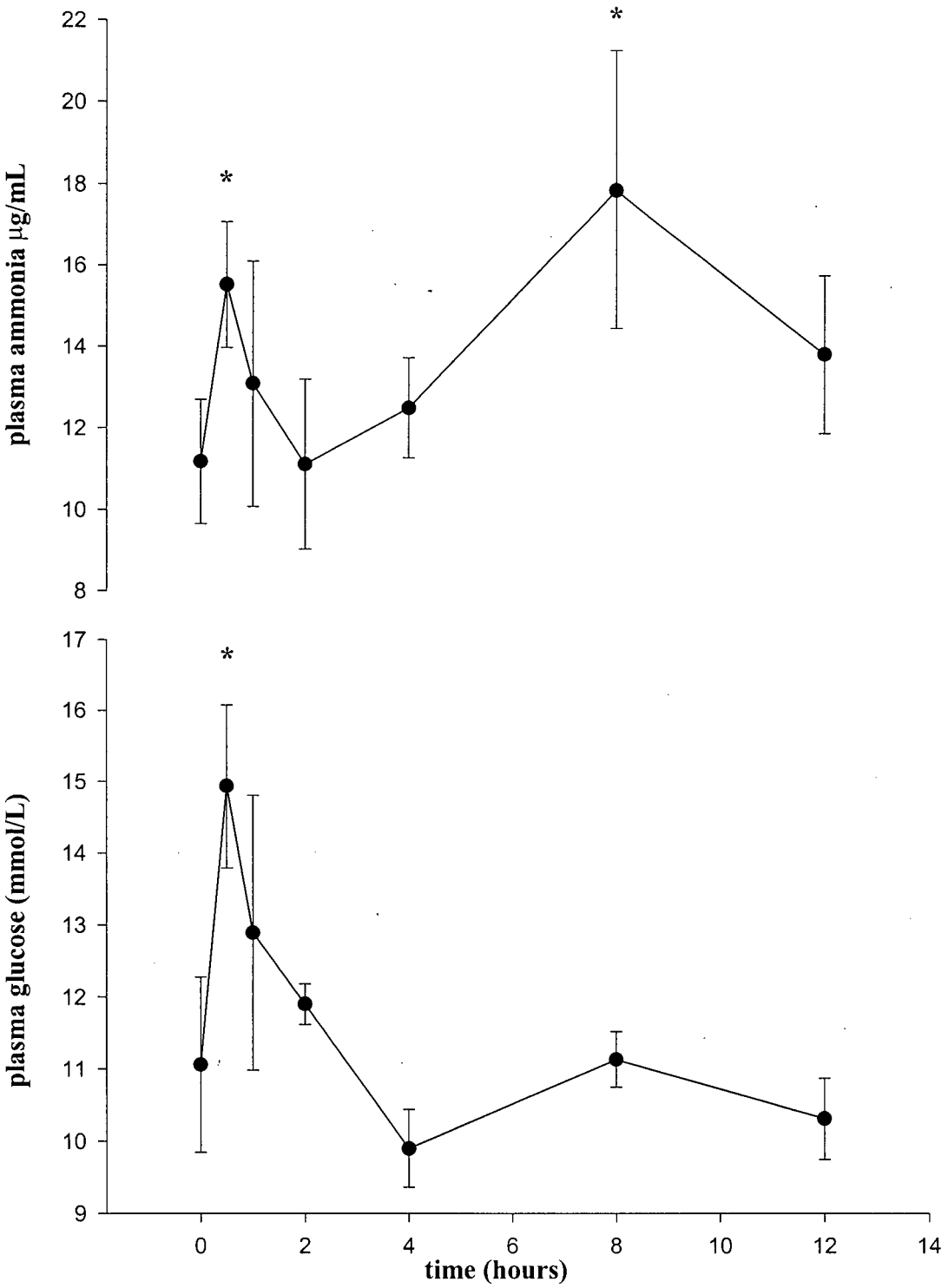

Figure 1. Postprandial ammonia (µg/mL) and glucose (mmol/L) pulse in rainbow trout (mean weight 45 g) plasma over a 12-hour period. Error bars indicate the standard error of the mean and * show significant differences ($p<0.05$, $n=8$) when compared to control (time 0).

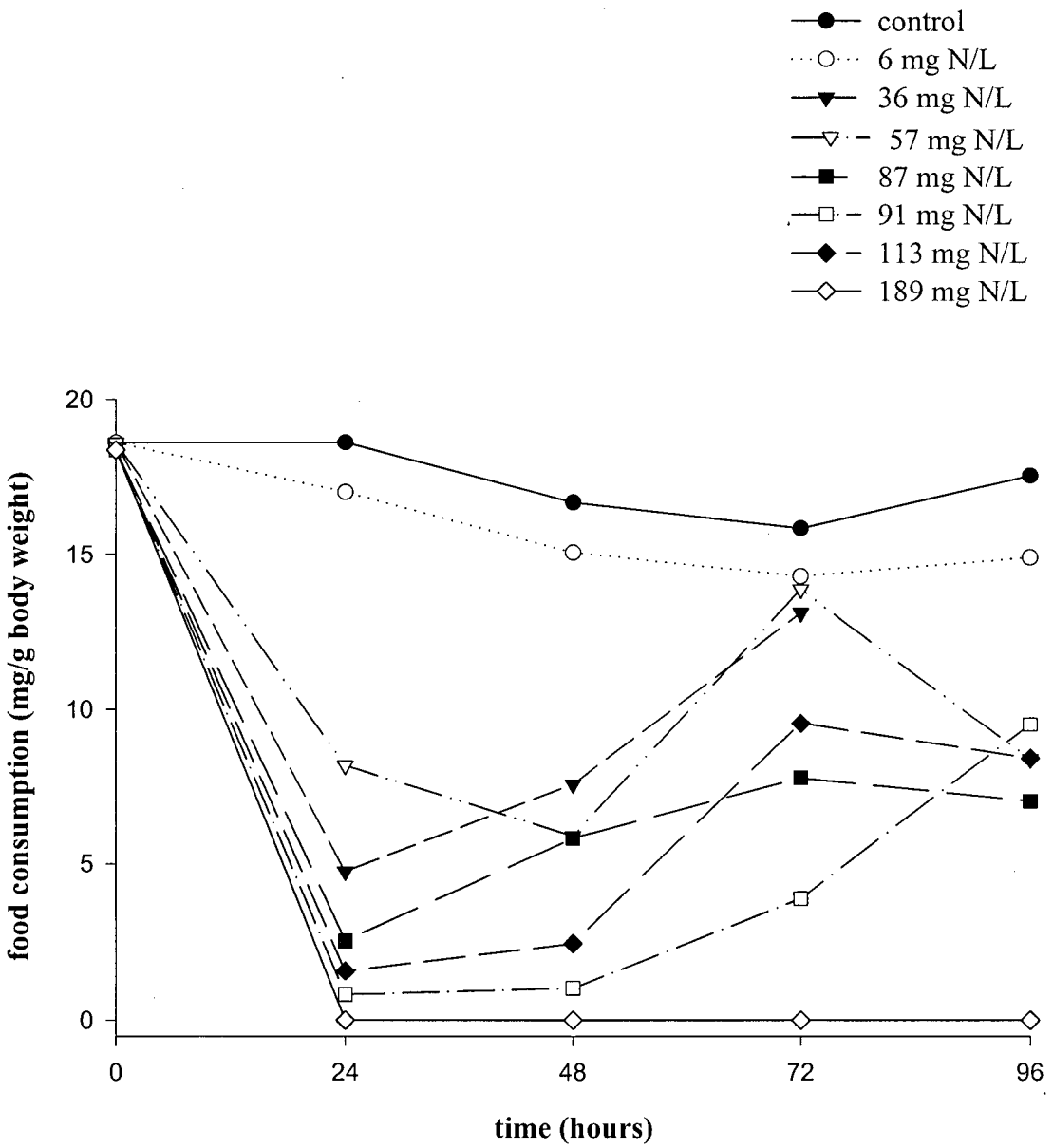

Figure 2. Daily food consumption (mg/g body weight) in rainbow trout exposed to elevate ambient ammonia (0-189 mg N/L, pH 7.2) for 96 hours.

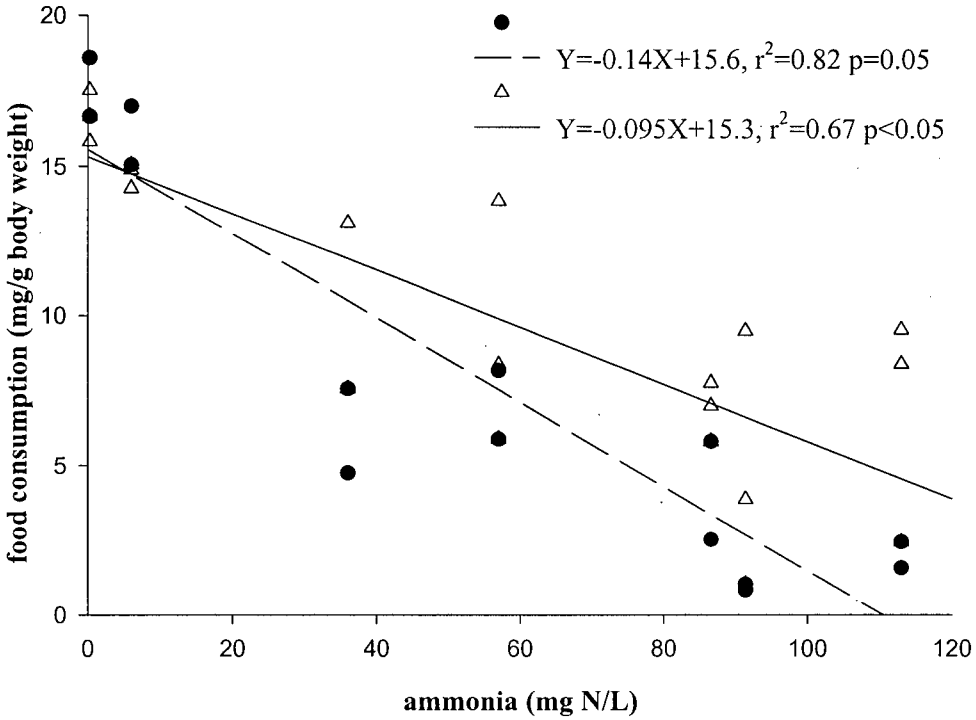

Figure 3. Daily food consumption (mg/g body weight) in rainbow trout exposed to elevated ambient ammonia (0-113 mg N/L). Regressions indicate both the first (solid circles) and the second 48 hours of feeding (hollow triangles) after start of ammonia exposure.

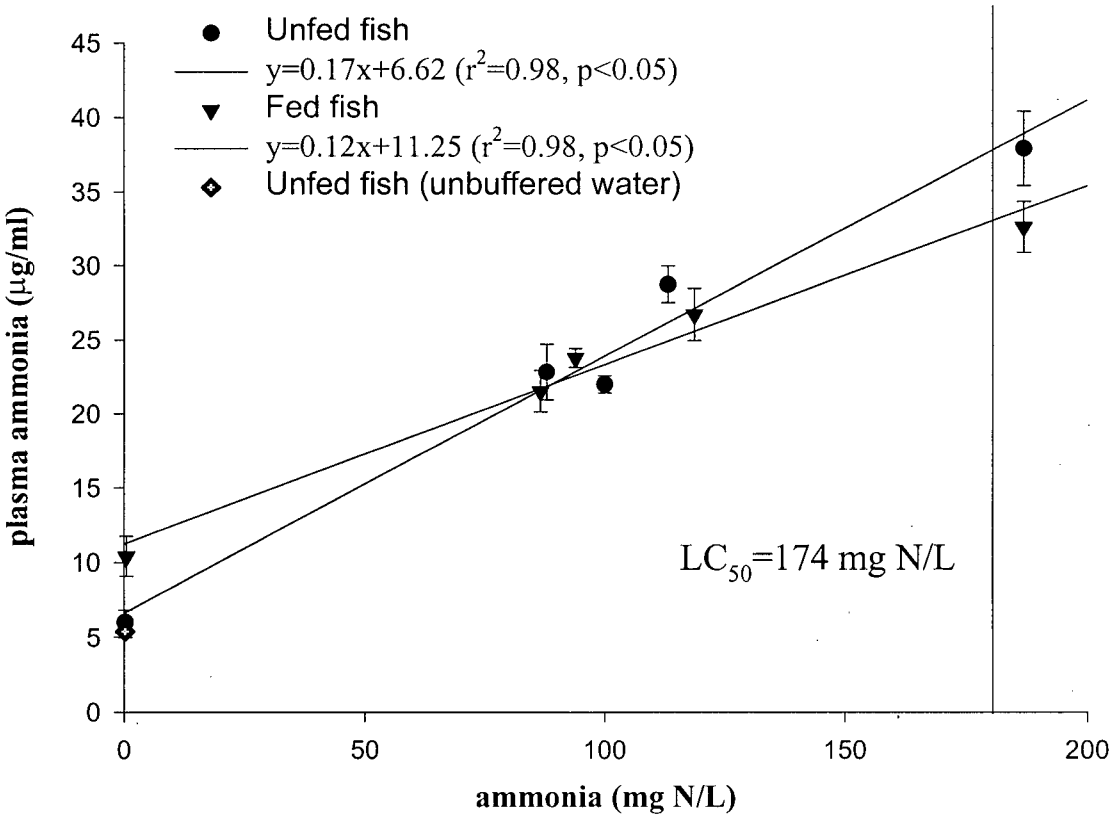

Figure 4. |Mean plasma ammonia levels (µg/ml) in unfed and fed rainbow trout exposed to elevated environmental ammonia (0-187 mg N/L) for 96 hours at pH of 7.2. The 96 hour LC_{50} value was 174 mg/L N for both unfed and fed trout. Bars indicate the S.E.M. and n=4-12.

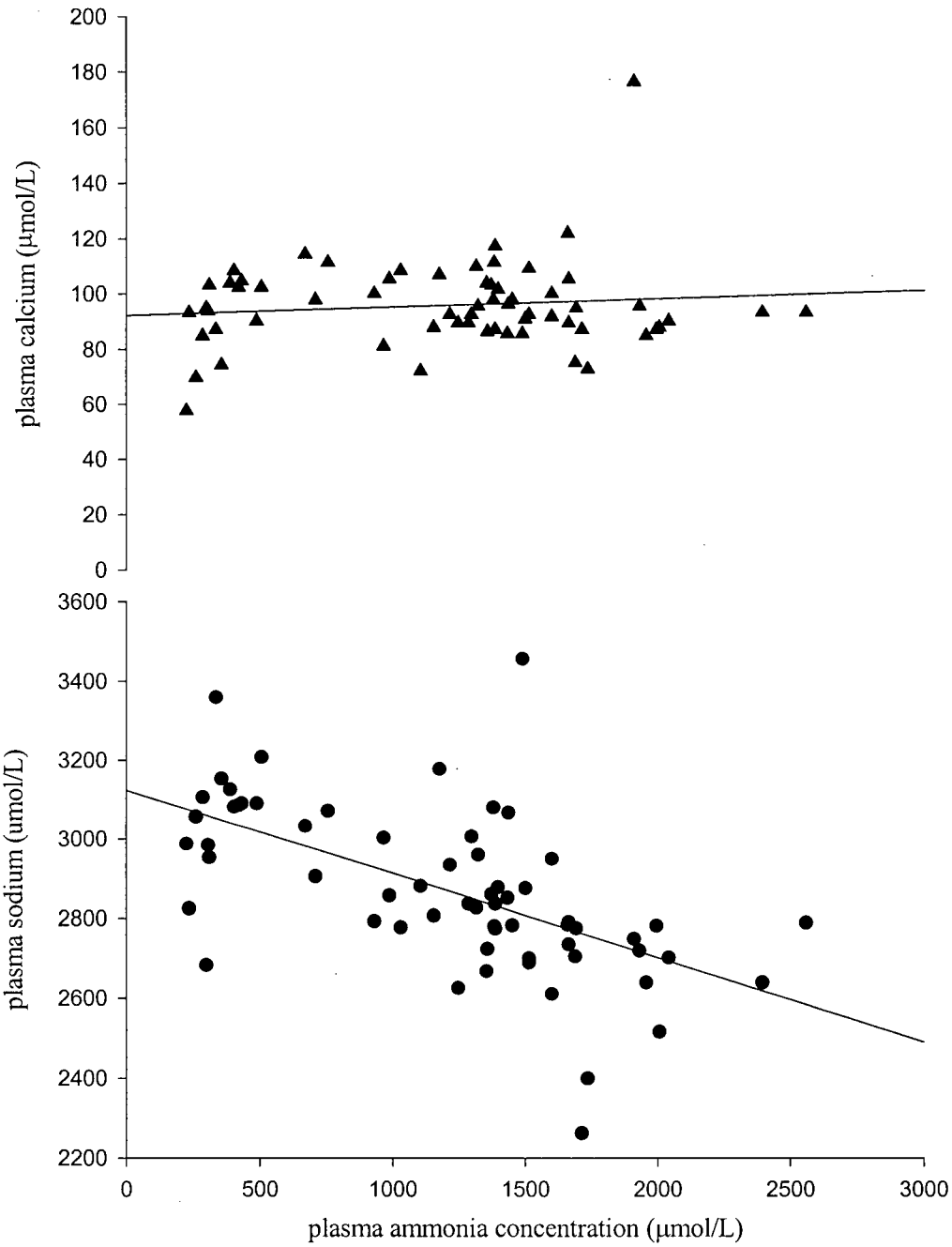

Figure 5. Plasma calcium and sodium levels (µmol/L) regressed on plasma ammonia (µmol/L) in rainbow trout exposed to elevated ambient ammonia (6-189 mg N/L) for 96 hours at pH 7.2.

● NH_3 µmol/L vs Na µmol/L

—— Na (µmol/L)=3123.2-(0.21*NH_3 µmol/L)

r^2=0.35, $p<0.05$

▲ NH_3 µmol/L vs Ca (µmol/L)

—— Ca (µmol/L)=0.03(NH_3 µmol/L)+9

r^2=0.01, $p<0.05$

Chapter 3. The effect of sub-lethal ammonia exposure on fed and unfed rainbow trout: the role of glutamine in regulation of ammonia.

1.0 Introduction

Much research has been done on ammonia toxicity in fish, however it is typically done according to standard test procedures so that tests are comparable. The requirements for toxicity tests are that fish not be fed for 24-h prior to or during tests. The acute increase in plasma ammonia of fish exposed to elevated ambient ammonia has been well documented (Cameron and Heisler, 1983; Wilson and Taylor, 1992; Vedel et al., 1998). A recent study found that in rainbow trout fed a high protein diet plasma ammonia levels reached 17.9 ±(3.4) μg/mL 6-h after feeding (Chapter 2). Surprisingly, although extensive literature is available on ammonia production and excretion rates in feeding fish (Leung et al., 1999; Dosdat et al., 1996; Gelineau et al. 1998; van Weerd et al., 1995), the physiological effect of this natural increase in internal ammonia during feeding has not been adequately investigated. Additionally, how such an increase could affect ammonia toxicity in fish exposed to elevated ammonia has not been considered. In a separate study it was found that feeding ameliorates ammonia toxicity in rainbow trout during the first 24-h of exposure, but the mechanism involved was not determined (Chapter 2).

Many mechanisms have evolved to cope with high internal ammonia in species of fish that live in high ammonia environments or in environments where ammonia excretion is impaired. Some documented mechanisms include conversion of excess ammonia to urea as in *H. fossilis* (Saha and Ratha, 1998) and *O. alacalicus grahami* (Randall et al., 1989) or to glutamine, as in *P. schlosseri* (Peng et al., 1998) and the marble goby, *O. marmortatus* (Jow et al., 1999). Other known mechanisms include active transport of ammonia out of the fish as in *P. schlosseri* (Randall et al., 1999). These types of adaptive mechanism are not known to exist in rainbow trout. It is known, however, that under ammonia loading conditions glutamine levels in the brain increase (Arillo et al., 1981;Vedel et al, 1998) causing a depletion of glutamate (Vedel et al, 1998). Ammonia converted to glutamine is catalyzed by mitochondrial glutamine synthetase. The metabolic pathway for glutamate/glutamine is depicted in Figure 1. Both glutamate and glutamine are non-toxic and can be transported readily throughout the circulatory system. The present study tested the effect that both elevated ambient ammonia and feeding

separately and in combination had on some physiological parameters involved with nitrogen metabolism in rainbow trout. The purpose of this study was to isolate the physiological changes that occur in fed and unfed fish exposed to sub-lethal ammonia at two time intervals and determine how feeding might protect rainbow trout from acute ammonia toxicity. The responses measured included, plasma ammonia and cortisol, and brain, liver, and muscle glutamate, glutamine, glutamine synthetase (GSase), glutaminase, glutamate dehydrogenase (GDH), alanine aminotransferase (ALT) and asparate aminotransferase (AST).

2.0 Methods

All rainbow trout used for this study were obtained from Spring Valley Trout Farm, Langley, British Columbia. After delivery fish were maintained in circular outdoor tanks supplied with dechlorinated Vancouver city water. On arrival, fish weight averaged 20 g. During the next three weeks, they were fed at a rate 3 % body weight daily to promote growth prior to moving them to indoor tanks at which point their average weight was 30 g.

Two weeks prior to the beginning of the experiment, 12 fish per tank were weighed and transferred to each of eight 60 L indoor tanks supplied with dechlorinated Vancouver city water. Once moved to indoor tanks, fish were only disturbed for feeding and tank cleaning. Light was controlled on a 12-h:12-h light/dark cycle. Each tank was supplied with precisely one L of aerated water per minute. Oxygen level was maintained above 8.7 mg/L. Water was delivered through a spray bar to create a slight current in the tank. A constant water flow was ensured through a system including a head tank and precision valves. Fish were hand fed daily at 0800 hr at a rate of 2 % body weight. Each tank of fish was allowed 30 minutes to consume food before excess feed was removed from a food trap and then each tank was cleaned. Five days prior to the beginning of each experiment, water was buffered and pH maintained at 7.3 with the addition of sodium bicarbonate, at a rate of 0.27 mmol/L, by peristaltic pump (Masterflex) to the head tank. Addition of bicarbonate was found to have no effect on the feeding rate of rainbow trout in a previous study using the same system and delivery rate (Chapter 2). One day prior to the start of the experiment food was withheld from 4 of the 8 tanks of fish. Each unfed tank of fish was paired with a tank of fed fish.

On the morning of the test, 4 tanks of fish were fed at 15-minute intervals starting at 0800 h. One hour after feeding ammonia treatment of the fed tanks commenced. Treatments consisted of fed and unfed tanks of fish exposed to 0 mg N/L and 10 mg N/L. Each treatment was replicated for a total of eight tanks. Ammonia treatment of the four unfed tanks of fish started at 0800 hr with start times staggered by 15 minutes. Ammonia levels in the tanks were increased instantaneously to 10 mg N/L with the addition of 2.48 g of NH_4Cl dissolved in 60 L of water. Ammonia levels were maintained by stock solutions of NH_4Cl delivered to each tank by peristaltic pump (Masterflex) at a rate that produced the required ammonia concentration. The ammonia added to the tanks decreased the pH from 7.3 to 7.2, which remained constant during the experiment. Fed fish were fed at 2% body weight daily (Moore-Clark Nutra fry, 48% protein) during ammonia exposure.

Water pH, temperature and dissolved oxygen were monitored daily in each tank during the acclimation period and during testing. Ammonia levels were measured six hours after the onset of treatment and at 1400 hr daily during the 48-h experiment. Water ammonia was measured using a modification of the Verdouw et al. (1978) method substituting nitroprusside (20 mg/L) for ferrocyanide.

Six fish were sampled from each tank at each time period, 6-h and 48-h, after ammonia exposure commenced. In addition sampling always took place 7-h after feeding. Fish were quickly netted from the tank, killed by concussion, weighed and measured and then blood was collected from the caudal vein. Brain, liver and muscle tissue was collected and immediately frozen in dry ice for later determination of glutamate, glutamine and enzymes. In fed groups the fullness of the stomach and size of gall bladder was qualitatively noted to establish if the fish had eaten prior to sampling. Sampling of each fish took no longer than 3 minutes, with the entire tank completed in less than 15 minutes. Blood samples were centrifuged to separate plasma and blood cells. Plasma was aliquotted and frozen at –80 ^{o}C for later measurement of cortisol and ammonia.

The blood variables, plasma ammonia, and plasma cortisol were measured using commercially available kits. The commercially available L-GLDH/NADH kit (Sigma 171-UV) was used for plasma ammonia and the Neogen (Lexington, Kentucky, USA) ELISA kit for plasma cortisol. Ether extraction was not performed on the plasma prior to measuring cortisol. From the12 fish sampled per treatment group, 6 fish were randomly selected and their tissues

were homogenized in 5 times their volume of cold 0.3 M perchloric acid. Homogenized tissue was then centrifuged at 6,000 x *g* at 4 °C for 3 minutes and then the supernatant was removed. The supernatant was neutralized with 1.5 M K_2CO_3, left on ice for 30 minutes, centrifuged for 30 seconds at 6, 000 x *g* and the supernatant removed and frozen at –80°C for determination of glutamate and glutamine. Glutamate and glutamine concentrations were determined enzymatically by deamination of L-glutamine and dehydrogenation of L-glutamate using a micro-modification of Sigma kit GLN-1. The modification of the commercially available kit was to reduce sample/reagents proportionally, so that reactions were completed in a micro-plate as opposed to test tubes. This required that volumes for all reagents be reduced to 25% of the original value with the exception of glutamate dehydrogenase, which was added to the plate at a rate of 5μL/well.

The tissue samples from the second replicate were homogenized for enzyme determinations using the following method. Frozen tissue samples were weighed and homogenized with a Polytron tissuemizer in 10 volumes of glycerol buffer, containing 10 μg aprotinin/mL (Mommsen and Hochachka, 1994). The homogenate was then subjected to a brief sonication. Homogenized tissue was then centrifuged at 6,000 x *g* for 30 seconds and the supernatant removed and frozen at –80 °C for later determination of GSase, glutaminase, GDH, ALT and AST.

GSase (reverse direction) was assayed using the transamidase activity of the enzyme, reacting glutamine with hydroxylamine and ADP to γ-glutamylhydroxamate in the presence of arsenite (Webb and Brown, 1976). The incubation medium consisted of 3 mM $MnCl_2$, 60 mM glutamine, 15 mM hydroxylamine, 0.4 mM ADP and 20 mM KH_2AsO_4 in 50 mM HEPES buffer, pH 6.7. Tissue homogenate was plated on round bottom microplates in quadruplicates and medium added to two experimental wells leaving two wells as controls. Brain tissue was incubated for 30 minutes, liver and muscle for 3 and 4-h, respectively. Both the reactions and controls (right after addition of incubation medium) were terminated by adding acid ferric chloride reagent (1:1:1 by volume of 10% $FeCl_3$ in 0.2 M HCl, 24% trichloroacetic acid and 50% HCl) to develop the γ-glutamylhydroxamate. The microplates were then centrifuged for 10 minutes at 2,500 x *g* and an aliquot of the supernatant was transferred to flat bottom plates and read at 540 nm on a Molecular Devices plate reader. The concentration of γ-glutamylhydroxamate produced was calculated using a standard curve produced using the same

methodology. Initially, time courses were established to ensure that the assay conditions were saturating and linear with time and volume of homogenate.

Glutaminase activity was measured by following the conversion of glutamine to glutamate and by assaying the quantity of glutamate formed. Briefly, diluted liver homogenates were plated on round bottom micro-plates (zero-time controls and experimentals in duplicates). For liver, the experimental wells were incubated for 90 minutes in an assay medium consisting of 60 mM Tris, 50 mM NaH_2PO_4, pH 8.0, 2 mM NH_4Cl, 5 mM glutamine. Muscle and brain homogenates were incubated for 90 and 30 minutes, respectively, in a medium consisting of 150 mM phosphate, 50 mM Tris (pH 8.6) and 20 mM glutamine. At the end of the incubation periods, the reactions were stopped by the addition of 17.5% perchloric acid to all the wells. The appropriate incubation media were added to the controls after the acid. The plates were cooled at 4°C for 15 minutes, and solutions neutralized with 2 M KOH, 0.5 M imidiazole, 40 mM EDTA, mixed on a plate reader and centrifuged for 15 minutes at 2,500 x *g*. Fifty μL of supernatant from each well was then replated on flat-bottomed micro-plates and 200 μL of a glutamate assay medium added. This medium contained Tris-EDTA-hydrazine buffer (0.1 M Tris: 2 mM EDTA: 0.63mol/L hydrazine, pH 9.0), Acetylpyridine dinucleotide (an analogue of NAD) (5mM) and ADP (100 mM) in a 100:10:1 ratio, and glutamate dehydrogenase (15 units/mL buffer). Samples were incubated for 1-h at room temperature and a reading was taken at 365 nm. Glutamate standards were included in the same protocol and were used to calibrate the method.

GDH, ALT and AST activities were determined in the homogenates of all tissues by continuous spectrophotometers in thermostated Molecular Devices plate readers. GHD was assayed directly, while ALT and AST were enzymatically coupled to produce a decrease in absorbance at 340 nm as NADH was oxidized to NAD. The specific media for these enzymes made up in imidiazole buffer pH 7.4 were as follows:

GDH : 250 mM ammonium acetate, 0.1 mM EDTA, 0.12 mM NADH, 1.0 mM ADP and 14 mM 2-oxoglutarate;

ALT : 200 mM L-alanine, 0.18 mM NADH, 25μM pyridoxal 5'-phosphate, 12 units/ml lactate dehydrogenase (in glycerol), 10.5 mM 2-oxoglutarate;

AST : 40 mM L-aspartate, 0.24 mM NADH, 25 μM pyridoxal 5'-phosphate, 8 units/ml malate dehydrogenase and 7.0 mM 2-oxoglutarate.

The design for this experiment was factorial, with three factors being tested at two levels for each factor. The factors tested were; ammonia (0 and 10 mg N/L), diet (fed at 2% body weight and unfed for two days prior to beginning of experiment) and time (6 and 48-h of ammonia exposure). Each factor was tested in combination with the other factor at all levels. Two replicates were tested per treatment combination. No significant difference was found between replicates when plasma ammonia and cortisol were compared and based on this, replicate tissue samples were randomly divided for measurement of either metabolites or enzymes. A factorial method makes it possible to consider the interaction effects of two or more factors, yet allows the main effects to be tested with accuracy if no interactions are found (Steel and Torrie, 1980). If significant interactions are found it is difficult to draw conclusions based on the main effects. In these cases the data were plotted as LS means averaged over any non-interacting factors and the significance of the interactions were considered. In the case of no interactions, but significant main effects, Bonferroni's test was used to detect the differences between groups. Mean values are presented ± the standard error of the mean (SEM) unless otherwise noted. Significant differences were noted when $p<0.05$.

3.0 Results

Mean water ammonia levels were measured at 9.93 ± (0.12) mg N/L and water pH remained constant at 7.2 during the course of the experiment. Fish continued to consume food at a rate of 2% body weight daily after the onset of ammonia treatment. Stomachs of sampled fish contained food and gall bladders were not enlarged. No mortality was noted at any of the treatment levels.

A significant interaction was noted between ammonia, diet and time ($p=0.001$) for plasma ammonia concentrations making unambiguous interpretation of the main effects difficult. Diet and ammonia showed a significant two-way interaction when measurements were averaged over both sampling times. Plasma ammonia was higher in fed than unfed fish at both ammonia levels ($p<0.05$, Figure 2A). It was determined that the diet and ammonia interaction

for plasma ammonia was significant ($p<0.001$) at 6-h; however, after 48-h there was no interaction ($p=0.393$). The mean plasma ammonia level in fed fish after 6-h was significantly higher in the ammonia treated fish than in the control fish at 14.43 ±(1.31) and 5.22 ±(0.53) μg/mL respectively ($p<0.05$, Figure 2B). Of the unfed groups, ammonia-exposed and control, the ammonia-exposed fish had significantly higher plasma ammonia levels after 6-h at 6.78 ±(0.57) than the control at 2.68 ±(0.19) μg/mL. Both treatment groups of unfed fish at the 6-h sampling time consistently had significantly lower plasma ammonia levels than the corresponding fed treatment groups (Figure 2B). After 48-h of ammonia exposure, the plasma ammonia of the exposed fed fish decreased significantly from 14.43 ±(1.31) to 5.85 ±(0.69) μg/mL (Figure 2B), a value that was not significantly different from the fed control or the unfed fish exposed to ammonia, which remained unchanged after 48-h at 5.48 ±(0.50) μg/mL (Figure 2B).

Plasma cortisol, after allowing for the effects of diet and time, was affected by water ammonia ($p<0.001$). Plasma cortisol significantly increased with ammonia exposure from 112.2 ±(5.2) ng/mL in the control fish to 186.1 ±(5.2) ng/mL in the ammonia-exposed fish when averaged over time and diet. It was determined that the effect of ammonia on plasma cortisol depended on the duration of exposure ($p<0.001$) but was not affected by feeding (Figure 3). After 6-h the plasma cortisol of fish exposed to ammonia was 78.2 ±(7.4) ng/mL but increased to 293.8 ±(7.4) ng/mL after 48-h. No relationship was noted between plasma ammonia and cortisol (Figure 3).

Combinations of diet, water ammonia and time did not affect brain glutamine and glutamate. Brain glutamine was however, affected individually by both diet and presence of ammonia in the water. The mean level of glutamine was significantly higher ($p<0.001$, Figure 4A) in ammonia-treated fish than control fish at 5.33 and 3.64 ±(0.1) μmol/g tissue, respectively, when averaged over fed and unfed fish. Glutamine was also significantly higher ($p=0.039$, Figure 4A) in fed than unfed fish when averaged over ammonia and control groups at 4.93 and 4.05 ±(0.1) μmol/g tissue, respectively. Brain glutamate showed the opposite trend with levels decreasing significantly ($p<0.001$, Figure 4D) in ammonia-treated fish. No relationship was noted for brain glutamate and feeding. Brain glutamate showed a non-significant ($p=0.07$) decrease in ammonia-treated fish with time. Glutamate levels decreased

from 6.16 ±(0.1) at 6-h to 4.97 ±(0.1) at 48-h in ammonia-treated fish averaged over all diet levels.

A significant interaction was noted between the level of diet and time ($p=0.005$, Figure 4B) and the glutamine in liver tissue. When measurements were averaged over all ammonia levels fed fish had significantly higher amounts of glutamine than unfed fish at 48-h but not at 6-h. Liver glutamine was significantly higher in fed fish, 2.96 ±(0.07) μmol/g tissue, as compared to the unfed fish, 2.01 ±(0.07), when averaged over all ammonia levels ($p=0.002$, $n=24$). Water ammonia had no significant affect on liver glutamine ($p=0.115$, $n=24$) but the trend indicates that glutamine increases in ammonia-exposed fish. A significant ($p=0.013$, $n=12$) interaction was noted however, between water ammonia and time for liver glutamine. At 48-h the ammonia-treated fish had significantly higher ($p=0.07$) liver glutamine at 2.98 than the control fish at 1.81 ±(0.1) μmol/g tissue when averaged over all levels of diet, but no significant differences were noted at 6-h. Liver glutamate was not significantly affected by any of the treatments tested (Figure 4E) and no interaction effects were determined for the treatments. It is worth noting that water ammonia caused a non-significant ($p=0.053$) increase in liver glutamate level when averaged over all other treatments (Figure 4E).

Muscle glutamine showed a significant increase from 0.292 to 0.423 ±(0.02) μmol/g tissue when control and ammonia-treated fish were averaged over all other treatments ($p=0.046$, Figure 4C), but glutamate levels did not change. There was an interaction between ammonia and diet for muscle glutamate (Figure 4F) with levels increasing in fed fish with increased ammonia exposure. In unfed fish, ammonia exposure caused no change in glutamate levels.

A summary of enzyme activities is given in Table 1. No significant change in activity was noted in the any of the tissues sampled for glutaminase or GDH. The activity of glutaminase was highest in the brain, then liver and finally muscle. For GDH, activity was highest in the liver, followed by brain and muscle. ALT activity was highest in the liver followed by brain and muscle. Activity of ALT in the liver increased significantly by 20% in fed compared to control fish when averaged over both ammonia and time. AST activity was highest in the brain, then liver and finally muscle. Activity of AST increased with ammonia exposure by about 20% in the brain, but decreased with ammonia exposure in the muscle by 50% when averaged over all levels of feeding and time. A significant interaction between feeding and ammonia was found for AST in muscle, with feeding and ammonia having a synergistic effect

on AST levels (Figure 5C). In fact AST activity decreased by half in fed fish exposed to ammonia and by only 10% in unfed ammonia exposed fish. GSase activity was highest in the brain, followed by liver and muscle. Activity of GSase in the brain increased significantly with exposure to ammonia by 38%, but did not change with feeding. A significant interaction was noted however between feeding and time for brain GSase, with GSase levels increasing with increased time without food and decreasing with increased time with food (Figure 5A). Liver GSase increased by over 100% with ammonia exposure and there was a significant interaction between ammonia level and duration of exposure (Figure 5B). Muscle GSase activity decreased significantly with feeding by more than 30%, but not with ammonia exposure with only a 20% increase.

4.0 Discussion

The plasma ammonia levels reported here fell into the range reported by other researchers (Vedel et al., 1998; Cameron and Heisler, 1983; Wilson and Taylor, 1992). These results indicate that fed fish exposed to ammonia have to deal with both the movement of ammonia into their bodies and/or the reduced ability to excrete ammonia, as well as excess ammonia produced during the catabolism of dietary protein. Ammonia exposure and feeding were shown to interact significantly with respect to plasma ammonia levels in the first 6-h of exposure, but not after 48-h.

The level of ammonia that the rainbow trout were exposed to in this study was sufficient to cause a stress response, as indicated by the increase in plasma cortisol after 48-h in both the fed and unfed fish. Contrary to what might be expected, after 6-h exposure, although plasma ammonia was elevated, there was no significant increase in plasma cortisol. Typically in fish exposed to an acute stress, plasma cortisol becomes elevated quite rapidly, in the order of minutes (Barton et al., 1980) and the increase is proportional to the level of the stress. In the present study both the fed and unfed fish exposed to ammonia showed no change in cortisol concentration after 6-h of exposure, but did after 48-h. Interestingly, the highest cortisol level was in the ammonia-exposed unfed fish. This suggests that either the elevated ammonia or increased metabolism due to feeding may inhibit the early cortisol response in these fish. Inhibition of cortisol would be beneficial under conditions when internal ammonia levels are

elevated as even small increases in cortisol are known to increase metabolic activity thus indirectly increasing internal ammonia (Mommsen et al., 1999).

Increased levels of brain glutamine and subsequent decreases in glutamate have been previously noted in fish exposed to elevated ammonia (Levi et al., 1974; Arillo et al., 1981; Iwata, 1988; Vedel et al., 1998). This is not surprising as the brain is the most sensitive organ to ammonia toxicity (Cooper and Plum, 1987), however it is interesting that brain glutamine levels increase under feeding conditions in rainbow trout suggesting they deal with detoxification of ammonia in the brain on a routine basis. This suggests that the amount of ammonia resulting from catabolism of ingested protein is not completely dealt with in the liver, resulting in elevated ammonia in the blood stream. Once the ammonia crosses the blood-brain barrier, the brain is then dealing with the ammonia by converting it to neutral glutamine. The conversion of excess ammonia to glutamate via GDH and then glutamine via GSase would allow fish to store the potentially toxic ammonia in a neutral form until circumstances allow the fish to excrete excess ammonia by diffusion or the glutamine is utilized in other metabolic pathways.

Corresponding to the increase in brain and liver glutamine the levels of GSase in these tissues also increase significantly with ammonia exposure and non-significantly with feeding. The interaction between feeding and time for brain GSase (Figure 8) suggests that feeding keeps GSase levels lower than noted in unfed fish when feeding stops for 48-h. It is probable that feeding causes sufficient increase in ammonia and glutamate concentrations in the brain and liver that synthesis of GSase is upregulated. The increased synthesis of GSase likely provides enough GSase to cope with the ammonia produced with feeding as well as the ammonia that accumulates when excretion is impaired. This type of response has been noted in the liver of rats fed high protein diets (Boon et al., 1999) with the concentration of GSase mRNA increasing with increasing protein levels in the diet. The upregulation of GSase allowing for increased storage of ammonia as glutamine and regulation of glutamate concentrations is the probable explanation for the protection of fed rainbow trout in an acute ammonia challenge noted in Chapter 2. It is also probable that once this system is either sufficient to cope with ammonia levels and/or is challenged that the plasma cortisol becomes elevated in order to provide more energy to meet the stress of an ambient ammonia challenge. The question remains in fish however whether the protein level in the diet is a key factor in this response

Increased internal ammonia concentrations could increase both the synthesis of glutamate via GDH and glutamine via GSase. Glutamate concentration in brain tissue decreased significantly in this study corresponding to increased GSase activity, in response to feeding and under conditions of elevated ambient ammonia that impair ammonia excretion. The connections between glutamate concentrations in the brain and ammonia toxicity are not clear. In a study conducted by Marcaida et al. (1992) where one type of glutamate receptor, the NMDA receptors were blocked in mice, ammonia toxicity was reduced, although the characteristic convulsions associated with ammonia toxicity still occurred. The activation of the NMDA receptors in the brain was associated with accumulation of glutamate (Albrecht, 1998), which caused Ca^{2+} release eventually resulting in cell death (Marcaida et al., 1992). If glutamate activation of the NMDA receptors is the mode of action for ammonia toxicity then preventing accumulation of glutamate by up-regulating GSase activity and converting excess glutamate to glutamine could prevent ammonia toxicity. This could however, create a second problem, as glutamate is required in the formation of GABA an inhibitory neurotransmitter (Stryer, 1988). Reduced levels of GABA in fish brains were noted with increased ammonia in a study by Arillo et al., (1981). Thus, it is apparent that there could be both an upper and lower threshold for glutamate concentrations in the brain and regulation of glutamate within these thresholds is a key factor in surviving ammonia exposure for fish.

In the liver, although glutamine levels increased significantly due to feeding and non-significantly with ammonia, glutamate levels did not decrease. It is possible this is due to the increase in glutamate provided by the conversion of alanine + oxoglutarate via ALT. This is supported by a significantly elevated concentration of ALT in the liver of fed fish (Table1) and a non-significant increase in ammonia-exposed fish. The limitations of this assumption need to be mentioned, as the concentration of alanine was not measured in this study. In addition, it is possible that GDH was upregulated and subsequently used to provide the glutamate. GDH mRNA levels increased with increased protein in a study on rats by Boon et al. (1999), supporting this hypothesis.

Muscle GSase activity decreased with feeding, but not with ammonia exposure. More importantly glutamine levels in the muscle increased significantly with ammonia exposure. Although, the glutamine concentrations were substantially lower in the muscle than either the brain or liver, the fact that muscle tissue makes up approximately 60% of the fish means the

increase represents a significant amount of glutamine storage. Using the example of a 40 g fish that is both feeding and exposed to 10 mg N/L for 48-h and approximate tissue proportions of: blood 3%, muscle 60%, liver 4% and brain 0.5% of whole body mass, the following calculations can be made based on the present data. Plasma ammonia would decrease 5.8 μmol in all tissues and glutamine would increase by 5.1 μmol. Since every μmol of glutamine formed requires 2 μmol of NH_4^+, the increase in glutamine would more than account for the decrease in circulating ammonia. ALT activity showed no change with ammonia or feeding. Similar results and activities for GSase and ALT were noted by Linton et al., (1998) in rainbow trout exposed to low levels of ammonia. Muscle AST activity however, decreased by more than 30 % with ammonia exposure in contrast to results found by Linton et al., (1998) who reported an increase in muscle AST with ammonia exposure after 420 days.

In summary, it appears that the liver and the brain of rainbow trout both have systems that can regulate detoxification of ammonia following feeding and exposure to ambient ammonia. This system appears to consist of increased activity of GSase in both the brain and liver, which allows excess ammonia and glutamate to be converted to glutamine, which is then transported to the muscle for temporary storage. This mechanism would allow rainbow trout to consume high protein diets without self intoxification and even protect them to some degree from increased ambient ammonia but, is by no means able to cope with the quantity of ammonia that *P. schlosseri* (Peng et al., 1998) or the marble goby, *O. marmortatus* (Jow et al., 1999) does.

Table 1. Activities (μmol min^{-1} g $tissue^{-1}$) of glutaminase, glutamate dehydrogenase (GDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glutamine synthetase (GSase) in the brain, liver and muscle tissues of fed and unfed (2% b.w., 48% protein, daily) rainbow trout exposed to ammonia (0 and 10 mg N/L at pH 7.2).

Tissues	Enzyme	Fed	Unfed	0 mg N/L	10 mg N/L	S.E.M
Brain	Glutaminase	0.869	0.801	0.869	0.789	0.01
	GDH	3.18	3.30	3.29	3.19	0.02
	ALT	2.64	2.50	2.50	2.64	0.03
	AST	62.7	65.8	58.8[a]	69.7[b]	0.6
	GSase	7.42	8.26	6.58[a]	9.10[b]	0.02
Liver	Glutaminase	0.144	0.141	0.138	0.148	0.002
	GDH	27.2	30.4	27.3	30.3	0.6
	ALT	60.1	50.6*	52.8	57.9	1.1
	AST	33.8	33.3	32.2	34.9	0.6
	GSase	0.0598	0.0753	0.0442[a]	0.0909[b]	0.0001
Muscle	Glutaminase	0.0505	0.0403	0.0519	0.0429	0.001
	GDH	1.01	0.915	1.03	0.889	0.04
	ALT	1.63	1.53	1.49	1.67	0.04
	AST	19.6	23.6	25.9[a]	17.2[b]	0.6
	GSase	0.00638	0.00846*	0.00673	0.00810	0.0002

Results represent the LS means averaged over 6 and 48 hour sampling times (n=24) with the corresponding S.E.M. * indicates a significant difference ($p<0.05$) between fed and unfed fish and [a] between 0 and 10 mg N/L. When analysis of variance (3-factor) indicated significant interactions (2 or 3 way) between ammonia concentrations, feeding or time, the results are indicated by bold typeface and further examined in the results section.

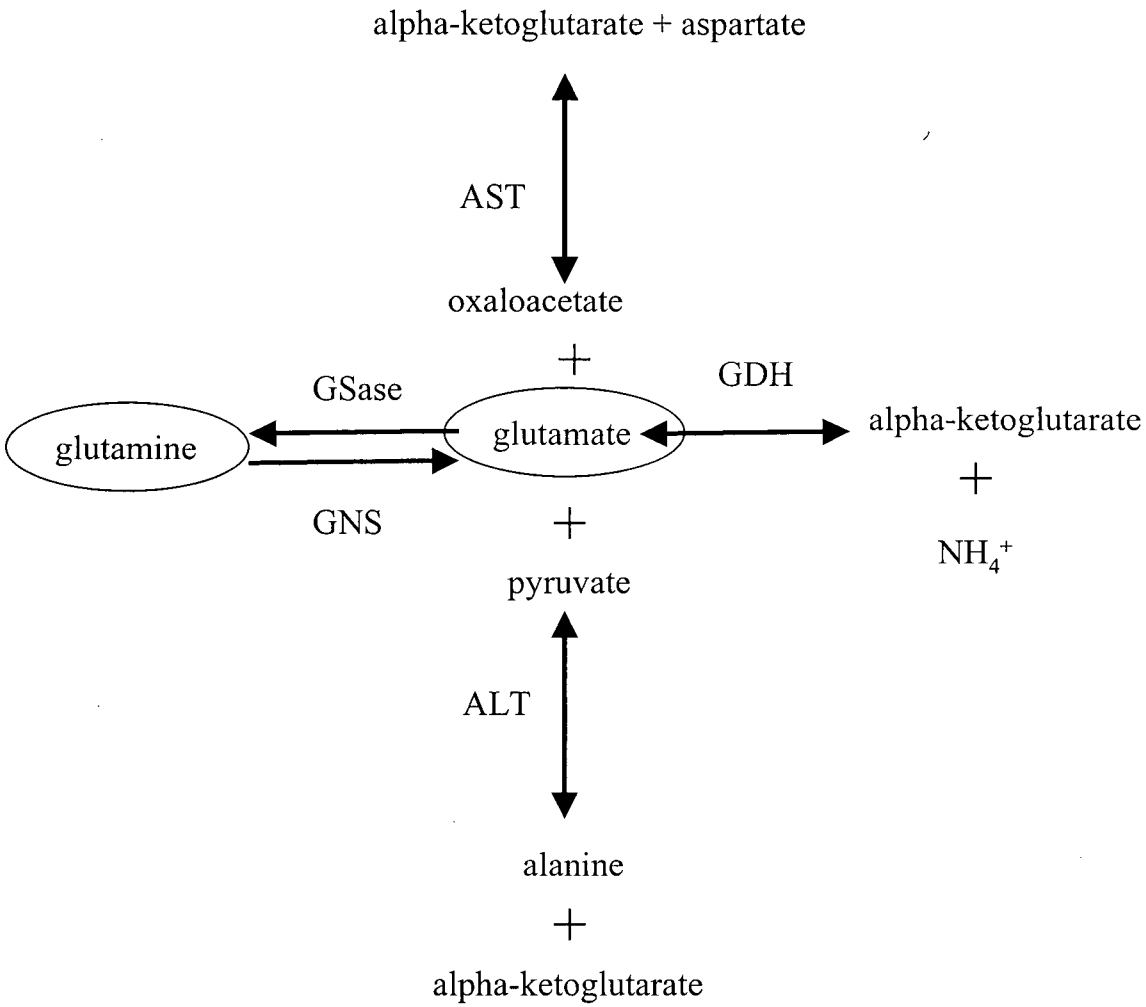

Figure 1. The metabolic pathways for glutamate and glutamine. The enzyme abbreviations are GNS-glutaminase, GSase- glutamine synthetase, GDH-glutamate dehydrogenase, ALT-alanine aminotransferase and AST-aspartate aminotransferase.

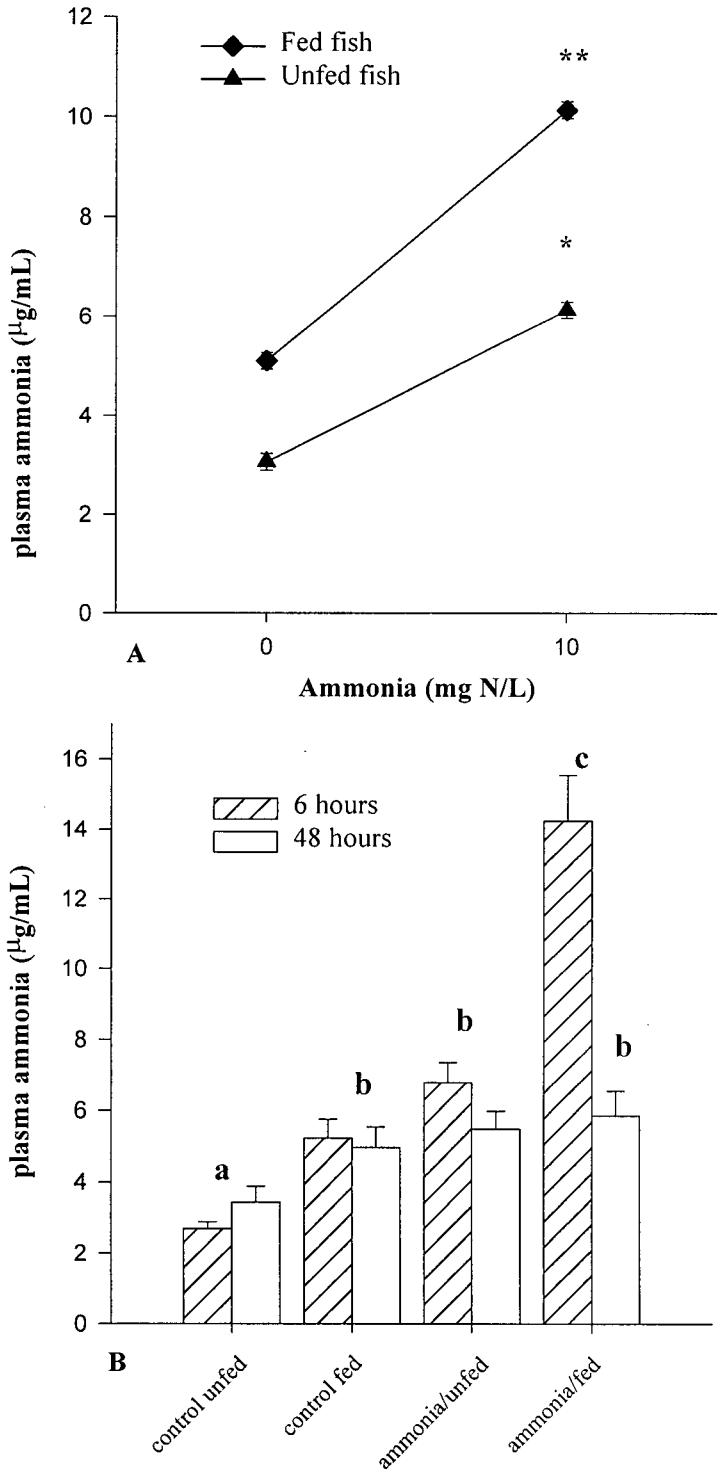

Figure 2. A) Mean plasma ammonia (μg/mL) in fed and unfed rainbow trout that were exposed to 10 mg N/L at pH 7.2 averaged over 6 and 48 hours. B) Mean plasma ammonia at 6 and 48 hours under the same conditions as Figure 1A. Bars indicate the standard error about the mean. Significant differences ($p<0.05$, $n=6$) between fed and unfed fish are indicated by *, by ** for control and 10 mg N/L in Figure 1A and by alphabetical letters for Figure 1B.

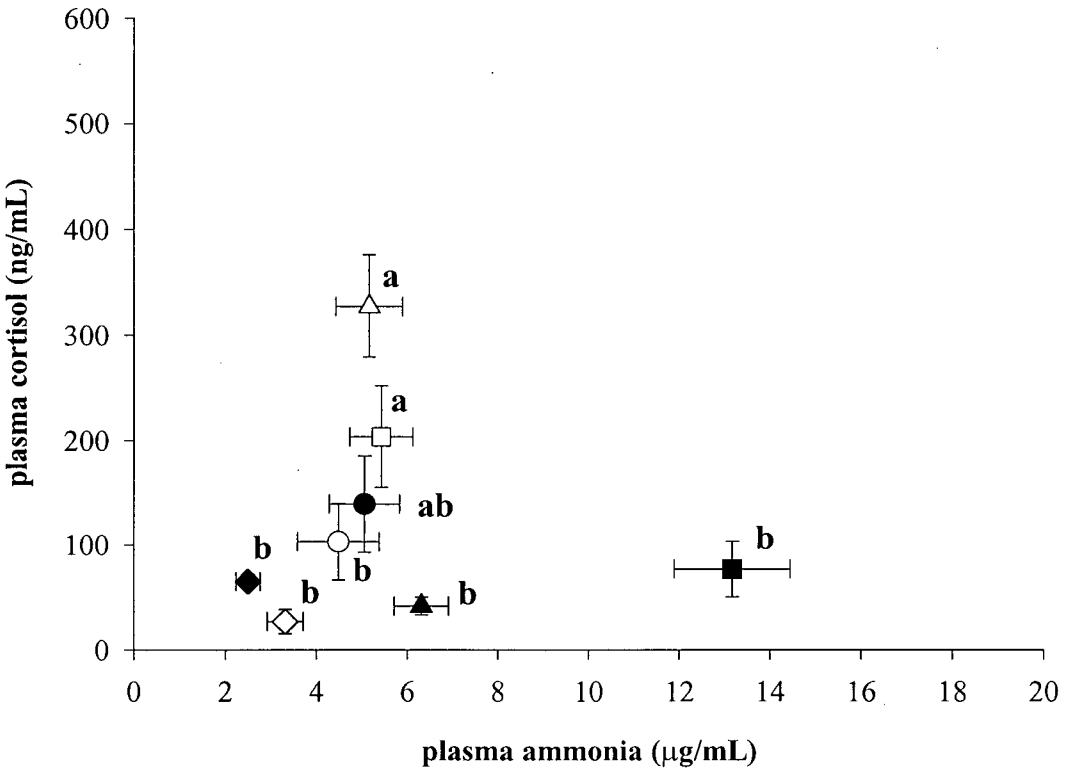

Figure 3. Relationship between plasma ammonia (μg/mL) and plasma cortisol (ng/mL) in fed and unfed rainbow trout exposed to ammonia (control and 10 mg N/L) for 6 and 48 hours at pH 7.2. Bars represent one S.E.M. Symbols represent the following treatments: squares-ammonia/fed, triangles-ammonia/unfed, circles-control/fed, diamonds-control/unfed and solid and hollow symbols were samples after 6 and 48 hours post ammonia exposure, respectively. Significant differences are indicated by a change in letter.

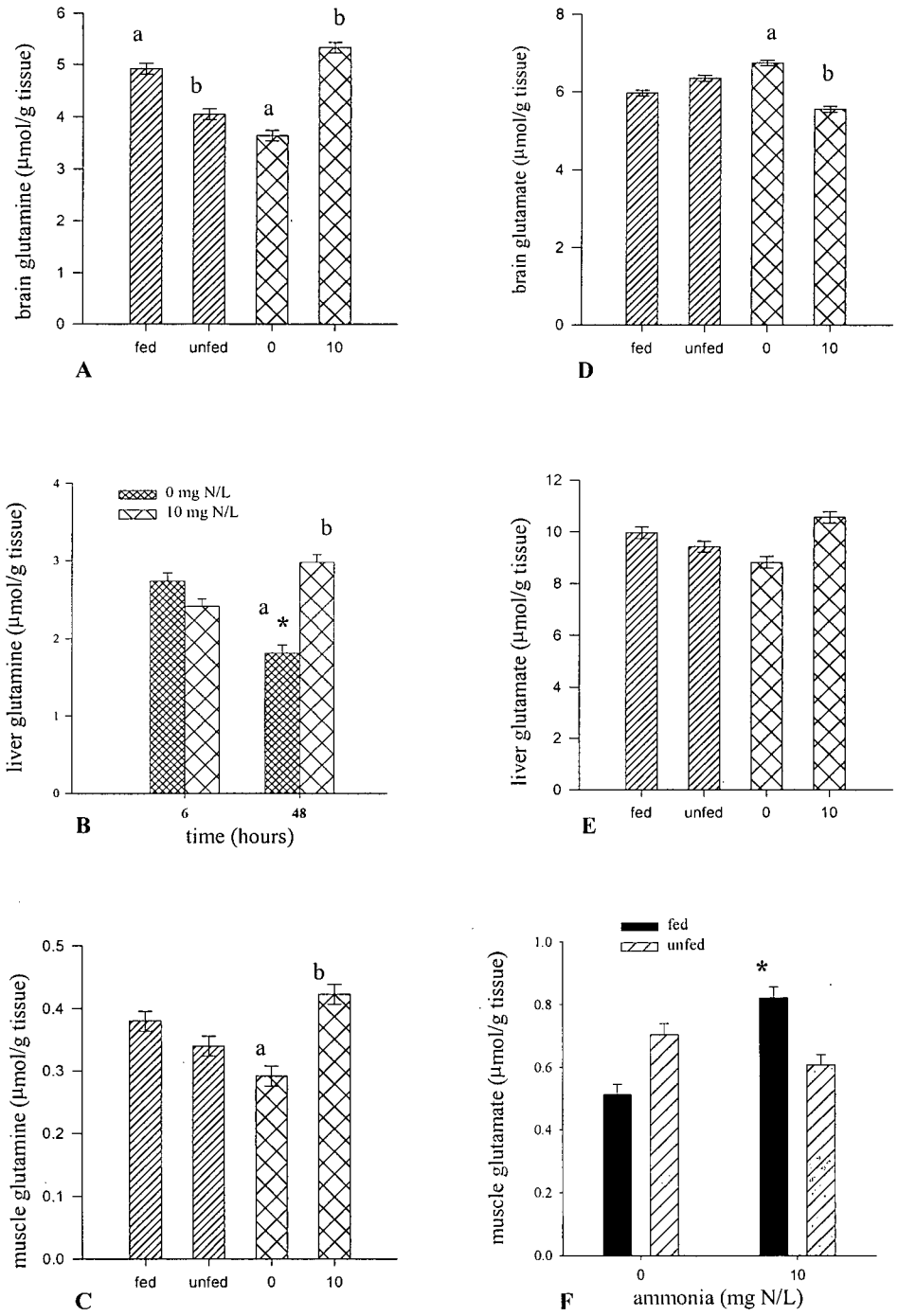

Figure 4. Brain, liver and muscle glutamine (µmol/g tissue) in rainbow trout exposed to ammonia (0 & 10 mg N/L, coarse) and either fed or not fed (fine fill). Bars are the LS mean averaged over sampling periods and either ammonia or feeding level for graphs A,C-E. Significant differences ($p<0.05$, n=24 (A,C-E) n=12 (B,F)) within treatments are indicated by a change in letter and between by *. B) LS mean for liver glutamine averaged over feeding levels. F) LS mean for muscle glutamate averaged over time.

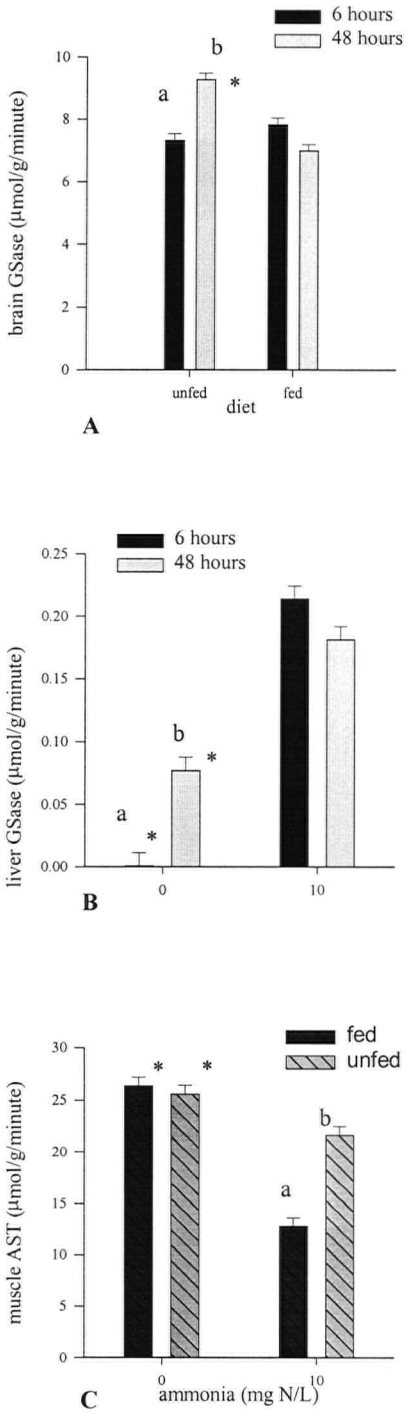

Figure 5. Interaction between feeding and time (6 & 48 hours) for brain GSase (µmol/g tissue/min.)(A) ammonia (0 & 10 mg N/L) and time for liver GSase (B) and feeding and ammonia for muscle AST (C) in ammonia-exposed rainbow trout. Data are presented as LS means averaged over the non-interacting factor. Bars represent the SEM and a significant differences ($p<0.05$, $n=12$) within comparable treatments are indicated by a change in letter and among treatments by *.

Chapter 4. The effects of dietary protein concentration on ammonia metabolism in rainbow trout under control and elevated ammonia conditions.

1.0 Introduction

There are two sources of protein catabolized in fish, protein provided by the ingestion of food and protein that comes from body tissues. The breakdown of both types of protein results in the production of amino groups. Amino groups in excess of the organism's requirements must be excreted as a waste product (Mommsen and Walsh, 1991). The majority of fish excrete this waste as ammonia across the gill membrane. This method of excretion is dependent on the existence of a concentration gradient between the fish and the water. Under conditions of elevated ambient ammonia fish still depend on diffusion to excrete nitrogenous wastes, consequently they can experience ammonia-loading conditions due to their inability to excrete waste and/or movement of ammonia from the environment into the fish. Ammonia loading from the environment depends on the ambient water conditions including ammonia concentration, pH, and hardness (Ip et al., 2001b) Endogenous ammonia concentrations depend on the rate of protein turnover (Medale et al., 1995), as well as the ability of the fish to rid itself of excess ammonia. The ammonia can be excreted or converted to less toxic compounds. Fish use a variety of mechanisms to decrease ammonia toxicity (Ip et al., 2001b) including conversion to glutamine (Levi et al., 1974; Arillo et al., 1981; Chapter 3). In rainbow trout, *Oncorhynchus mykiss*, the level of brain glutamine increases when fish are exposed to elevated ambient ammonia (Vedel et al., 1998) or following feeding (Chapter 3). The increase in glutamine coincides with an increase in glutamine synthetase (GSase) activity suggesting that rainbow trout are capable of detoxifying ammonia in the brain to some degree.

Due to the economic importance of rainbow trout as an aquaculture species, much research has been conducted on their dietary requirements in order that maximum growth with minimal water fouling is achieved. The suggested protein content of a salmonid diet is between 40 and 55% crude protein (Jobling, 1994). This requirement assumes adequate and appropriate sources of non-protein energy and an optimal ratio of digestible protein to digestible energy in the diet. For rainbow trout, diets containing 40% protein have been shown to be optimal, but higher protein can be used if feeding is restricted (Cho et al., 1976). The protein content of the commercial diet used in the previous study was 48% (Chapter 3) which, was well within the

suggested guidelines, but above the suggested optimum; yet fish fed this diet at a rate of 2% body weight daily experienced toxic ammonia concentrations. The purpose of the present research was to determine how the level of dietary protein alone and in combination with elevated ambient ammonia affected amino acid synthesis particularly the GSase pathway in rainbow trout.

2.0 Methods

The rainbow trout used for this study were purchased from a local fish farm, Spring Valley Trout Farm, Langley, British Columbia. Fish were maintained in circular outdoor tanks for 3 weeks prior to being moved to 60 L indoor tanks. Both indoor and outdoor tanks were supplied with aerated, dechlorinated Vancouver city water. Fish were maintained in the indoor experimental tanks, with 12 fish (36 ±2.75 g) in each of eight tanks, for two weeks before the experiment commenced. Food (Nutra Fry, Moore-Clark, 48% crude protein) was provided at a rate of 2% body weight at 8:00 am daily. Fish were allowed 30 minutes to consume food and then tanks were cleaned and excess food was removed through food traps. Light was controlled on a 12:12 hour light/dark cycle.

The indoor tank system was designed to provide precisely one litre of water/minute to each of the 12 experimental tanks. A current was created in the tanks using a spray bar to deliver the water across the surface. The desired experimental water pH was achieved by buffering the water supply in the head tank with sodium bicarbonate. Water pH was maintained at 7.3 for 5 days prior to the commencement of the ammonia treatments. Addition of sodium bicarbonate was shown not to affect rainbow trout in a previous study (Wicks and Randall, 2001; Chapter 2). Water temperature was 9 ^{o}C and water dissolved O_2 was never below 87 % saturation.

Two days prior to the commencement of the experiment a custom diet was prepared in the laboratory. A food base was created using a 1:1 ratio of carboxyl methylcellulose and food grade cornstarch. A Bradford protein assay was performed on this food base and it was determined that the content of protein/amino acids in the base was below the level of detection. The base was mixed with an appropriate quantity of ground commercial food (Nutra Fry, Moore Clark, 48% crude protein) to obtain a dry mixture containing either 1% or 40 % crude protein. The dry mix was then formed into a dough by addition of about equal parts of distilled H_20 and

then hand rolled, cut into pellet sized pieces and left to air dry for 48-h. This diet had been previously tested for palatability on a non-experimental group of rainbow trout. Test fish showed no preference for pellet type. Note that this is not a diet that would be formulated for aquaculture studies, but is thought to be appropriate for a short-term physiological study.

Food was withheld from the fish 24-h prior to the beginning of the experiment. On the day the experiment commenced, 4 tanks of fish were fed the 1% protein diet and 4 tanks of fish were fed the 40 % protein diet. Feeding commenced at 0800 hr with each tank staggered by 15 minutes. Fish were given 30 minutes to consume food and then excess was removed through food traps to minimize disturbance. Excess pellets were counted and recorded to determine feeding rates. Ammonia treatments in the fed tanks commenced between 0900 and 1000 hr, corresponding to one hour after feeding. The four tanks of unfed fish started ammonia treatment at 0800 hr with start times staggered by fifteen minutes. Ammonia levels in the tanks were increased instantaneously to 10 mg N/L with the addition of 2.48 g of NH_4Cl dissolved in 60 L of water one hour after feeding. Ammonia was added to two replicates of each diet (1 and 40% protein). The remaining 4 tanks were controls, 2 for each diet. The concentration of the ammonia in the tanks was held constant by the addition of NH_4Cl by peristaltic pump (Masterflex). The ammonia added to the tanks decreased the pH from 7.3 to 7.2, which then remained constant during the experiment.

Water pH, temperature and dissolved oxygen was monitored daily in each tank during the acclimation period and during testing. Ammonia levels were monitored daily, always 6-h after feeding, corresponding to peak excretion, (Chapter 2; Gelineau et al., 1998) in order that maximum ammonia levels were reported. Water ammonia was measured using a modification of the Verdouw et al., (1978) method substituting nitroprusside (20 mg/L) for ferrocyanide.

Terminal sampling of 6 fish per tank occurred at 6 and 48-h after ammonia exposure commenced. This corresponded to 7-h after feeding. Fish were killed by concussion, weighed and measured, and then blood was collected from the caudal vein. Brain, liver and muscle tissue was collected and immediately frozen in dry ice for later determination of glutamate, glutamine and enzymes. Stomach content and the size of the gallbladder were qualitatively noted to establish if the fish had eaten prior to sampling. The 6 fish from each tank were sampled in less than 15 minutes with as little disturbance as possible. From the blood samples, plasma was

collected and alliquotted for determination of cortisol and ammonia. Both the tissue and plasma samples were frozen at –80 °C for later measurement of parameters.

Plasma ammonia was measured using the L-GLDH/NADH kit (Sigma 171-UV) and plasma cortisol using a modification of the Neogen (Lexington, Kentucky, USA) ELISA kit. Ether extraction was not performed on the plasma prior to measuring cortisol. Six tissue samples were randomly selected from the pooled treatment replicates for determination of glutamate and glutamine and the remaining 6 were used for enzyme measurements. For determination of glutamate/glutamine, tissue samples were homogenized in 5 times their volume with cold 0.3 M perchloric acid for 45 seconds and then centrifuged at 6000 x *g* at 4 °C for 3 minutes. The supernatant was removed and neutralized with 1.5 M K_2CO_3 and then cooled on ice for 30 minutes. The neutralized supernatant was then centrifuged for 30 seconds at 16,000 rpm and the subsequent supernatant removed and frozen at –80°C for later determination of glutamate and glutamine. Glutamate and glutamine concentrations were determined enzymatically by deamination of L-glutamine and dehydrogenation of L-glutamate using a micro modification of Sigma kit GLN-1.

The 6 remaining tissue samples from each treatment combination were homogenized for enzyme determinations using the following method. Frozen tissue samples were weighed and homogenized in 10 volumes of glycerol buffer and 1 μL/mL of buffer of 10 mg/mL aprotinin according to Mommsen and Hochachka (1994). The homogenate was then sonicated and centrifuged at 6, 000 x *g* for 30 seconds. The supernatant was removed and frozen at –80 °C for later determination of GSase, glutaminase, GDH, ALT and AST. GSase (reverse direction) was assayed using the arsenolysis of glutamine to γ-glutamyl hydroxamate measured at 540 nm. Glutaminase activity was measured following the conversion of glutamine in the sample to glutamate and measuring the quantity of glutamate formed. GDH, ALT and AST activities were determined in the homogenates of all the tissues by continually measuring the decrease in absorbance (340 nm) as NADH was converted to NAD. For complete methods for all enzyme measurements see (Chapter 3).

The experiment was constructed as 2x2x2 factorial array. Factorial treatments had either fish fed a diet containing 1 or 40 % crude protein, which were then subjected to either 0, or 10 mg N/L for a period of 6 or 48-h. Two replicates (n=6 per replicate) were tested per treatment combination. Since no significant difference was found between ammonia levels in the tanks or

between replicates when plasma ammonia and cortisol were compared, tissue samples were pooled between replicates and then randomly divided for measurement of either metabolites or enzymes. A 3-way ANOVA was performed for each set of data to determine significant main effects and interactions. When interactions between factors were noted, data were plotted as LS means averaged over any non-interacting factors and the significance of the interactions were considered. In the case of no interactions, but significant main effects, Bonferroni's test was used to detect the differences between groups. Mean values are presented ± the standard error of the mean unless otherwise noted. Significant differences were noted when $p<0.05$.

3.0 Results

No mortality occurred as a result of any of the treatments. Water parameters were maintained constant for the duration of the experiment at the following levels; temperature 9.2°C, pH 7.19, dissolved O_2 87% saturation, ammonia treated tanks, 10.6 ±0.32 mg N/L, control tanks, 0.16 ±0.003 mg N/L. Food consumption was not altered by the addition of ammonia. The intestines of sampled fish contained food and the gall bladders of all but 3 fish were empty at the time of sampling indicating that the majority of the fish consumed the homemade diets under both elevated ammonia and control conditions.

Plasma ammonia concentration was significantly elevated by exposure to ammonia. The plasma ammonia level was non-significantly higher in fish fed a diet containing 40% crude protein 6-h after the onset of ammonia exposure, relative to those fed the diet with 1% crude protein (Figure 1A). Both ammonia-exposed groups had plasma ammonia levels that were significantly higher than the corresponding control groups at 6-h. After 48-h of ammonia exposure, the plasma ammonia levels in the 10 mg N/L ammonia/high protein group decreased significantly (Figure 1A). The fish fed a low protein diet and exposed to ammonia showed a non-significant decrease in plasma ammonia with time, but this level was significantly higher than observed in both the high protein/ammonia-exposed fish and the corresponding control group. A significant interaction was noted between dietary protein level and time (Figure 1B). At the 6-h sample period fish fed a diet with 40% crude protein had significantly higher plasma ammonia than noted for fish fed the diet with1% protein; however, after 48-h there was no significant difference between the groups of fish. The plasma ammonia levels in fish fed the

high protein diet decreased from 8.55 to 4.46 ±0.21 μg/mL between the 6 and 48-h sampling period when averaged over ambient ammonia levels yet daily food consumption did not change. The plasma ammonia levels of fish fed 1% protein did not change with time.

It was determined that a significant interaction existed between both time and ammonia and dietary protein level and ammonia for the measured plasma cortisol concentrations. The concentration of plasma cortisol increased significantly with time when fish were exposed to ammonia. After 6-h of ammonia exposure, plasma cortisol was 66.5, but increased to 119.8 ±5.6 ng/mL after 48-h. There was no significant difference in the cortisol concentrations in the control fish with time. The interaction between dietary crude protein concentration and ammonia exposure for cortisol is depicted in Figure 2. No significant differences were found between cortisol concentrations in control fish fed diets with different levels of crude protein, with the concentrations of 76.4 and 95.1 ±5.6 ng/mL for fish fed diets with 1 and 40 % crude protein respectively. In ammonia-exposed fish, cortisol levels were significantly higher ($p<0.05$, $n=24$), 128.1 ±5.6 ng/mL, in the fish fed a diet containing 1% crude protein, than in the fish fed the diet with 40% protein (58.2 ±5.6 ng/mL). In addition, fish fed a diet containing 40 % crude protein showed no significant change in cortisol level with ammonia exposure, yet the fish fed the diet with 1% protein showed a significant increase from 76.4 to 128.1 ±5.6 ng/mL.

Glutamine levels were found to be highest in brain tissue, followed by liver and then muscle. (The following descriptions for tissue glutamine and glutamate refer to the main effects and are LS means averaged over all levels of the factors not being discussed. Interaction effects are described where appropriate). Under high ammonia conditions, the glutamine levels in the brain increased significantly from 3.75 to 5.19 ±0.062 μmol/g tissue (Figure 3A), but did not change with the level of dietary protein. Liver glutamine concentration increased with both ammonia exposure and increased level of dietary protein (Figure 3B). Under high ammonia conditions, the glutamine levels in the liver increased significantly from 1.78 to 2.70 ±0.069 μmol/g tissue. The increase due to dietary protein content was not as substantial with levels increasing from 1.96 in fish fed the diet with 1% protein to 2.52 ±0.069 in those fed the diet with 40% protein, but the increase was still significant. There were no significant main effects for muscle glutamine (Figure 3C), however, there was a significant interaction between ammonia exposure and dietary protein level. In fish fed a diet with high protein content there

was no change in glutamine levels between ammonia-exposed and control fish, with concentrations of 0.557 and 0.514 ±0.019 μmol/g tissue, respectively. In fish fed a diet with low protein content glutamine levels decreased significantly with ammonia exposure, from 0.547 in the control group to 0.327 ±0.019 μmol/g tissue in the ammonia-exposed group.

The glutamate levels measured were highest in the liver followed by the brain and then the muscle tissue. Glutamate levels in the brain decreased significantly with ammonia exposure and increased significantly with dietary protein level (Figure 3D). The decrease in brain glutamate due to ammonia exposure corresponds to the increase in brain glutamine already noted. There was no change in liver glutamate with either ammonia exposure or change in dietary protein level (Figure 3E). There were no significant main effects for muscle glutamate, but a significant interaction existed between ammonia and dietary protein content (Figure 3F). In control fish, the highest glutamate level, 1.02 ±0.031 μmol/g tissue, was found in fish fed the diet with 1% protein. The control/high protein fish had a significantly lower muscle glutamate level, 0.417 ±0.031 μmol/g tissue. When these 2 groups of fish were exposed to elevated ambient ammonia the opposite trend was observed. Fish fed the diet with high protein content under elevated ammonia conditions had more than twice the concentration of muscle glutamate, 1.00 ±0.031 μmol/g tissue, than those fed a low protein diet at 0.487 ±0.031 μmol/g tissue (Figure 3F).

Table 1 provides a summary of the main effects and the occurrence of interactions between factors for the enzyme data for all tissues. A significant interaction was noted between protein and ammonia for brain AST (Figure 4A). AST activity was significantly higher in fish fed the diet with 40% protein than in fish fed the diet with 1% protein under control conditions. Under elevated ammonia conditions the opposite effect was observed (Figure 4A). In liver tissue, no significant change was noted in GDH, ALT, AST and GSase activity with any of the treatments. Liver glutaminase decreased significantly from 0.145 to 0.126 ±0.002 μmol/g tissue/minute when dietary protein level was decreased from 40 to 1%. Muscle glutaminase, AST and GSase showed no significant change in activity with any of the treatments. Both ALT (Figure 4B) and GDH (Figure 4C) activity in the muscle showed an interaction effect between ammonia level and dietary protein. Under control conditions, there was no significant difference between levels of dietary protein for both muscle ALT and GDH activity. When fish were exposed to elevated ammonia and fed a diet containing 40 % protein there was a synergistic

effect on ALT and GDH activity, while no change was noted in fish fed 1% protein and exposed to ammonia. The muscle ALT activity in the ammonia/40% protein group increased from 1.37 in the control ammonia group to 1.86 ±0.05 μmol/g tissue/minute. The GDH activity in the muscle increased from 0.757 in the control/40% protein group to 1.02 ±0.03 μmol/g tissue/minute in ammonia-exposed fish fed a diet with 40% protein.

4.0 Discussion

The present study was conducted in an effort to understand how the level of crude dietary protein affects the glutamine synthetase ammonia detoxification pathway in rainbow trout. Chapter 3 which considered the effect of feeding and fasting in combination with elevated ambient ammonia on nitrogen metabolism via glutamine formation, suggested that feeding up regulates the glutamine synthetase pathway in both the liver and brain tissues, resulting in the detoxification of ammonia produced during protein catabolism. It has been demonstrated in rats that high dietary protein concentration increases the level of both GDH and GSase mRNA in liver tissue to prevent excess ammonia from entering the circulatory system (Boon et al., 1999) and crossing the blood-brain barrier. Prior to recent experiments, detoxification of endogenous ammonia in teleost fish had only been identified in the marble goby, *Oxyeleotris marmoratus* (Jow et al., 1999). This fish activates glutamine synthetase in the liver to cope with ammonia produced during periods of air exposure by converting endogenous ammonia to glutamine. It is this glutamine synthetase pathway that could protect feeding rainbow trout exposed to elevated ambient ammonia (Chapter 2,3) and excessive dietary protein from ammonia toxicity.

In both this study and a previous one (Chapter 3), it was determined that the exposure of rainbow trout to 10 mg N/L ammonia at a pH of 7.2 was sufficient to cause significant increases in plasma ammonia and cortisol concentrations, indicating that these fish were experiencing conditions that impaired ammonia excretion and caused stress. In teleost fish the stress response typically involves activation of the hypothalamus-pituitary-interrenal axis, which results in the release of cortisol into the circulation (Donaldson, 1981). Exposure to 10 mg N/L was sufficient to cause an increase in plasma cortisol levels, a good indication of stress (Mommsen et al., 1999), after 48-h, signifying that this level of ambient ammonia caused stress. Interestingly, cortisol levels only increased in fish that were fed a diet containing 1% protein and, not in fish

fed a diet with 40% protein. There are two possible explanations for this observation. It is possible that fish ingesting high levels of protein have sufficient protein to provide substrates to the Krebs cycle, after deamination, to meet the energetic costs required to cope with the stress. The second possibility is that the higher level of dietary protein has up regulated the glutamine synthetase pathway, which detoxifies ammonia to glutamine. It is apparent that these fish are able to cope with ammonia resulting from both endogenous and exogenous sources thereby eliminating the stressor.

The two levels of crude dietary protein tested in this study, 1% and 40%, fed at a rate of 2% body weight daily, did not cause significantly different plasma ammonia concentrations (3.93 ±0.38 and 5.31 ±0.669 μg/mL respectively), although a non-significant increase was noted in the 40% protein group in the first sample period. In contrast, a previous study (Chapter 3) found that rainbow trout fed a diet consisting of 48% crude protein at a rate of 2% body weight daily caused a significant increase, 2.68 ±0.19 to 5.22 ±0.53 μg/mL, in plasma ammonia 6-h after feeding when compared to fasted fish. Although there was no significant difference between the plasma ammonia of fish fed diets containing 1 and 40 % protein in the present study, the postprandial plasma ammonia levels in unfed fish (Chapter 3) and fish fed the diet with 1% protein in the present study were both lower than fish fed diets with 40 and 48% protein (Chapter 3). In both studies plasma ammonia levels in fish fed the higher protein diets (40 and 48%) decreased in both the control and ammonia-exposed groups after 48-h even though the fish showed no decrease in food consumption with ammonia exposure. This is likely due to an upregulation of the GSase pathway.

The brain is the most sensitive organ to elevations in ammonia (Cooper and Plum, 1987). Due to the sensitivity of the brain to ammonia the highest levels of GSase exists in this tissue. This has been confirmed in rainbow trout in an early study by Walton and Cowey (1982). The high activity of GSase is thought to catalyze conversion of excess ammonia to glutamine by the following pathway:

$$\text{Glutamate} + NH_3 + \text{ATP} \Rightarrow \text{Glutamine} + \text{ADP} + P + H^+$$

(Mommsen and Walsh, 1992). In the present study glutamine levels increased in both the brain and liver in fish exposed to elevated ammonia. A similar result was found in Chapter 3,

signifying that exposure to 10 mg N/L at pH 7.2 caused excessive internal ammonia levels that were handled by detoxifying ammonia to glutamine. Corresponding to the increased glutamine levels in the brain a significant decrease in glutamate was noted with increased ammonia. The glutamate levels in these fish do not drop as low as the threshold of 2.37 μmol/g wet weight proposed by Vedel et al., (1998) suggesting that these fish could cope with higher ammonia levels before neurological impacts were noted. Due to the importance of glutamate as a neurotransmitter and a substrate for the formation of GABA it would be detrimental to fish to deplete glutamate completely and this is the proposed explanation for the threshold (Vedel et al., 1998). With respect to dietary protein, neither the fish fed diets with 1 or 40 % protein showed any change in brain glutamine concentration; however in a previous study (Chapter 3) a significant increase was noted when unfed and fish fed a diet containing 48% protein were compared. In fact the mean glutamine levels in rainbow trout fed the diet with 48 % protein were higher than any other diet treatment group in the present and previous study. The increases in both the GSase activity and glutamine concentrations likely accounts for the decreased plasma ammonia levels after 48-h of ammonia exposure in fish fed high dietary protein content.

When the glutamine/glutamate ratios were calculated in brain tissue from the LS means (averaged over the 6 and 48-h sampling periods) (Figure 5) from both studies (present and Chapter 3) an interesting trend was noted. If the control group is considered, it is apparent that the glutamine/glutamate ratio increases with increased dietary protein. At 48 % protein, the ratio decreases suggesting that a threshold has been reached. The decreased ratio is the result of a simultaneous increase in both the mean level of glutamine and glutamate as opposed to only an increase in glutamine in fish fed diets with 1 and 40% protein. It is possible that the glutamate is either produced in the brain via,

$$\alpha\text{-ketoglutarate} + NH_4^+ + NADH \Rightarrow \text{glutamate} + NAD + H_20$$

catalyzed by GDH or transported to the brain via the circulatory system from other tissues such as liver. This extra glutamate would prevent the fish from potential neurological consequences due to the depletion of glutamate (Randall et al., 1997), while allowing fish to continue to form glutamine. Under elevated ambient ammonia conditions, once again the glutamine/glutamate

ratio is higher than in the control group and increases with increased protein intake. The threshold is however, reached at a lower protein level, 40% as compared to 48% in the control group. At 48% protein the ratio increases again, likely due to the up regulation of GSase activity as indicated in Table 1 allowing for more glutamine formation. It is only at the 40% protein level that there is no difference between the control and ammonia-exposed fish. In ammonia-exposed fish muscle, GDH and ALT activity increase when fish are fed the diet with 40% crude protein (Table 1). ALT can catalyze the formation of glutamate by the following pathway:

$$\alpha\text{-ketoglutarate} + \text{Alanine} \Leftrightarrow \text{Pyruvate} + \text{Glutamate}$$

The increased activity of both GDH and ALT in muscle corresponds to the 50% increase in muscle glutamate (Figure 3F) that can then be used as a substrate for glutamine production in the muscle or be transported to other tissues.

Vedel et al., (1998) noted that rainbow trout exposed to elevated ambient ammonia reached a threshold for both the upper limit for glutamine at 7.91 μmol/g wet weight and the lower limit for glutamate at 2.37 μmol/g wet weight. These results were similar to an earlier study by Arillo et al., (1981). The results of both the present study and Chapter 3 were similar to results of the Vedel et al. (1998) study showing that elevated ambient ammonia at sub-lethal levels causes increased glutamine levels in the brain.

Muscle tissue appears to be an important sink for excess ammonia in rainbow trout even though; a non-significant increase ($p=0.057$, $n=24$) in muscle glutamine was noted in this study, whereas in the previous study (Chapter 3) muscle glutamine levels showed a significant increase. In both experiments levels increased by 25% or more, resulting in an actual increase of more than 0.15 μmol/g tissue. If the proportion of muscle in a rainbow trout is approximately 60% of its body weight then the fish used in these experiments (36 g mean weight) had an increase of 2.7 μmol/fish of glutamine in the muscle alone. This is in fact more than the brain and about the same as the liver.

In summary, rainbow trout appear to be able to detoxify endogenous ammonia by activation of the glutamine synthetase pathway. This pathway seems to be upregulated after feeding depending on dietary protein concentration. High dietary protein levels, 48 % a common level of crude protein in commercial fish foods, appear to up-regulate the synthesis of GSase so

that excess ammonia resulting from protein catabolism can be detoxified to glutamine. A protein level of 40% may only prime this pathway but ammonia levels do not become elevated sufficiently to require conversion to glutamine and thus normal GSase activity in the animal is sufficient. The up regulation or priming of the GSase pathway is the probable explanation for the brief protection feeding evokes in rainbow trout exposed to acute ambient ammonia (Chapter 2 and 3). It is also possible that a diet containing 48% crude protein may exceed the requirements of rainbow trout and in fact cause physiological stress.

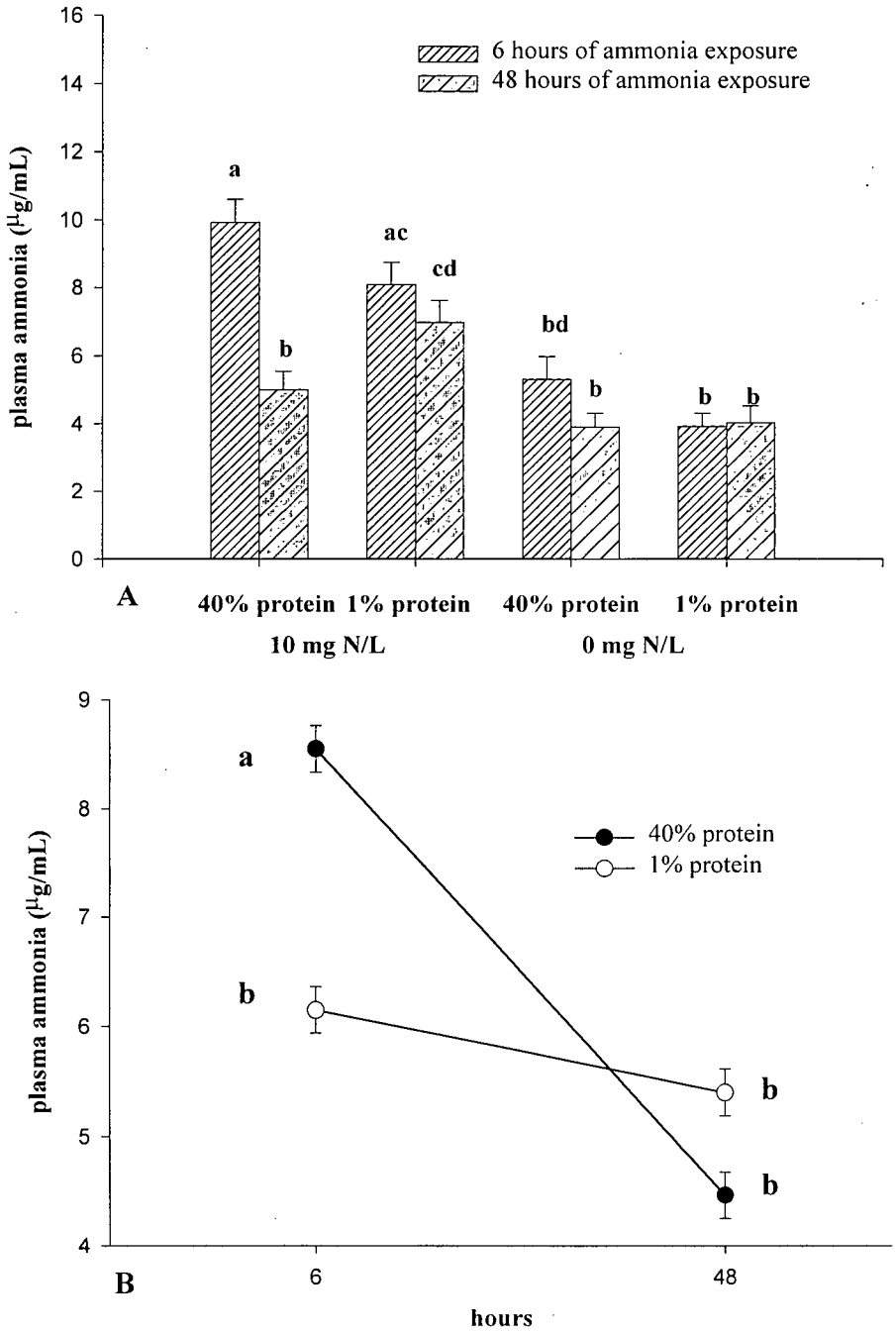

Figure 1. A) Plamsa ammonia (µg/mL) in rainbow trout fed diets with different crude protein levels (1% and 40%) and exposed to ammonia (0 and 10 mg/L N) at pH 7.2 for a period of 6 and 48 hours. B) Interaction between dietary protein and time for plasma ammonia. Significant differences ($p<0.05$) are indicated by a change in letter and bars represent mean (A) and LS mean (B) +/- SEM.

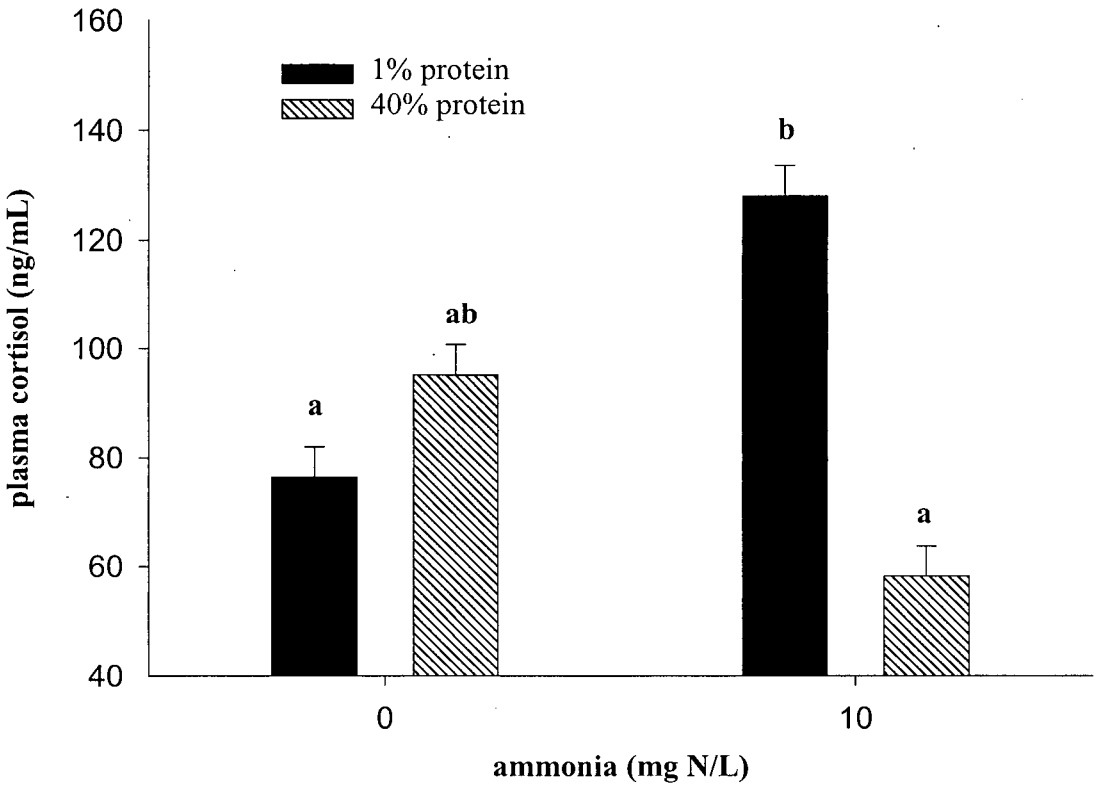

Figure 2. Plasma cortisol (ng/mL) in rainbow trout fed diets containing different levels of crude protein (1 and 40%) and exposed to elevated ammonia (0 and 10 mg N/L). Symbols represent LS means (n=24) +/- SEM averaged over 6 and 48 hours. A change in letter indicates a significant difference ($p<0.05$) between means.

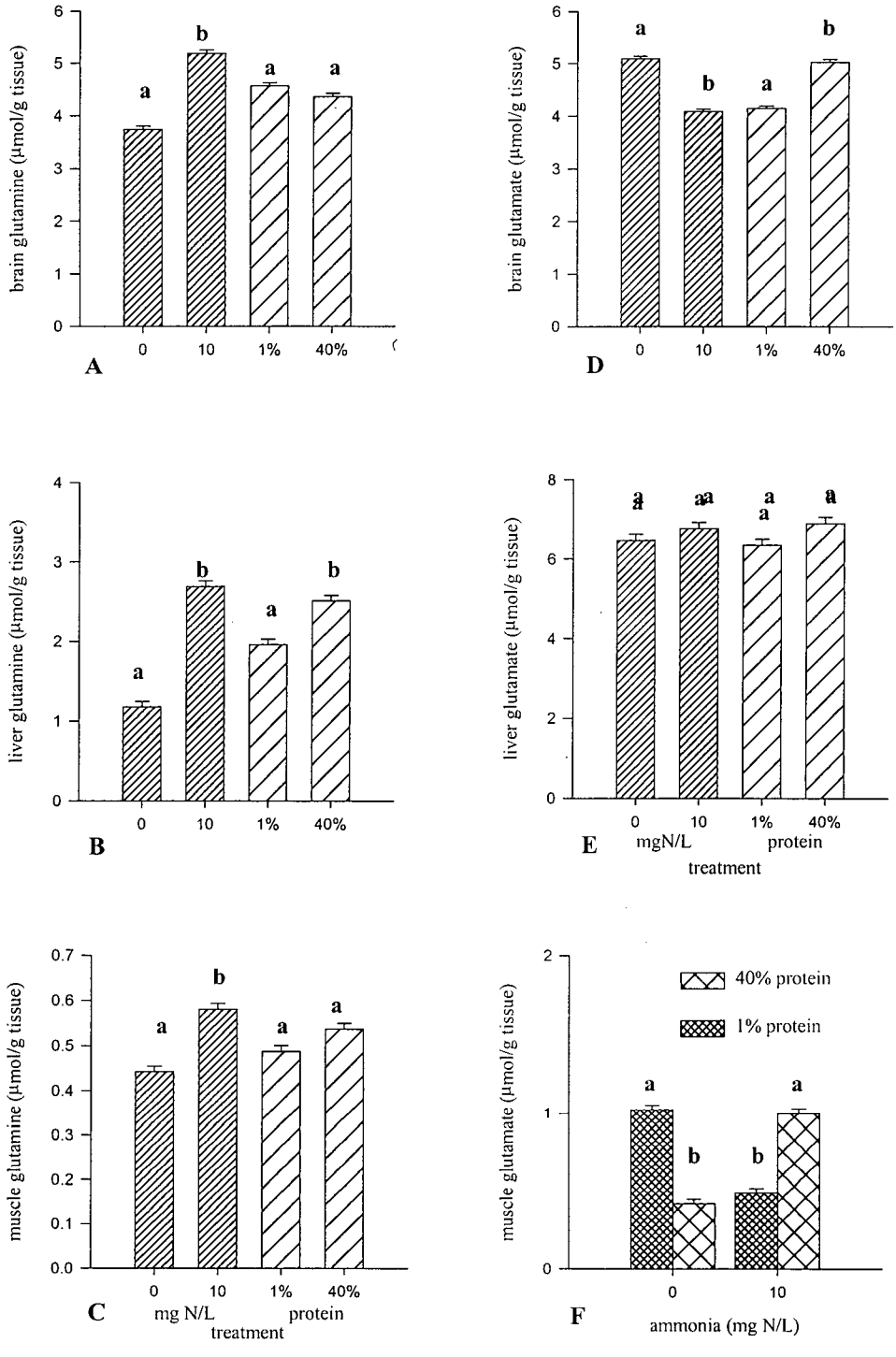

Figure 3. Brain, liver and muscle glutamine and glutamate concentrations (µmol/g tissue) in rainbow trout exposed to ammonia (0 &10 mg N/L, fine fill) and fed different levels of dietary protein (1 & 40%, coarse fill). Bars represent the LS means averaged over 6 and 48 hour sampling periods and either ammonia or protein level for graphs A-E. Significant differences ($p<0.05$, n=24(A-E), n=12(F)) between treatments are indicated by a change in letter. F) LS mean for muscle glutamate averaged over time only.

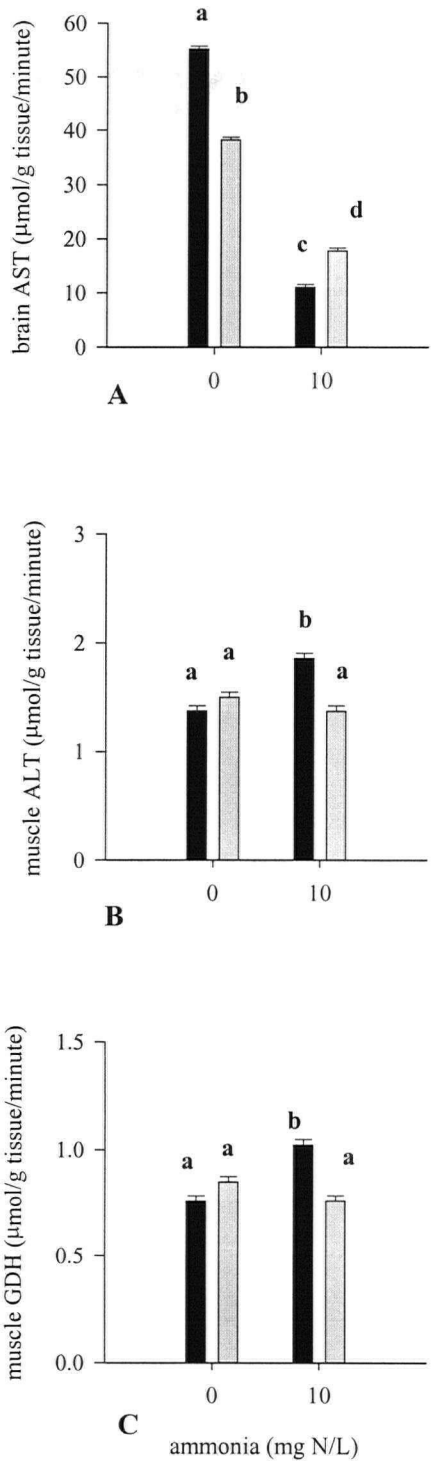

Figure 4. Interaction effects for brain AST (A) and muscle ALT (B) and GDH (B) i rainbow trout fed diets with different levels of protein (2% body weight daily of 1 & 40% crude protein) and exposed to ammonia (0 & 10 mg N/L). Bars, black- 40% protein and stippled- 1% protein, represent the LS means averaged over the 6 and 48 hour sampling periods. Error bars are the SEM. Significant differences ($p<0.05$, $n=12$) are indicated by a change in letter.

Table 1. The main effects for activities (μmol min^{-1} g $tissue^{-1}$) of glutaminase, glutamate dehydrogenase (GDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glutamine synthetase (GSase) in the brain, liver and muscle tissues of rainbow trout fed either a diet containing of 1% or 40% protein (2% b.w. daily) and exposed to ammonia (0 and 10 mg N/L at pH 7.2).

Tissues	Enzyme	1%	40%	0 mg N/L	10 mg N/L	S.E.M.
Brain	Glutaminase	1.02	1.05	1.02	1.05	0.01
	GDH	3.01	3.12	2.99	3.14	0.03
	ALT	2.36	2.35	2.43	2.28	0.03
	AST	27.9	33.1*	46.7 [a]	14.4 [b]	0.4
	GSase	6.91	7.59	6.75 [a]	7.76 [b]	0.02
	Glutaminase	0.145	0.126*	0.129	0.142	0.002
Liver						
	GDH	37.6	34.5	36.9	35.2	0.5
	ALT	38.7	40.4	38.3	40.8	0.9
	AST	28.1	25.2	27.9	25.4	0.6
	GSase	0.0799	0.0738	0.0629	0.0909	0.0005
	Glutaminase	0.0194	0.0251	0.0199	0.0244	0.0009
Muscle						
	GDH	0.805	0.890	0.804	0.891	0.02
	ALT	1.44	1.62	1.44	1.62	0.04
	AST	30.7	31.7	28.8	33.6	0.9
	GSase	0.00960	0.0109	0.0103	0.0102	0.0005

Results represent the LS means (n=24) averaged over time and/or dietary protein or ammonia concentration with the corresponding SEM. * indicates a significant difference ($p<0.05$) between level of dietary protein and [a] between 0 and 10 mg N/L. When analysis of variance (3 factor) indicated a significant interaction (2 or 3 factor interaction) between any of the factors, ammonia concentration, feeding and time the enzyme is presented in bold typeface and the interaction is further examined in the results section.

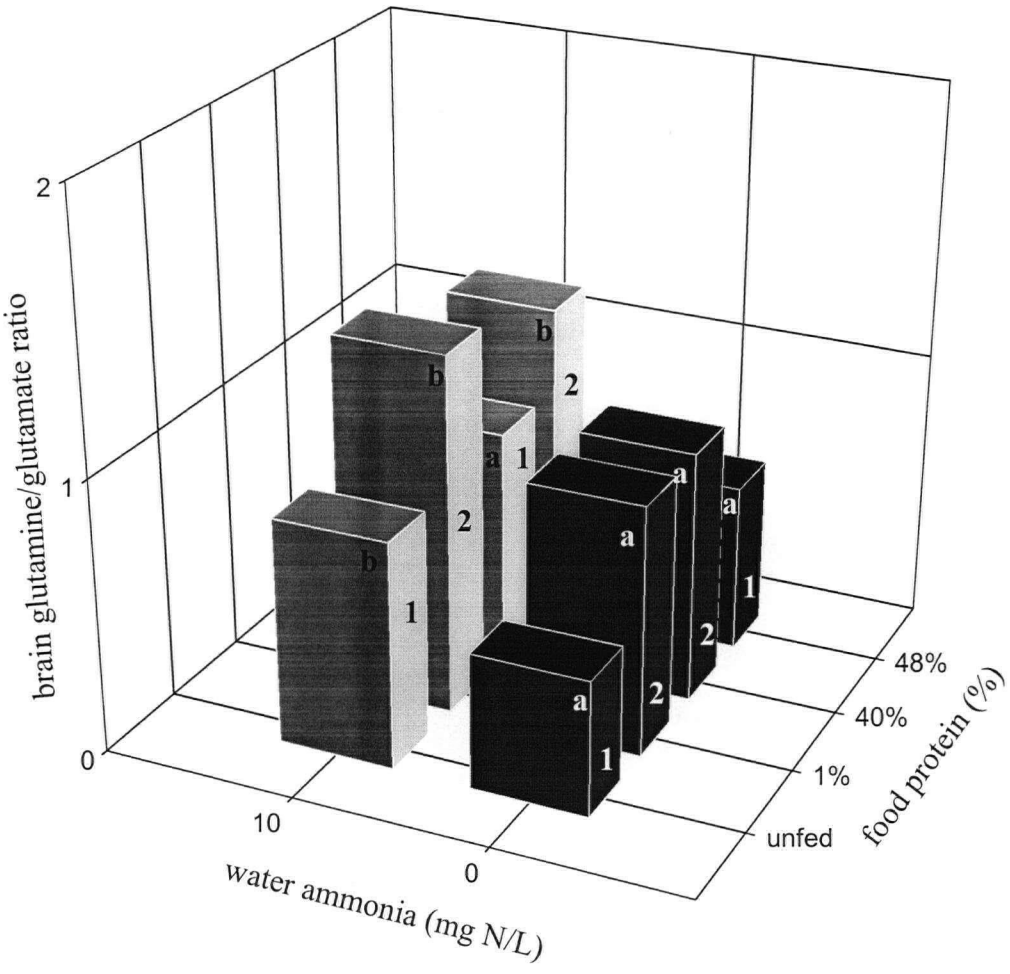

Figure 5. The ratio of brain glutamine/glutamate in rainbow trout fed different protein levels (control, 1, 40 and 48 %) and exposed to elevated ambient ammonia (0 and 10 mg N/L, pH 7.2) for 48 hours. Fish were fed at a rate of 2% body weight daily and sampling was 6 hours postprandial. Significant differences (n= 12, $p<0.05$) between ammonia and protein levels are indicated by a change in letter and number respectively.

Chapter 5. The effect of stress on acute and sub-lethal ammonia exposure in rainbow trout: does cortisol exacerbate ammonia toxicity?

1.0 Introduction

Fish in their natural environment and under certain aquaculture conditions experience stress. Stress can be caused by physical, social or environmental factors such as handling, forced swimming, starvation, confinement, extreme changes in temperature or salinity, disease, high density or chemicals that have been added to the environment. When fish are exposed to stress either an internal change or an external stimulus evokes a chain of physiological reactions that compensate for and resist the change. In teleosts, the stress response typically involves activation of the hypothalamus-pituitary-interrenal axis, resulting in the release of cortisol into the circulation (Wendelaar Bonga, 1997). The main effects of cortisol have been observed in the liver, gills and intestine and involve changes in osmoregulation and energy metabolism (Wendelaar Bonga, 1997). Cortisol also affects intermediary metabolism, stimulating glycogenesis and gluconeogenesis (Vijayan et al., 1997). Substrates needed in these metabolic pathways require the breakdown of amino acids, which could hypothetically result in increased ammonia production (Mommsen et al., 1999).

A cortisol-mediated increase in protein catabolism and ammonia production (Mommsen et al., 1999) could exacerbate ammonia toxicity in fish encountering elevated ambient ammonia concentrations. Actively swimming fish exposed to environmental ammonia have to cope with a net influx of ammonia from the water as well as ammonia accumulation in muscle due to deamination of adenylates during exercise (Mommsen and Hochachka, 1988). As a result, the LC_{50} values for salmon swimming at 60% U_{crit} in a respirometer under conditions of elevated ambient ammonia were significantly lower than those of resting fish (Wicks et al., 2001). A similar response may occur with stressed fish.

Rainbow trout are able to up-regulate glutamate/glutamine metabolism under conditions of elevated ambient ammonia (Chapter 3 and 4) and following the consumption of high protein food, which also results in increased internal ammonia (Chapter 3). A critical enzyme in this pathway is glutamine synthetase (EC 6.3.1.2) which catalyzes the ATP-driven conversion of glutamate + NH_3 $\rightarrow$ glutamine, a neutral, non-toxic amino acid. Previous research has indicated that glutamine synthetase (GSase) synthesis is upregulated by the synthetic corticosteroid

dexamethasone (Mommsen et al., 1991) and cortisol (Walsh and Milligan, 1995; Vijayan et al., 1996). As a result, the conversion of excess ammonia to glutamine is promoted at the expense of tissue glutamate and ATP. Indirectly linked though the ubiquitous mitochondrial glutamate dehydrogenase, cellular α-ketoglutarate concentrations and mitochondrial redox status could also be affected.

The first goal of these studies was to establish if exposure to stress exacerbates acute ammonia toxicity in rainbow trout and if so, if this could be due to the action of cortisol. The second goal was to determine how rainbow trout cope with ammonia produced as a result of stress and/or cortisol in combination with exposure to elevated ambient ammonia.

2.0 Methods

2.1 Fish husbandry and experimental set up.

Rainbow trout (*Oncorhynchus mykiss*, 30 to 50 g) were obtained from Sun Valley Fish Farm, Langley, BC. Fish were held in a round 1000 L outdoor tank supplied with dechlorinated Vancouver tap water for a minimum of 4 weeks. Fish were fed at 1% body weight, 5 times per week with commercial trout chow (Moore-Clark, Nutra Fry).

After having been moved to 8 oval indoor tanks (60 L capacity each), fish were given 2 weeks to acclimate prior to the onset of experiments. The feeding regime was the same as previously mentioned. Five days prior to the onset of an experiment, water was buffered to pH 7.5 in the LC_{50} studies and pH 7.3 in the sub-lethal studies by addition of sodium bicarbonate so that pH 7.2 would be achieved during the experiment. The addition of bicarbonate has no effect on rainbow trout (Chapter 3).

Dechlorinated Vancouver tap water was aerated in a head tank supplying all 8 tanks with precisely 1 L/min of water though a spray bar directed at the water surface to provide a slight current. Tank oxygen levels never dropped below 85% saturation. The water temperature was 7.5 ± 1.6 °C. Water ammonia levels were increased gradually to the appropriate concentration by addition of a stock NH_4Cl solution *via* peristaltic pump (Masterflex) and tubing connected by a one-way valve to the water supply for each tank. Water variables (temperature, pH, dissolved oxygen, total ammonia) were tested 6-h after the start of each experiment and daily thereafter.

Fish condition was closely monitored and individuals showing loss of equilibrium and unresponsive to touch were removed immediately and sacrificed by concussion.

2.2 Experimental Design

Four different experiments were performed. Experiment 1 was a flow-though 96-h acute ammonia toxicity test that investigated the effect of high-density stress on ammonia toxicity. Ten rainbow trout (31 ± 3.6 g) were placed in each of 8 tanks and allowed to acclimate for 2 weeks prior to the experiment. Fish were fed until 3 days before the test when water levels were decreased in 4 tanks to increase loading density. The final loading densities were <5g/L (low-density) and >30g/L (high-density), respectively. The tanks were paired, one low and one high density and ammonia concentrations were increased gradually in 6 of the 8 tanks to 120, 150 and 180 mg N/L. These ammonia levels were established in a previous study using fed and unfed rainbow trout under the same experimental conditions (Chapter 2). The remaining pair of tanks was used as controls. During the 96-h test morbid fish were removed from the tanks and counted.

Experiment 2 tested the effect of artificially elevated cortisol on acute ammonia toxicity. A preliminary study determined the level of plasma cortisol produced by injecting rainbow trout with 100 μg/g body mass of hydrocortisone suspended in a hydrogenated coconut oil/vegetable oil mixture. Results indicated that plasma cortisol levels of 975.8 ±393.9 ng/mL were reached 24-h post injection and remained elevated for at least 96-h. Despite large individual variation, all cortisol-injected fish had significantly higher plasma cortisol levels than sham-injected fish. Plasma cortisol levels in sham-injected (vehicle only) fish were not significantly different than control fish after 48 h.

Sixty rainbow trout were moved from the outdoor tanks and randomly divided among 8 indoor tanks. Once acclimated, fish from 4 tanks were weighed, rapidly anaesthetized in 100 mg tricaine methanesulphonate (MS 222)/L buffered with an equal mass of $NaHCO_3$, weighed and given a single interperitoneal injection of 100 μg/g body mass of hydrocortisone suspended in hydrogenated coconut oil/vegetable oil mixture (40 mg hydrocortisone/mL of oil mixture). Fish in the remaining 4 tanks were sham-injected with vehicle only to act as controls. Fish were given 48-h to recover after injection. Tanks were again paired, one cortisol and one sham-

injected, and each pair was exposed to either 0, 75, 100 and 130 mg N/L ammonia for a 96-h acute ammonia toxicity test. The ammonia levels tested were chosen after an exploratory test; the protocol used in experiment 2 was the same as that for experiment 1.

Experiment 3 determined the effects of stressors on plasma ammonia and cortisol and glutamate metabolism in fish exposed to exogenous ammonia. Twelve fish were placed in each of the 8 tanks and left to acclimate. Thee days prior to ammonia exposure, food was withheld and in 2 tanks water levels were reduced to create high-density conditions (>40 g of fish/L). In 2 other tanks, 3 ppm hydrocortisone was added to the water *via* peristaltic pump. After 72 hours, ammonia levels were raised in 4 tanks, including one high-density and one cortisol tank, to 10 mg N/L by addition of NH_4Cl. This level was subsequently maintained by continuous addition of NH_4Cl. One tank served as an ammonia exposure control and one as a control without ammonia or stress. In the remaining 2 tanks (ammonia-treated and control) the fish were exposed to air for 1 minute daily by removing the water from the tank after ammonia treatment started. The water was replaced after 1 minute at either the control or 10 mg N/L level. The 8 treatments will be referred to as: control/control, ammonia/control, control/density, ammonia/density, control/cortisol, ammonia/cortisol, control/air and ammonia/air.

Six fish from each treatment group were terminally sampled 6 and 48-h after the onset of ammonia exposure. The air exposure always occurred 2-h prior to the sampling time. Fish were killed by concussion and blood was obtained by caudal puncture. Blood was immediately centrifuged and plasma was collected, aliquotted, and frozen at –80°C for later measurement of plasma ammonia and cortisol. Brain, liver and white muscle samples were also collected from each fish. The white muscle was taken from above the lateral line just posterior to the dorsal fin to avoid any red muscle. Tissue samples were frozen immediately on dry ice and then transferred to –80°C for later measurement of enzymes. The sampling of each fish was completed in less than 3 minutes.

In experiment 4, the effect of blocking the action of plasma cortisol on nitrogen metabolism was investigated under control and elevated ammonia conditions. Twelve rainbow trout were again placed in each of 8 indoor tanks and left to acclimate. Food was withdrawn 72-h prior to the ammonia exposure. 48-h before the experiment began, fish were netted from the tanks, anaesthetized in buffered MS 222, weighed and injected (2.5 µl/g) with one of the following compounds suspended warm coconut oil/vegetable oil (1:1, v/v): 40 mg/mL

hydrocortisone, 40 mg/mL RU486 (Mifepristone), vehicle (sham) or 40 mg/mL RU486 followed 12-h later by 40 mg/mL hydrocortisone. RU486, an antiglucocorticosteroid, was injected to block glucocorticoid receptors and therefore the effect of the injected cortisol (Mommsen et al., 1999). Fish were sampled as described for experiment 3.

2.3 Analysis

Mortality rates and measured water ammonia concentrations from experiments 1 and 2 were used to determine the LC_{50} values and confidence intervals for each type of treatment. The actual water ammonia levels were determined using a modification of the Verdouw et al. (1978) method substituting nitroprusside (0.2 mg/L) for ferrocyanide. The LC_{50} and 95 % confidence intervals were calculated on software available though the US EPA using the Spearman-Karber method. Values were considered different when the 95% confidence intervals did not overlap.

In experiments 3 and 4, plasma ammonia concentration was determined with a kit that uses L-glutamate dehydrogenase and NADH (Sigma Chemical Co., 171-UV). Plasma cortisol was measured with an ELISA kit (Neogen, Lexington, KY, USA). It was previously determined that ether extraction need not be performed on the fish plasma prior to measuring cortisol when using this method.

For the determination of enzyme activities, tissue samples from experiments 3 & 4 were weighed and homogenized (Ultra Turrax) in 10 volumes phosphate buffered glycerol, containing 10 μg/mL aprotinin (Mommsen and Hochachka, 1994). Homogenates were then sonicated briefly before centrifugation at 12,000 x g for 30 seconds. The supernatants were removed and frozen at –80 °C for later determination of enzyme activities.

Glutamine synthetase (GSase, reverse direction) was assayed in a timed assay using the transferase activity, by quantifying γ-glutamyl hydroxamate (Chapter 3). Glutaminase activity was measured by enzymatic quantification of glutamate (Chapter 3). Glutamate dehydrogenase (GDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined by coupled enzymatic methods (Chapter 3). Enzyme activities are expressed in units (U/g tissue), where 1 unit is the amount of enzyme converting 1 μmole substrate to product per minute under saturation conditions.

Experiments 3 and 4 had a 2x2x4 factorial design. With factors of ammonia (0 and 10 mg N/L) and time (6 and 48-h) having two levels and being common between the two experiments. The third factor for experiment 3 was stressors (density, air, 3 ppm cortisol or control) and experiment 4 has a third factor, injection type (cortisol, RU486, RU486 + cortisol, sham). For each variable measured, a 3-way analysis of variance was performed, after data had been checked for normality and equal variance. The cortisol data from experiment 4 were normalized by log transformation. Untransformed values are presented for clarity. Main effects were considered significant if $p<0.05$; a Bonferroni multiple comparison test was done to determine where the differences occurred (Steel and Torrie, 1980). When interactions were noted between factors, the main effects were not considered and the interaction was further examined. All values are presented as the least squares (LS) means ±S.E.M. averaged over the unreported factors unless otherwise noted.

3.0 Results

3.1 Interactions between density and cortisol and exogenous ammonia.

The 24-h LC_{50} (136 ±14.5 mg N/L) (experiment 1) was significantly lower in the high-density fish than at low density (177 ±12.1mg N/L), while no significant difference was noted in the 96-h LC_{50} concentration (161 ±19.6 mg N/L) for trout held under high or low-density conditions.

For cortisol-injected fish (experiment 2), the 96-h LC_{50} was significantly higher than for the sham-injected fish. There was no significant difference between the 24 and 96-h LC_{50} for cortisol-injected fish (86.0 ±20.2 mg N/L). The LC_{50} values for the sham-injected fish could not be calculated as the mortality rates were very low over the range of ammonia concentrations tested. This result was predictable based on experiment 1. The LC_{50} for high-density fish from experiment 1 was significantly higher than that for the cortisol-injected fish from experiment 2.

3.2 Effects of stressors on fish exposed to exogenous ammonia.

3.21 Effects on plasma ammonia and cortisol

No mortalities occurred during the experiment at any treatment level. Water ammonia levels remained constant (10.0 ±0.47 mg N/L) in ammonia-treated tanks and were below the level of detection in the control tanks.

Plasma ammonia concentrations showed a significant interaction between the stressors and ambient ammonia. A synergistic effect on the plasma ammonia concentration was noticeable when density and ammonia exposure were combined (Figure 1). Plasma ammonia concentration in the high-density control fish (2.72 ±0.13 μg/mL) was significantly lower than either the control or cortisol exposed fish. When fish were exposed to ammonia, all treatment groups experienced significant increases in plasma ammonia concentrations (Figure 1). In the density-exposed group, the combination of density stress and exposure to ammonia had a synergistic effect with plasma ammonia concentrations increasing significantly. This plasma ammonia concentration was the highest among all the groups.

Plasma cortisol was significantly elevated by exposure to both ammonia and to the stressors. Under control conditions, plasma cortisol increased from 65.0 ±4.68 ng/mL in control/control fish to 98.9 ±4.68 ng/mL in the high-density treatment group and increased significantly in the air-exposed group to 119.3 ±4.68 ng/mL (Figure 2). In ammonia-exposed fish, cortisol concentrations were significantly elevated in the ammonia/control and ammonia/air treatment groups (104.1 and 192.1 ±4.65 ng/mL, respectively) when compared to the control/control group (Figure 2). The high density and the cortisol-exposed fish showed no significant differences when exposed to elevated ammonia. When plasma ammonia was regressed against plasma cortisol (Figure 3) the regression was not significant ($p=0.099$, $r^2=0.0287$, $n=96$).

3.22 Effects on glutamate metabolism.

Brain: A significant interaction was noted between stressors and ammonia exposure for glutaminase, GDH and GSase (Table 1). Glutaminase activity was unaffected by stress, but decreased with exposure to ammonia in all treatment groups except fish exposed to 3 ppm cortisol, which showed no change (Figure 4A). Brain GDH was highest in the control/control fish and decreased with exposure to stress (Figure 4B). Control levels for brain GDH were determined to be 3.09! 0.03 U/g tissue and decreased to a similar level in fish exposed to

cortisol, air and density stress, respectively. GDH activity in the brain increased in fish exposed to the combined effects of both stress and ammonia, but decreased significantly in the control/ammonia group (Figure 4B). GSase activity in the brain did not change with exposure to stressors alone (Figure 4C), but when ambient ammonia was increased, activity decreased significantly in both the control and the air-exposed fish. When compared to the control/control group brain GSase activity decreased from 6.75 ±0.03 U/g tissue to 1.78 in the control/ammonia group, 3.55 in the air/ammonia group and to 4.73 ±0.03 U/g tissue in the density/ammonia group (Figure 4C).

ALT activity in the brain showed a 3-way interaction between ammonia, stressor and time. The ammonia/stressor interaction depended on the sampling time. At the 6-h sampling period there was no interaction between ammonia and stressors, but the interaction was significant after 48-h. At the 6-h sampling period all stressors caused a significant decrease in ALT activity with the activity measured at, air/control 2.20, density/control 2.24 and cortisol/control 2.22 ±0.02 U/g tissue when compared to the control/control sample group at 2.63 ±0.02 U/g tissue. There was no ammonia effect at this sampling time. After 48-h, ammonia exposure had a synergistic effect when combined with air stress resulting in the activity of ALT decreasing to an even lower value of 1.94 ±0.02 U/g tissue in this group. There was no change in brain AST activity at any treatment level.

Liver: Liver glutaminase showed significant ammonia, stressor and time interaction (Table 1). No effect of either ammonia or stress was noted at the 6 and 48-h sampling period; however there was a synergistic effect when ammonia and density stress were combined. The combination of ammonia and density stress caused a significant decrease in the mean glutaminase activity from 0.301 in the density/control group to 0.181 U/g tissue in the density/ammonia group. Both ammonia and stressors affected the GDH activity in liver. Exposure of fish to ammonia caused a significant decrease in GDH activity (from 52.5 in controls to 43.5 ±0.33 U/g tissue in ammonia exposed fish). Exposure of fish to 3 ppm cortisol lead to a 10% increase in GDH activity.

The ALT activity was not altered by stress, but a significant interaction occurred between ammonia and time. No effect of ammonia was noted for ALT at the 6-h sampling period with an activity of 24.5 ±0.3 U/g tissue, but activity increased to 32.2 ±0.3 U/g tissue

after 48-h. AST activity decreased significantly in the liver tissue with ammonia exposure from 30.8 to 26.5 ±0.3 U/g tissue, but was not affected by exposure to stressors.

GSase activity showed a significant interaction between ammonia and stressor (Figure 4D). There was no effect of stressors on GSase activity under control conditions; however, when ammonia levels were increased, only the cortisol-exposed fish showed no change in GSase activity. In the air/ammonia and control/ammonia groups liver GSase activity increased to levels significantly higher than the control/control group. Liver GSase activity in the density-stressed fish exposed to ammonia decreased significantly when compared to control/density activity, but the decrease was not significant when compared to the activity in the control/control group.

Muscle: Glutaminase activity in white skeletal muscle decreased significantly in fish exposed to ammonia (Table 1). Stress exerted no effect on the activity of glutaminase. There was a significant interaction between ammonia and time for the activity of GDH. Under control conditions, the GDH activity was not significantly different at the 6 and 48-h sampling period respectively. Under conditions of elevated ammonia, the GDH activity increased significantly after 6-h to 0.67 when compared to the controls and increased significantly again after 48-h to 0.84 ±0.006 U/g tissue, more than double the control level.

For muscle ALT a significant 3-way interaction existed between ammonia, stressor and time. The ammonia/stressor interaction was significant at only the 48-h sampling period. The mean (±S.E.M) ALT activity decreased significantly ($p<0.05$, $n=6$) in air-stressed fish exposed to ammonia at the 48-h sampling period. The muscle GSase activity dropped significantly ($p<0.05$, $n=48$) from 0.011 in control fish to 0.0093 ±0.00014 U/g tissue in the ammonia-exposed fish. There was no effect of stressors on muscle GSase.

3.3 Effects of cortisol on fish exposed to exogenous ammonia.

3.31 Plasma ammonia and cortisol

Experiment 4 considered the effects of cortisol injections and exogenous ammonia exposure on glutamate metabolism in rainbow trout. No mortalities were noted at any treatment level. The water ammonia levels remained constant (10.1 ±0.38 mg N/L) in ammonia-treated tanks and the control tanks showed only nominal ammonia concentrations. Both cortisol and

cortisol/RU486 injections caused a significant increase in plasma cortisol concentrations (798.5 and 698.1 ±13.5 ng/mL, respectively), compared to the sham-injected fish (48.5 ±13.5 ng/mL). The RU486-injected fish had an intermediate mean cortisol concentration of 252.0 ±13.5 ng/mL which was significantly higher than noted for the sham group, but lower than found for fish given the other two injections. Ammonia had no effect on plasma cortisol concentrations.

The relationship between plasma ammonia and plasma cortisol (Figure 5) was significant (p=0.002), but the relationship between these variables was weak with less than 10% of the variation in plasma ammonia being explained by changes in plasma cortisol ($r^2 = 0.095$). No significant change was noted in the plasma ammonia concentrations due the injection type (Figure 6). However, plasma ammonia concentrations were significantly elevated by ammonia exposure in all treatment groups except the RU486-injected fish (Figure 6). Levels became elevated after 6-h of exposure and remained elevated for the duration of the experiment. The cortisol and cortisol/RU486-injected fish exposed to ammonia showed a synergistic increase in plasma ammonia when the injection was combined with ammonia exposure (Figure 6). The increase in the plasma ammonia due to ammonia exposure alone (sham/ammonia) was 3.06 μg/mL. In the injected fish, the increase was significantly higher at 3.50 and 4.41 μg/mL for cortisol and cortisol/RU486-injected fish, respectively.

3.32 Glutamate metabolism

In brain, a significant interaction was noted between stressor type and ammonia exposure for glutaminase, ALT and GSase (Table 2). The brain glutaminase activity was not significantly changed in the control fish injected with different treatments. The lowest activity was noted in the sham-injected fish with levels increasing with the different stressors (Figure 7A). When ammonia exposure and cortisol injections were combined, the glutaminase activity decreased significantly from 1.07 under control conditions to 0.758 ±0.015 U/g tissue. The opposite trend was noted for the ammonia-exposed fish injected with the sham treatment and the RU486/cortisol, with glutaminase increasing in the both the sham-injected fish from 0.884 to 1.16 ±0.015 U/g tissue and RU486/cortisol-injected fish from 0.980 to 1.20 ±0.015 U/g tissue (Figure 7A). The ALT activity in the brain was significantly higher in the control/cortisol fish at 2.55 and the control/RU486 fish at 2.55 fish than the control/sham-injected fish (Figure 7B).

Under conditions of elevated ambient ammonia, there was no significant difference between injection types. The activity of GSase in the brain was significantly elevated under control conditions by injection with either cortisol or RU486 (Figure 7C). When the injections were combined with exposure to elevated ammonia, the level of brain GSase in the sham-injected fish more than doubled. A similar doubling occurred for the RU486/cortisol-injected fish compared with controls after ammonia exposure. The opposite trend was noted in the cortisol-injected fish, where brain GSase activity significantly decreased from 8.9 U/g under control conditions to 5.9 U/g tissue under conditions of elevated ammonia.

A significant 3-way interaction between ammonia, injection and time was found for brain AST. The main interaction was between ammonia and injection (data not shown). Under control conditions, sham-injected fish had brain AST activity of 10.8 ±0.68 U/g tissue; however the RU486/cortisol fish had more than twice the activity at 25.7 U/g tissue ($p<0.001$, $n=12$). Cortisol and RU486-injected control fish had AST levels more than 4 times higher than the sham-injected fish at 44.2 and 46.0 ±0.68 U/g tissue, respectively. When fish were exposed to elevated ammonia, the sham-injected fish showed a significant increase in brain AST activity. Cortisol and RU486-injected fish had a significant decrease in AST activity when compared to control conditions with levels decreasing to 11.7 and 24.3 ±0.68 U/g tissue, respectively. No significant changes were noted in brain GDH activity with either injection or ammonia exposure.

In liver, there was a significant interaction between injection and time for glutaminase and GSase activity (Figure 7D&7E). At the 6-h sampling time the sham-injected fish had the highest liver glutaminase activity, but it was only significantly higher than the RU486-injected control fish. A similar result was noted after 48-h for liver GSase activity which showed no significant differences due to injection at the 6-h sample time (Figure 7E); however the GSase activity in the RU486/cortisol-injected fish decreased significantly with time, from 0.285 after 6-h to 0.069 ±0.005 U/g tissue at 48-h. Cortisol injection was accompanied by a significant increase (30%) in the mean liver GSase activity when averaged over time and ammonia (Table 2) as compared to all other injections.

GSase activity in the muscle tissue had a significant interaction between injection type and ammonia exposure. Under control conditions, no effect of injection type on GSase activity was apparent (Figure 7F); however when water ammonia levels were elevated, the GSase

activity increased significantly in the RU486/cortisol-injected fish. GDH activity in muscle was only affected by injection type with activity increasing from 0.385 ±0.005 in the sham-injected fish to 0.490 U/g tissue in cortisol-injected ones. Muscle ALT and AST activities were not affected significantly by any of the treatments.

4.0 Discussion

4.1 Ammonia and cortisol regulation

In this study the general hypothesis was that stress would exacerbate ammonia toxicity due to cortisol-mediated increases in ammonia production by the fish. Cortisol is known to increase the metabolic rate, which corresponds to an increase in ammonia excretion (Chan and Woo, 1978), since fish, especially rainbow trout, show a preference for amino acids as oxidative substrates (Van den Thillart, 1986). Higher mortality rates in rainbow trout held at high density and exposed to a range of ammonia support the hypothesis that density stress increased ammonia production and thus exacerbated ammonia toxicity. The lack of a significant difference between mortality rates after 96-h may be due to the removal of morbid fish, resulting in lower stocking density. Evidence that cortisol plays a role in exacerbating ammonia toxicity is found in the significantly lower LC_{50} concentration in the 96-h LC_{50} study using cortisol-injected fish. The fish mortality rate in that study did not change with time, as compared to the sham injected fish and suggests that cortisol or the effects of cortisol in fact play a role in exacerbating ammonia toxicity.

The implication of increased mortality with stress or cortisol injection combined with ammonia exposure is that metabolism increased prior to ammonia exposure to meet the energetic requirements to cope with the stress (Vijayan et al., 1993; 1997), adding a second ammonia assault though internally generated ammonia. In experiments 3 and 4, the treatments resulted in some degree of cortisol release. The stressors from experiment 3 not unexpectedly caused a smaller increase in plasma cortisol than the cortisol injections in experiment 4; however, independent of the level of circulating cortisol, no measurable relationship was noticeable between cortisol and plasma ammonia. The absence of a relationship between cortisol and ammonia could be for a similar reason to the one demonstrated by Hopkins et al. (1995) in gulf toadfish, *Opsanus beta*. Under control conditions, these fish showed a significant

linear increase in nitrogen excretion with increased plasma cortisol; however when the toadfish were stressed (confinement) and plasma cortisol concentration exceeded 15 ng/mL, no correlation existed between plasma concentrations of cortisol and ammonia. In the present study, plasma ammonia concentrations were used as opposed to excretion rate. It is possible, therefore, that under normal environmental conditions ammonia excretion rates increased to compensate for increased ammonia production. The overall result would be an unaltered concentration of internal ammonia. This theory however, would not hold true under ammonia loading conditions, where there would be a cumulative increase in plasma ammonia due to impaired excretion of metabolic waste. Increased plasma ammonia concentration, in fact, was noted in the high density/ammonia-exposed fish, which had the highest plasma ammonia levels of any treatment group (Figure 1).

Interestingly, plasma cortisol in the density-stressed fish was elevated under the control conditions, but contrary to the hypothesis not with ammonia exposure, which is also a stressor. In contrast, the air-stressed fish demonstrated a synergistic response to the combination of repeated air exposures and ammonia exposure as cortisol levels doubled relative to those of fish held under control conditions. The conflicting results are likely due to differences between these two stressors. In this regard, the density stress was started 3 days prior to the onset of the experiment and was maintained for the duration of the experiment whereas the air stress was a brief daily stress incurred during the experiment. It is likely that cortisol levels peaked within 1-h of the fish being exposed to the high-density stress and they probably remained elevated for the whole period as noted by Barton et al. (1980). The air-stressed fish experienced an acute stress just prior to ammonia exposure and increased cortisol level 1-h later, returning to control levels in about 4-h (Barton et al., 1980). Perhaps fish held at high densities experienced a change in metabolic activity prior to the ammonia stress/sampling periods in contrast to the secondary and temporary stimulus of air exposure.

Although, there was no measurable linear relationship between plasma cortisol and ammonia in either experiment 3 or 4, the combination of density stress, cortisol and cortisol/RU486-injections and ammonia exposure resulted in plasma ammonia concentrations that were higher than the respective control fish under conditions of elevated ammonia. It has been well documented that elevated cortisol causes increased metabolic activity in fish as indicated by the up-regulation of some key enzymes and mobilization of amino acids (Vijayan

et al., 1991; 1996; Milligan, 1997). An increase in metabolism could increase ammonia production (Mommsen et al., 1999) as amino acids are degraded and provided as intermediates for the TCA cycle (Walton and Cowey, 1982). The present results support previous research, and further, agree with the hypothesis that cortisol mediated increases in metabolic ammonia waste exacerbate ammonia toxicity in fish under elevated ammonia conditions.

4.2 Ammonia metabolism in brain

Results from previous studies indicate that rainbow trout, which experience elevated plasma ammonia after feeding and during ammonia exposure, detoxify the excess by converting it to glutamine, which corresponds to increased GSase synthesis (Chapter 3 and 4). From previous studies on the effects of ammonia in fish, it is clear that the brain is the most sensitive tissue to elevated ammonia (Vedel et al., 1998; Arillo et al., 1981), as in mammals (Cooper and Plum, 1987). Under control ammonia conditions there was no significant changes in enzyme activities (GSase, glutaminase, GDH, ALT and AST) in brain tissue or in plasma ammonia concentrations resulting from high-density, air or dissolved cortisol exposure. It was evident that the stressors did not change the metabolism in a way that would cause an ammonia threat on the brain.

A different result for glutamate metabolism in the brain was noted when stressors were combined with ammonia exposure. The activity of neither glutaminase nor GSase was affected by stress alone, yet when stressors were combined with ammonia exposure GSase and glutaminase activity decreased in all but cortisol-exposed fish. These results are contrary to those in Chapter 3 and 4 where fed fish were exposed to elevated ammonia, causing increased GSase activity. The absence of a change in enzyme activity in the cortisol-exposed fish was likely due to continuous exposure of the fish to cortisol, which never allowed self-regulation. The down-regulation of GSase in the other stress-treatment groups would prevent the fish from depleting brain glutamate stores when ammonia concentrations increased. The decrease in glutaminase activity would decrease the conversion of glutamine to glutamate. The combination of the two enzymes, GSase and glutaminase, help regulate glutamate concentrations in the brain. Glutamate is required as an excitatory neurotransmitter and for the production of GABA, an inhibitory neurotransmitter. Reduced GABA formation has been demonstrated in fish when glutamate levels are low as a result of ammonia exposure (Arillo et al., 1981). Low

concentrations of GABA in the brain of mammals are known to cause seizures (Taylor et al., 1999), which are also characteristic of ammonia toxicity. It may be that the combination of ammonia exposure and stress caused a more extreme ammonia insult than ammonia exposure alone and thus depleted brain glutamate levels to the lowest possible threshold. To prevent further depletion of glutamate concentrations in the brain decreased activity of GSase occurred. This in turn decreased capacity for glutamine formation and consequently ammonia detoxification. This is the likely explanation for the increased ammonia toxicity in LC_{50} experiments performed on stressed and cortisol-injected fish in the present study. When feeding fish were exposed to only ammonia making the ammonia insult less severe (Chapter 3 and 4), the mechanism may be different and focus on preventing glutamate accumulation in the brain by converting excess ammonia and glutamate to glutamine. This response may protect the fish from adverse effects of both excess ammonia and glutamate. Accumulation of glutamate in the brain activates the NMDA receptors in the brain (Marcaida et al., 1992) causing release of Ca^{2+} and eventually cell death (Albrecht, 1998). It may be that the regulation of glutamate in the brain at concentrations between the upper and lower thresholds is critical to prevent toxic effects of ammonia. Changes in glutamate concentrations within these thresholds are likely of no physiological consequence and allow some degree of ammonia regulation without adverse effects.

Control fish held at high density and control ammonia-exposed fish showed a suppression of GDH activity in the brain, which alone could result in decreased glutamate production. The combination of stressors and ammonia caused brain GDH activities to return to control levels while brain GSase activity decreased significantly in all but the cortisol exposed fish. This again reflects the importance of glutamate regulation; increased GDH and increased GSase activity would prevent glutamate accumulation in the face of elevated ammonia level.

Cortisol has been implicated as a factor inducing GSase synthesis (Mommsen et al., 1992; Walsh and Milligan, 1995). In the present study, although large individual variation makes it difficult to determine significant differences between circulating cortisol levels, it is still apparent that stressors in combination with ammonia exposure cause decreased GSase activity. The present results for brain GSase activity are contradictory to previous studies on glutamate metabolism in feeding fish when they were exposed to the single stressor, ammonia. In Chapters 3 and 4 it was reported that brain GSase activity increased in response to ammonia

exposure, when fish were feeding. It is probable that cortisol is just one of many substances involved in modulating glutamate metabolism in the brain as noted in mammals (Mommsen et al., 1999), and circulating ammonia and glutamate play a key role. This hypothesis is supported by the fact that a single stress will up regulate GSase synthesis; however when a second stressor is added and circulating ammonia and likely glutamate increases, GSase activity decreases. Unfortunately glutamate was not measured in this study and thus its role can only be inferred. It is important to note that comparable single stressor experiments were conducted on fish that were either feeding or had been recently fed and it was determined that feeding played a fundamental role in ammonia metabolism (Chapter 3 and 4). In contrast, the fish in the present experiments were fasted for 3 days prior to and during the experiments, indicating that the dietary status and/or more significantly glutamate concentrations may be crucial in the ability of a fish to respond to stress.

To further investigate the role of cortisol in glutamate metabolism, the results from the injection experiment need to be considered. Brain enzyme activities were altered under control conditions based on the type of injections. Interestingly, the sham-injected fish had very low activities for brain GSase, and these were approximately half that of the control fish in the stress experiment and the values measured in previous studies (Chapter 3 and 4). This may reflect the nutritional status of the fish as described above or that the oil injections have some indeterminable effect on this enzyme. The increased activity of brain GSase in cortisol and RU486-injected fish suggests that cortisol does play a role in modulating the synthesis of GSase, which catalyzes the removal of excess ammonia and glutamate from the brain. Since RU486 effectively blocks the glucocorticoid receptors and therefore any feedback to the hypothalamic-pituitary axis (Mommsen et al., 1999), it is unlikely that the control of GSase is through this channel. Interestingly, RU486 injection followed by cortisol 12-h later did not result in any increase in GSase activity. It has been documented that RU486 is not always a cortisol antagonist and in fact can cause changes in some metabolic enzymes (Mommsen et al., 1999). This is the first time that a relationship between circulating cortisol and GSase induction in the brain has been demonstrated in rainbow trout.

4.3 Ammonia metabolism in liver

Hepatic metabolism is the major source of ammonia in fish and the liver is a target organ for cortisol (Wendelaar Bonga, 1997). The increase in circulating cortisol due to exposure to stressors in experiment 3 did not affect liver glutamate metabolism; however, when the stressors were combined with ammonia exposure, changes occurred. It has been suggested that cortisol modulates GSase activity in fish (Vijayan et al., 1996, Walsh and Milligan, 1996). The present results for liver GSase activity support the hypothesis that stressors resulting in increased circulating cortisol cause upregulation of GSase mediated by cortisol. Again, the important differences in the data can be accounted for by the nature of the stressors. Cortisol increased in both the control and air-stressed fish exposed to ammonia, which are examples of immediate stress, yet in the high-density ammonia-exposed fish the activity decreased. It is probable that GSase was upregulated by cortisol in the high-density fish prior to ammonia exposure when the stress began 3 days prior to the ammonia treatment. Down-regulation of GSase by decreased synthesis or accelerated degradation most likely occurred when the ammonia exposure commenced as a means to conserve glutamate. Additionally, the cortisol-exposed fish did not respond to ammonia exposure with an increase in GSase activity, again likely due to the cortisol made available to them though the water in advance of ammonia exposure. Any effects of cortisol could have occurred well before ammonia exposure and sampling. It is apparent that the nature and timing of the initial stressor is important when combined with ammonia exposure. The longer lasting stressors appear to suppress GSase activity and the acute stressors up-regulate GSase activity under conditions of elevated ambient ammonia. This evidence suggests that the nature of the stressor and the subsequent cortisol release play a key role in the regulation of glutamate metabolism.

Further support of the role of cortisol in glutamate metabolism is found in the injection experiments. Fish injected with cortisol alone and RU 486 plus cortisol show almost twice the activity for GSase in the liver than that of the control fish not exposed to ammonia. This suggests again that cortisol does in fact induce GSase synthesis in the liver as supported by results from Vijayan et al., (1996); however the mechanism on how this occurs remains to be elucidated. However, using mammalian GSase as a model, a number of mechanisms can be postulated for fish. For, instance, the (single) mammalian GSase gene, contains glucocorticoid and additional steroid responsive elements (de Groot, et al., 1987) as well as other regulatory elements in the 5'-untranslated region (Lie-Venema et al.,1997). Interestingly, the rainbow trout

expresses at least six different glutamine synthetase genes, belonging to four different clades (Murray et al., 2001), making an analysis of the regulation of this octameric enzyme in this species a rather complicated endeavour.

4.4 Ammonia metabolism in white muscle.

White muscle is the largest tissue by mass, accounting for more than 60% of the live mass of the trout. As a result, relatively small changes in metabolism in this tissue could result in significant changes on the whole animal level. Also, the mode of action of ammonia toxicity in muscle is likely due to the depolarization of the muscle (Wicks et al., 2001) rather than the stimulation of NMDA receptors that result from increased glutamate (Albrecht et al., 1998) as predicted in brain tissue. In the present study, neither exposure to stressors or exposure to ammonia had much effect on glutamate metabolism in this tissue. However, when the fish were injected with cortisol, a significant increase in GSase activity was noted and the increase was further enhanced when the fish were exposed to ammonia. If glutamate regulation is not of significance in muscle tissue, then the upregulation of GSase could indicate that muscle acts as an ammonia sink though storage of glutamine. The RU 486/cortisol-injected fish also showed a substantial increase in GSase activity with ammonia exposure, again suggesting that ammonia, in addition to cortisol, is involved in controlling the turnover of GSase.

4.5 Conclusions

Exposure of trout to stressors clearly exacerbates acute ammonia toxicity. This effect is probably attributable to the release of cortisol into the circulation as part of the stress response. Although the present studies show only a weak correlation between exposure to stress and internal ammonia levels, it is most likely that the heightening of ammonia toxicity is due to increased production of nitrogenous wastes when metabolic activity is stimulated by cortisol. This hypothesis was supported by the aggravation of ammonia toxicity in cortisol-injected fish.

In previous sub-lethal studies on feeding and ammonia toxicity in rainbow trout protection from ammonia toxicity is evoked by feeding because GSase is upregulated and α-ketoglutarate is likely not limiting the formation of glutamate and glutamine (Chapter 3 and 4).

The uncharged, innocuous glutamine is accumulated in brain, liver and muscle, thus removing ammonia from circulation. Using the same protocols, but fasted fish exposed to stressors (high-density, air-exposure and 3 ppm ambient cortisol) and then exposed to ammonia, a different response was obtained. Fasted trout were unable to regulate internal ammonia when ammonia exposure commenced. Further, the down-regulation of GSase and glutaminase and up-regulation of GDH indicate this is likely due to a limitation in α-ketoglutarate available for glutamate formation. The main effect of ammonia exposure and cortisol injections is an upregulation of brain, liver and muscle GSase activity, one of the key enzymes in glutamate metabolism and thus ammonia regulation. However, it is apparent from these studies that cortisol is not solely responsible for the regulation of GSase and that increased levels of circulating ammonia and/or glutamate may also be involved. Tissue specific responses to ammonia may occur and more research is required in this area before the mechanisms are fully elucidated.

Table 1. Activities (U/g tissue) of glutaminase, glutamate dehydrogenase (GDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glutamine synthetase (GSase) in rainbow trout exposed to the following stressors; high density and ambient cortisol (3ppm) for four days prior to and during ammonia exposure, 1 minute daily of air exposure and a control group. Fish were exposed to ammonia (0 and 10 mg N/L at pH 7.2).

	Ammonia	**mg N/L**			**Stressor**	**type**		
Tissue Enzyme	0	10	SEM	control	density	3 ppm cortisol	air exposure	SEM
Brain								
Glutaminase	0.909	0.738*	0.005	0.813	0.818	0.844	0.818	0.0007
GDH	2.78	2.89	0.01	2.87	2.76	2.87	2.85	0.02
ALT	2.23	2.34	0.01	2.46	2.19	2.22	2.26	0.01
AST	45.4	46.6	0.4	43.5	47.6	47.2	45.7	0.5
GSase	5.82	3.97*	0.02	4.22[a]	5.15[abc]	5.57[b]	4.64[ac]	0.03
Liver								
Glutaminase	0.138	0.150*	0.0005	0.137	0.140	0.153	0.145	0.0004
GDH	52.5	43.5*	0.3	48.7[ab]	43.5[a]	53.2[b]	46.6[ab]	0.5
ALT	23.9	28.3*	0.2	26.8	24.1	28.8	24.7	0.3
AST	30.8	26.5*	0.3	26.7	26.5	31.1	30.3	0.4
GSase	0.278	0.296	0.003	0.309	0.250	0.2665	0.317	0.004
Muscle								
Glutaminase	0.0281	0.0226*	0.0004	0.0283	0.0229	0.0264	0.0237	0.0005
GDH	0.409	0.752*	0.006	0.602	0.589	0.543	0.588	0.008
ALT	1.22	1.23	0.006	1.28	1.20	1.23	1.19	0.008
AST	25.8	24.3	0.2	26.6	25.5	23.8	24.2	0.3
GSase	0.0112	0.00929*	0.0001	0.0109	0.0104	0.00921	0.0104	0.0002

Results represent the LS means averaged over the 6 and 48 hour sampling period and the factor not being analyzed (n=48 for ammonia and n=24 for stressors) with the corresponding SEM. Significant differences ($p<0.05$) between the main effects are indicated by * for ammonia levels and by a change in letters for stressor type. When analysis of variance (3-factor) indicated a significant interaction (2 or 3 factor) between ammonia concentration, stressor type and time the results are indicated by bold typeface and further examined in the results section.

Table 2. Activities (U/g tissue) of glutaminase, glutamate dehydrogenase (GDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glutamine synthetase (GSase) in rainbow trout injected with a 100μg/g body weight of either cortisol, RU486, RU486 followed 6 hours later with a cortisol injection or a sham injection. Fish were then exposed to ammonia (0 and 10 mg N/L at pH 7.2) for 6 and 48 hours.

	Ammonia	**mg N/L**			**Injection**	**type**		
Tissue Enzyme	0	10	SEM	cortisol	RU486	cortisol/ RU486	sham	SEM
Brain								
Glutaminase	1.02	1.01	0.08	0.914	1.03	1.09	1.03	0.01
GDH	3.02	3.09	0.02	3.01	3.22	2.97	3.03	0.02
ALT	2.33	2.28	0.01	2.34 ^{ab}	2.47 ^{a}	2.45 ^{ab}	2.13 ^{b}	0.02
AST	31.7	32.8	0.3	27.9	35.1	35.9	29.9	0.5
GSase	6.24	7.25*	0.04	7.42 ^{a}	7.34 ^{a}	6.83 ^{ab}	5.40 ^{b}	0.05
Liver								
Glutaminase	0.0962	0.0547*	0.002	0.0820^{a}	0.0433 ^{b}	0.0923 ^{a}	0.0842 ^{a}	0.002
GDH	29.1	28.4	0.2	30.6 ^{a}	26.6 ^{b}	29.4^{ab}	28.5^{ab}	0.2
ALT	27.5	29.8	0.3	29.1	30.3	29.4	25.9	0.5
AST	42.7	40.3	0.4	42.2	39.9	42.8	41.1	0.5
GSase	0.199	0.175	0.003	0.289 ^{a}	0.147 ^{b}	0.178 ^{b}	0.138 ^{b}	0.006
Muscle								
Glutaminase	0.0355	0.0317	0.002	0.0254	0.0416	0.0408	0.0263	0.002
GDH	0.448	0.412	0.004	0.490 ^{a}	0.440 ^{ab}	0.405 ^{b}	0.385^{bc}	0.005
ALT	1.77	1.60	0.02	1.84	1.70	1.74	1.45	0.03
AST	28.1	25.8	0.3	27.4	30.1	25.9	24.5	0.4
GSase	0.0160	0.0214*	0.0003	0.0214	0.0170	0.0201	0.0163	0.0004

Results represent the LS means averaged over the 6 and 48 hour sampling period and the factor not being analyzed (n=48 for ammonia and n=24 for injections) with the corresponding SEM. Significant differences ($p<0.05$) between the main effects are indicated by * for ammonia levels and by a change in letters for injection type. When analysis of variance (3-factor) indicated a significant interaction (2 or 3 factor) between ammonia concentration, injection type and time the results are indicated by bold typeface and further examined in the results section.

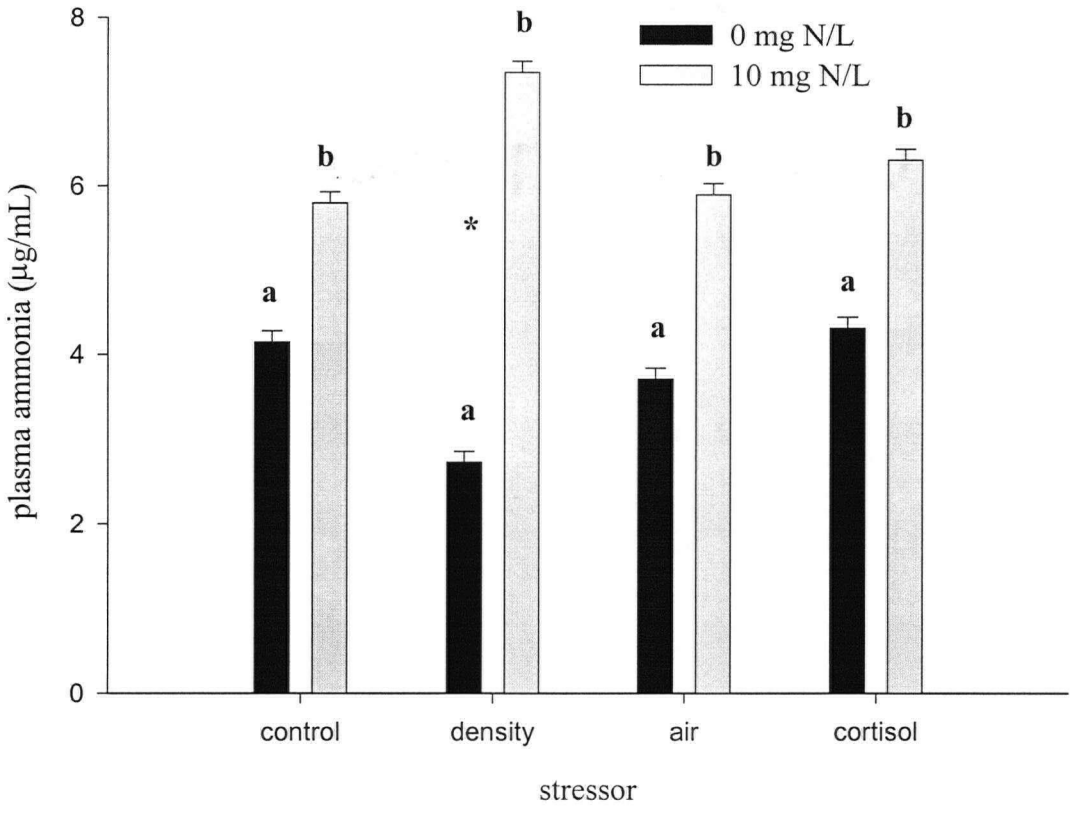

Figure 1. Experiment 3. Least square means for plasma ammonia and stressors in rainbow trout exposed to elevated ammonia (0 and 10 mg N/L) at pH 7.2 for 48 hours. Symbols represent LS means +/- S.E.M. and significant differences ($p<0.05$, $n=12$) between ammonia levels are indicated by a change in letter and by * for stressor.

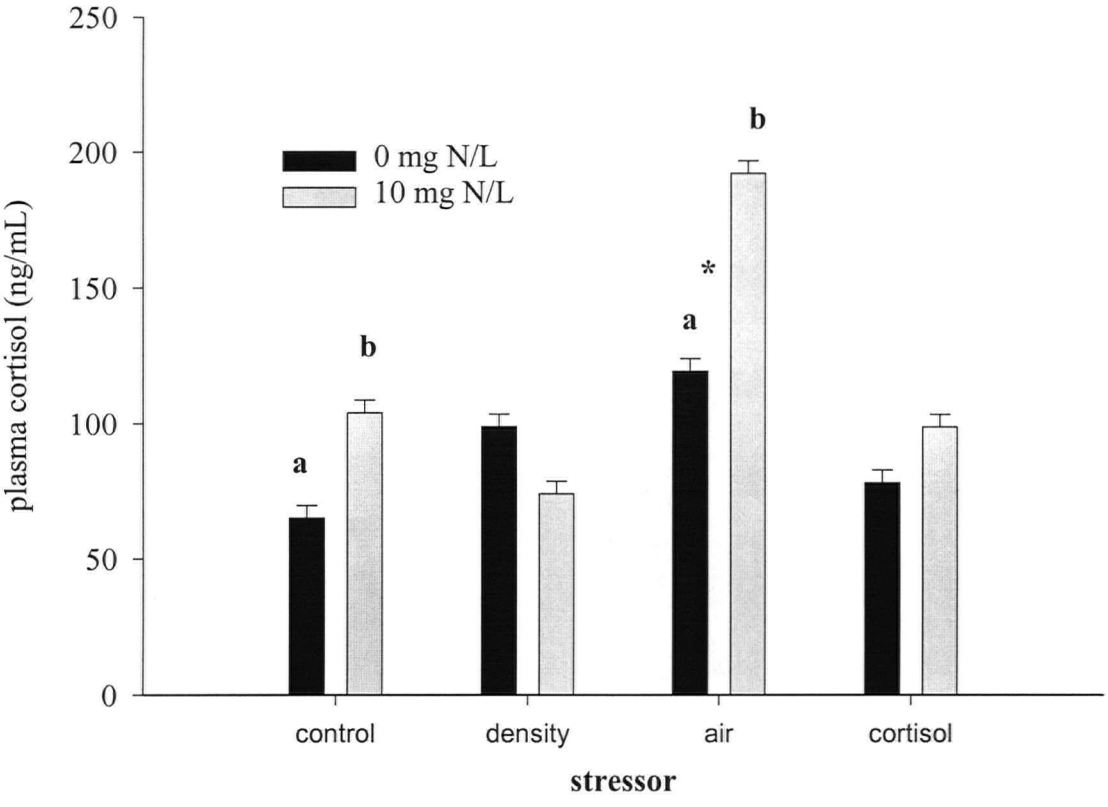

Figure 2. Experiment 3. LS means for plasma cortisol (ng/mL) in ammonia exposed rainbow trout exposed to stressors including (density, air exposure, 3 ppm cortisol and control) and then exposed to ammonia, 10 mg N/L. The means have been averaged over the 6 and 48 hour sampling period. Error bars represent the S.E.M. and significant differences ($p<0.05$, $n=12$) are indicated by a change in letter for ammonia level and * for stressors.

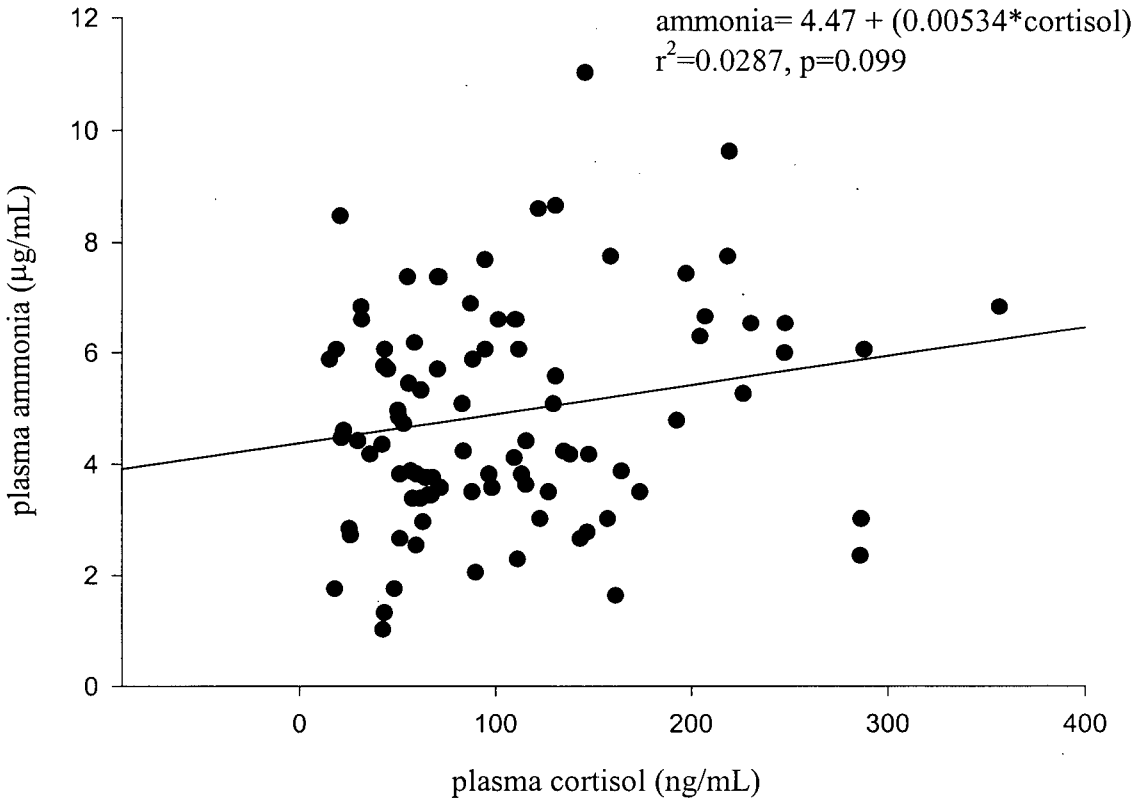

Figure 3. Experiment 3. The concentration of plasma ammonia (µg/mL) regressed on plasma cortisol (ng/mL) in rainbow trout exposed to 0 and 10 mg N/L at pH of 7.2.

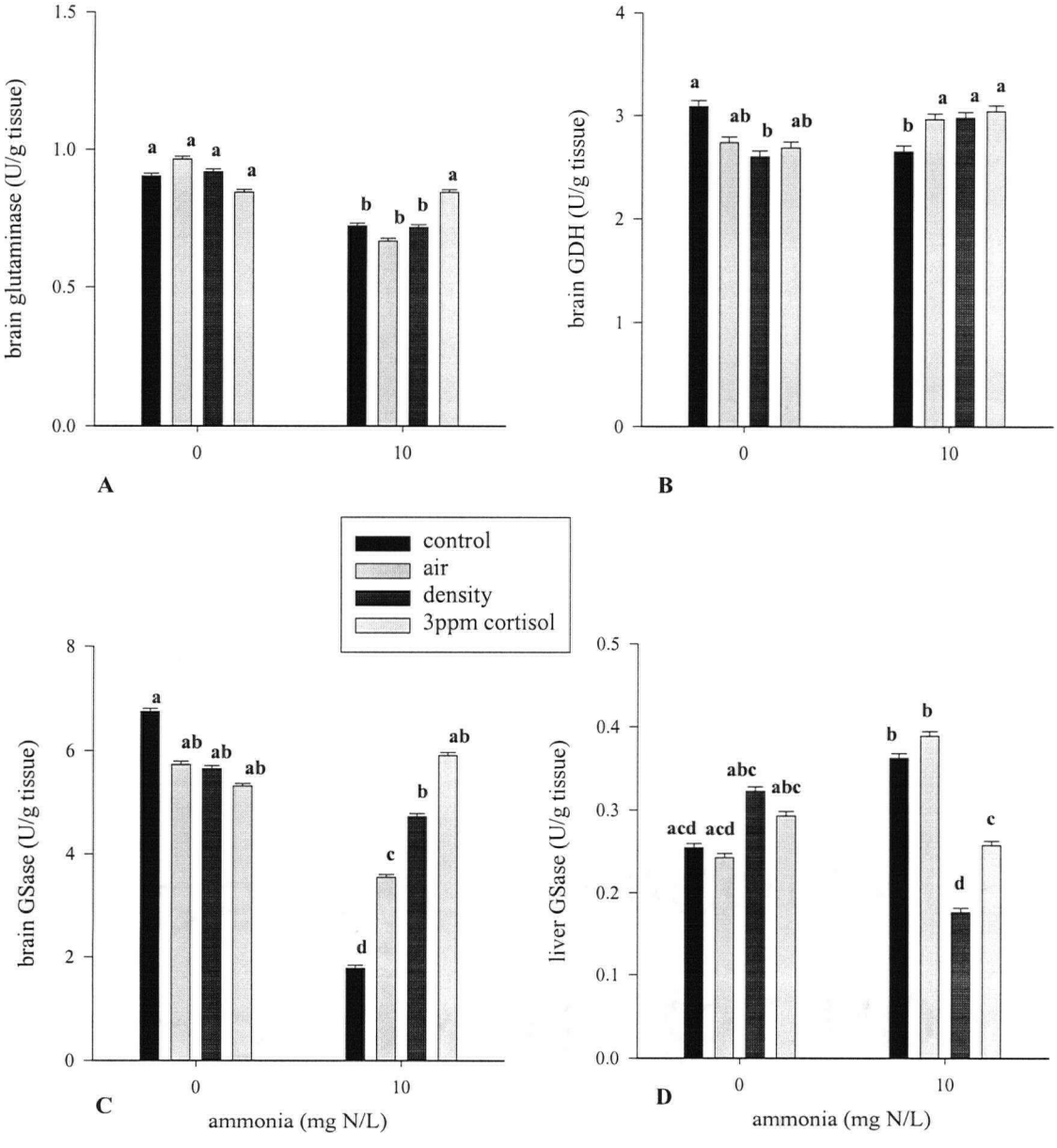

Figure 4. Experiment 3. LS means for brain glutaminase and GDH and brain and liver GSase activities (U/g tissue) in rainbow trout exposed to stressors in combination with exposure to 0 and 10 mg N/L. Values are averaged over 6 and 48 hour sampling periods. Significant differences between means are indicated by a change in letter ($p<0.05$, $n=12$) and bars represent the S.E.M.

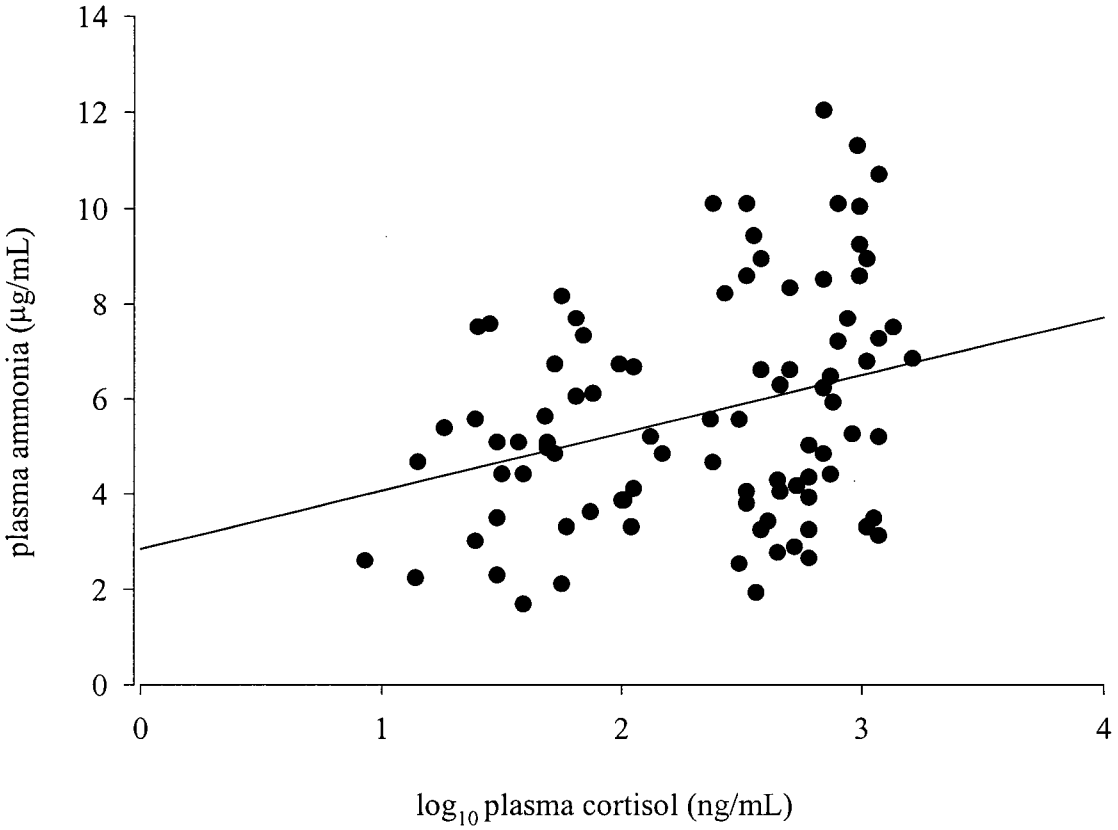

Figure 5. Experiment 4. The regression of plasma ammonia (μg/mL) on $\log_{10}$ plasma cortisol (ng/mL) for rainbow trout injected with cortisol, RU486, cortisol/RU486 and sham and exposed to 0 and 10 mg N/L. The equation of the regression is ammonia=2.847 + (1.220*$\log_{10}$cortisol), p=0.002, r^2=0.095 and n=96.

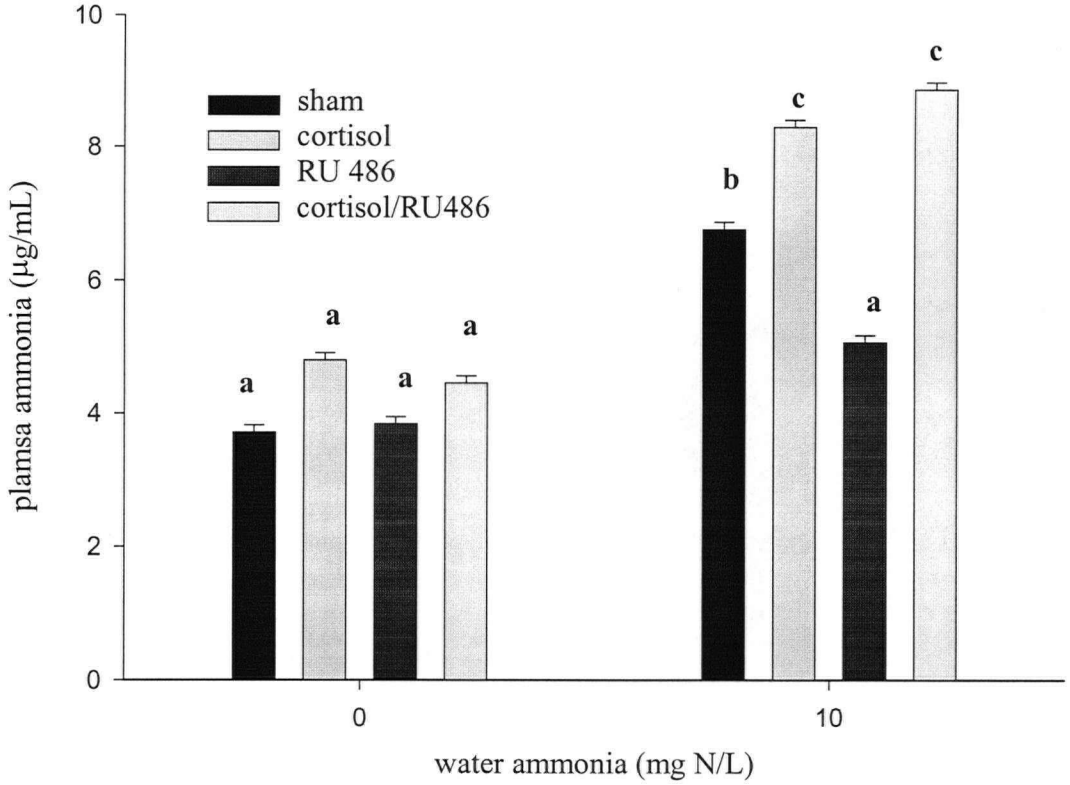

Figure 6. Experiment 4. LS means for plasma ammonia (μg/mL) in rainbow trout injected with cortisol, RU486, RU486/cortisol and sham (oil only) and then exposed to 0 and 10 mg N/L at pH 7.2. LS means are averaged over 6 and 48 hour sampling times and bars represent SEM. Significant differences are indicated by a change in letter ($p<0.05$, $n=12$).

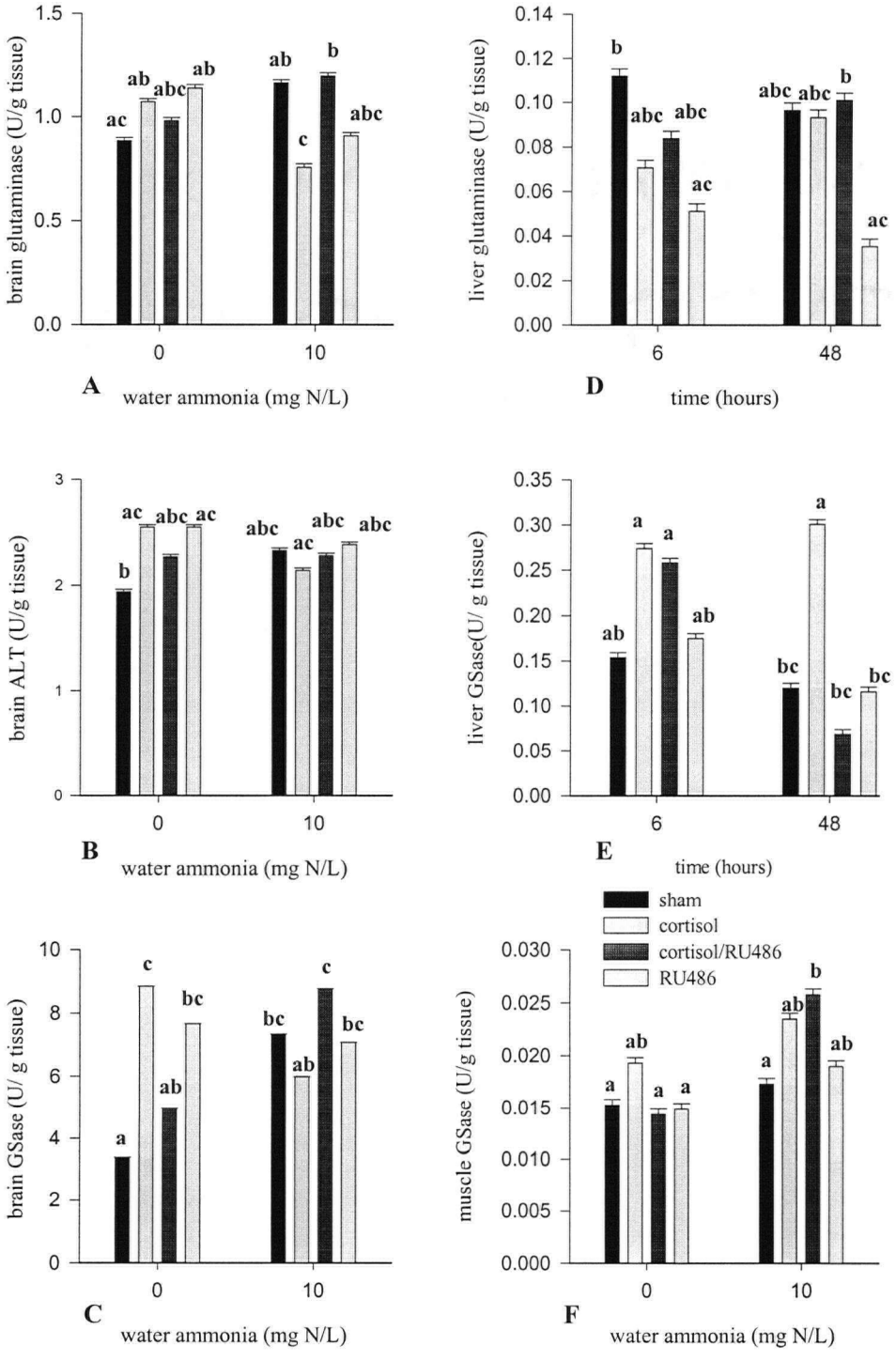

Figure 7. Experiment 4. LS means +/- SEM for brain glutaminase, ALT and GSase, liver glutaminase and GSase and muscle GSase activities (U/ g tissue) in rainbow trout injected with 100 μg/g body weight of either sham, cortisol, RU486 followed by cortisol or RU486 in combination with exposure to ammonia (0 or 10 mg N/L) for a period of either 6 or 48 hours. Significant differences are indicated by a change in letter ($p<0.05$, $n=12$).

Chapter 6. General Conclusions

One of the main reasons for conducting the present research was to assess the validity of the criteria used for the prevention of ammonia toxicity in fish living in fresh water. As discussed in the introduction, ammonia is produced naturally in fish during swimming, feeding and stress. These are all conditions that are avoided when using standard toxicity testing protocols. It has been previously demonstrated that salmonids that are actively swimming and exposed to elevated ambient ammonia have both reduced swimming potential and higher mortality rates than a "resting" fish (Wicks et al., 2001) and that the present US EPA standard may not be protective under swimming conditions.

Some of my studies consider both acute ammonia toxicity tests and tests performed at sub-lethal ammonia levels under other natural conditions where fish are either feeding or stressed. All of the toxicity testing conducted in the present studies was done in water maintained at a pH of 7.2. The CMC and the CCC value set forth by the US EPA (1998, 1999) are respectively, 19.7 and 2.8 mg N/L at a pH of 7.2 when salmonid fish are present in a body of water. The CMC value is 29.5 mg N/L when salmonids are absent. In the acute toxicity tests that used rainbow trout either stressed by high loading density or fed just prior to ammonia exposure, it was evident that the present standards were protective; however, a large difference existed between feeding, which ameliorated acute toxicity, and stress, which exacerbated it. These differences, caused by natural variables experienced by fish, indicate that the present toxicity testing protocols used to collect data for development of US EPA ammonia water quality criterion are not appropriate with respect to ammonia. The protocols from the present study that utilize both stressed and fed fish should be integrated with the recommended methods for toxicity testing. With the use of more appropriate testing methods the present criteria can be adjusted according to the true nature of the toxicant.

In the sub-lethal studies, rainbow trout exposed to 10 mg N/L at pH 7.2, a concentration intermediate to the CCC and the CMC standard, experienced stress as indicated by an increase in circulating cortisol. Although this stress was not lethal, it could conceivably compromise the fish's immune system and thereby result in reduced survival due to other factors such as disease or a second stressor such as metal exposure. This type of physiological data should continue to

be collected and be used to make amendments to present water quality criteria as required. Recently, the US EPA incorporated the effects of temperature on early life-stages of fish into the ammonia criteria (EPA, 1999). A new significantly lower chronic criterion for ammonia was established at temperatures below 15 ^{o}C when early life stages of fish are present (US EPA, 1999). This is an example of how the nature of the toxicant has been considered by the US EPA. In addition, in 1998 the results from a long-term mesocosm study were incorporated into the US EPA water quality document indicating that organisms held at CCC ammonia concentrations showed no decrease in biomass supporting the present standard. The examples given above and the sub-lethal data collected for this thesis help to provide a more complete understanding of ammonia toxicity in the aquatic environment. This type of toxicological research needs to continue and the data be used for re-evaluation of the procedures used to establish criteria and the criteria themselves.

A second reason for the research in this thesis, resulting from studies on ammonia toxicity, was to provide insight to toxic mechanisms and ammonia handling in tissues, especially the brain which is a target for ammonia toxicity. Excretion of nitrogenous wastes is a common problem among all living things. When ammonia excretion is impaired for whatever reason and internal ammonia concentrations increase, toxic effects can be seen. In the mammalian model, ammonia toxicity appears to manifest itself in the brain and results in symptoms such as convulsions, coma and death. Similar symptoms have been noted in fish with convulsions and hyperventilation being the most notable. In mammalian models these effects have been attributed mainly to interference with energy metabolism and a substantial reduction in neural transmitters in the brain (Cooper and Plum, 1987). Recently, the ionotropic glutamate receptors, and more specifically the NMDA receptors in the brain have been implicated in mediating ammonia toxicity. Injection of NMDA receptor agonists protects mice from acute ammonia toxicity (Marcaida et al., 1992). The involvement of these receptors appears to be related to an accumulation of extra synaptic glutamate caused by either a release from the neurons and/or reduced uptake (Albrecht, 1998). This accumulation of glutamate activates the NMDA receptors and causes subsequent Ca^{2+} release eventually ending in cell death (Marcaida et al., 1992). Based on this animal model, it is apparent that the loss of control of glutamate regulation in brain tissue could be a key factor in controlling ammonia toxicity. It is worth

noting, however, that although blocking the NMDA receptors does prevent death from ammonia toxicity it does not prevent the symptomatic convulsions (Marcaida et al., 1992).

Nothing is known about the compartmental distribution of glutamate in fish, but overall glutamate is produced from α-ketoglutarate + NH_4^+ via glutamate dehydrogenase and removed by combining with NH_3 to form glutamine via glutamine synthetase (Figure 2B). Using data from Chapter 3, the mean concentrations of glutamate and glutamine synthetase activity in the brain show an interesting relationship (Figure 1). The data suggest that as the activity of glutamine synthetase increases the concentration of glutamate decreases. Figure 2A depicts a proposed model of how ammonia and glutamate regulation occurs in the brain of rainbow trout exposed to sub-lethal concentrations of ammonia while feeding. The model proposes upper and lower thresholds for glutamate concentrations between which changes in concentration have little or no physiological consequence. In this model neither substrates nor enzymes (Figure 2B) are limiting and therefore it enables the fish to convert ammonia to glutamine, without neurological impairment.

A different model is proposed when rainbow trout are fasting and exposed to both ammonia and high-density stress (Figure 3). In this model, the fish are able to regulate internal ammonia that results from an initial stressor (density); however, when the stressed fish are exposed to ammonia, regulation is no longer possible and plasma ammonia increases. Based on this model the fish attempt to balance glutamate concentrations by reducing GSase activity, thus slowing glutamine formation and ammonia detoxification. Reduction of glutaminase activity also occurs which would slow down conversion of glutamine to glutamate and prevent more ammonia production (Figure 2B). A very interesting aspect of the model is that GDH activity decreases with the initial stressor and then increases again with the second ammonia stress. This change in activity may be attributable to limited availability of α-ketoglutarate for glutamate formation in the brain making it a good candidate as the rate-limiting substrate. It is possible that when fish are feeding (Figure 2A) α-ketoglutarate may not be in such short supply. In fact, feeding animals are likely to have ample supply of α-ketoglutarate from the diet, including some derived from dietary amino acids, such as arginine or histidine, initially dealt with in the liver. The question remains how α-ketoglutarate may reach the brain without using glutamate or glutamine as carriers. These models support the hypothesis that regulation of brain glutamate is

critical to avoid potential toxic effects, and that glutamate rather than ammonia may be regulating the synthesis of glutamine synthetase. They may of course both be important.

My studies show that glutamine is accumulated in muscle tissue when fish are exposed to ammonia and, even though the quantity of glutamine accumulated is small per gram of tissue when compared to the brain, the proportion of the total body mass for this tissue is high making muscle the most important ammonia sink in rainbow trout. A similar strategy to cope with a much larger quantity of ammonia has been adopted by the marble goby when excretion is impaired during air exposure (Jow et al., 1999). The marble goby activates glutamine synthetase in the liver, thus enabling rapid conversion of ammonia to glutamine, which is then stored in muscle tissue until more favourable conditions are encountered. In comparing ammonia metabolism in brain and muscle tissue, it is important to note that different modes of action for ammonia may exist. As previously described, an accumulation of glutamate resulting from increased ammonia appears to play a key role in ammonia toxicity in the brain. In muscle, it is likely that the depolarization of muscle membranes (Wicks et al., 2001) and depletion of glycogen stores, NADH and adenylates (Arillo et al., 1981; Wang et al., 1994) is problematic when ammonia is elevated, not the glutamate levels. Since the potential for over activation of the NMDA receptors is limited to the central nervous system, glutamate regulation is less important in muscle tissue, making muscle a good option for ammonia storage. The importance of the removal of excess ammonia in muscle tissue should not be understated however, as increased ammonia has been documented to decrease swimming performance (Wicks et al., 2001). The fate of the stored glutamine was not determined in this study, although it is probable that it is transported to the liver and deaminated by glutaminase (Mommsen and Walsh, 1992) after the ammonia insult subsides. Alternatively it may be transported out of the body through feces. This is an area that requires further investigation.

It has been shown that rainbow trout fed a high protein diet also unregulated glutamate/glutamine metabolism to protect the brain from high levels of circulating ammonia or excess glutamate. The priming of this pathway in fish fed a high protein diet in fact ameliorated the effects of acute ammonia exposure. The upregulation of glutamate/glutamine metabolism was directly related to the protein content of the diet the fish consumed. The diet containing 40% crude protein did not cause a measurable increase in glutamine formation, while the diet containing 48% did. These results indicate that commercial fish food manufactures may want to

reassess the protein content of the various salmonid diets as well as feeding regimes, as the high protein levels of commercially available food is energetically costly to rainbow trout as well as economically costly to the manufacturers.

With respect to stressed fish exposed to ammonia it was determined that stressors exacerbated ammonia toxicity. It was evident from the data that the nature of the stressor, whether it was either an acute stress such as handling or chronic stress such as high loading density, is an important consideration in fish that may be exposed to ammonia. The importance of the type of stressor is related to the demonstrated role that cortisol plays in increasing the synthesis of glutamine synthetase. It was suggested that cortisol increased ammonia toxicity by increasing intermediary metabolism and thus nitrogenous waste production. Although stress/cortisol increased ammonia toxicity, increased cortisol was involved in up regulation of glutamine synthetase, a key component of a pathway proposed to ameliorate ammonia toxicity. It was apparent however that cortisol was not solely responsible for control of glutamine synthetase synthesis and that the level of circulating ammonia or glutamate concentrations may be involved. The work contained in this thesis regarding the role of cortisol in ammonia metabolism and the enzymes involved does not fully explain the nature of these relationships and demonstrated that this is an area that needs to be more fully explored.

In conclusion, the research contained in this thesis has resolved some aspects of ammonia toxicity in the rainbow trout. Based on my data I propose that although the present ammonia criteria are protective of rainbow trout the procedures for determining these criteria need to be re-evaluated to include more realistic environmental conditions. It is likely if different toxicity-testing protocols, incorporating feeding and stressors are implemented that the present ammonia criteria will change. In addition, I have confirmed that rainbow trout are capable of detoxifying internal ammonia to some degree by storing it as glutamine. This ability is dependent on the both the nutritional status and level of stress in the fish. I have proposed two models for ammonia metabolism in the brain, one for fed fish and one for fasting stressed fish. The differences between these two models emphasize the importance of the environmental conditions and physiological status of fish with respect to ammonia metabolism and toxicity. The combination of physiological and toxicological information contained in my thesis as it pertains to ammonia toxicity in fish provides some new insight into an old topic.

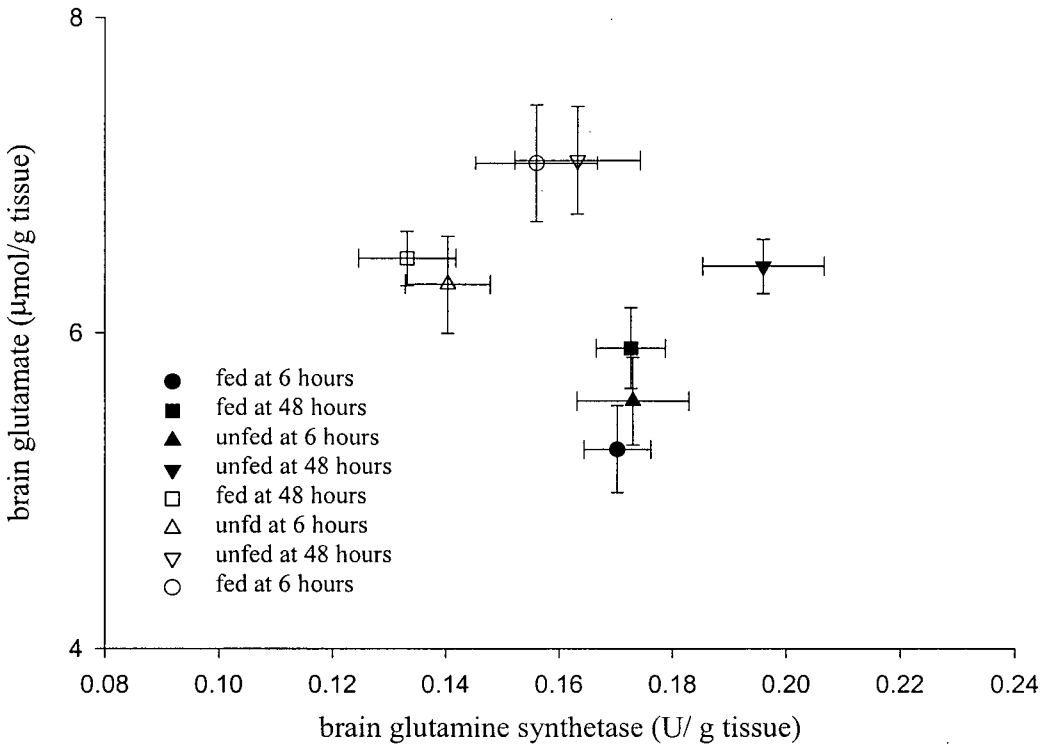

Figure 1. Relationship between mean glutamine synthetase activity and mean glutamate concentration in brain tissue of fed and unfed rainbow trout exposed to control (hollow symbols) and 10 mg N/L ammonia (solid symbols) for 6 and 48 hours. Bars represent the S.E.M. and n=6.

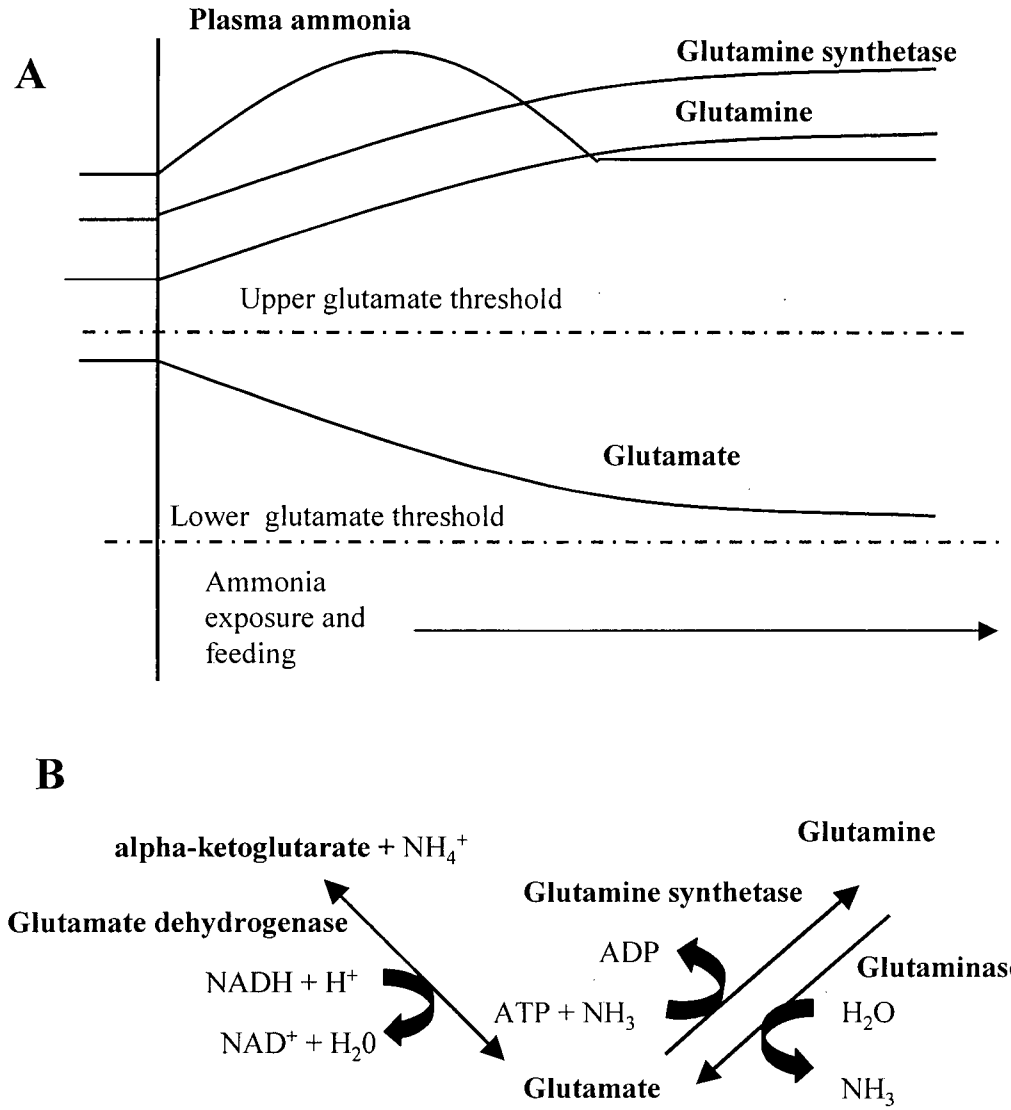

Figure 2. A) Proposed model for the regulation of glutamate and ammonia in the brains of fed rainbow trout exposed to sub-lethal ambient ammonia. B) Pathway for glutamate and glutamine metabolism.

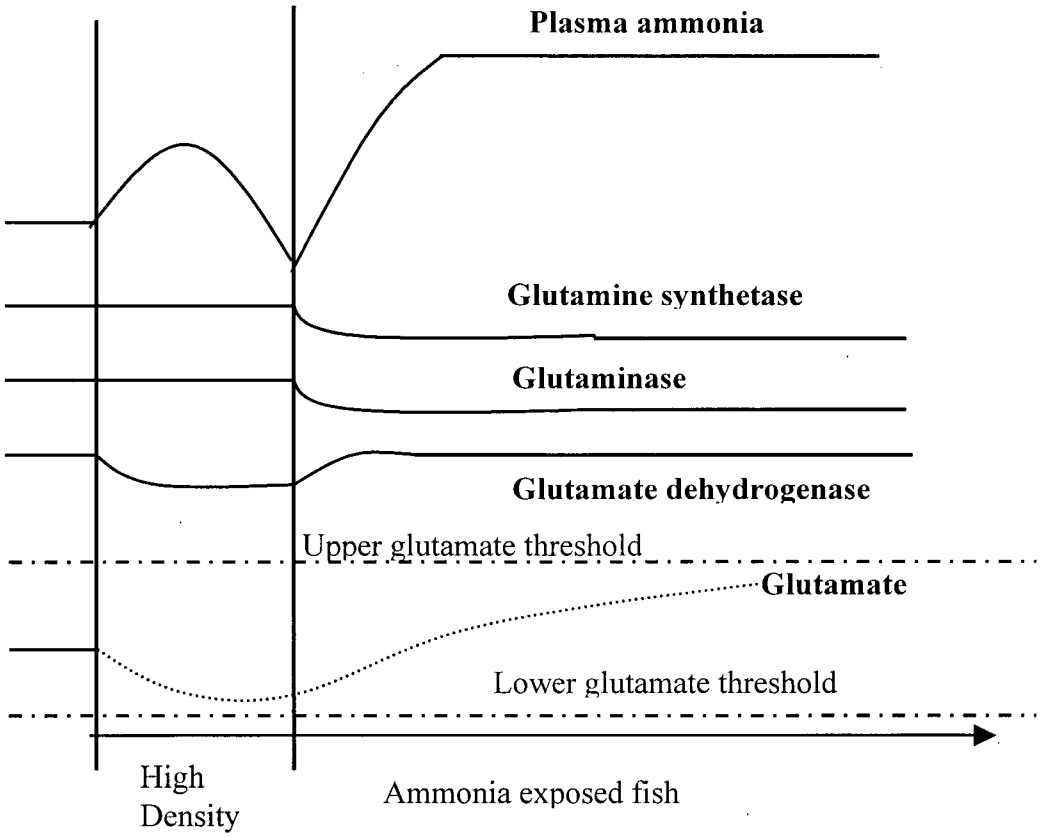

Figure 3. Proposed model for the regulation of glutamate and ammonia in the brain of fasted rainbow trout exposed to a 48-hr density stress prior to sub-lethal ammonia exposure.

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