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THE COMPARATIVE PHYSIOLOGY OF FATHEAD MINNOWS AND RAINBOW TROUT: INSIGHTS INTO THE TOLERANCE OF THE TWO SPECIES TO TOXICANTS

By

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A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree
Doctor of Philosophy

McMaster University
February, 2001

A PHYSIOLOGICAL COMPARISON OF FATHEAD MINNOW AND RAINBOW TROUT

DOCTOR OF PHILOSOPHY (2000) McMASTER UNIVERSITY

(BIOLOGY) Hamilton, Ontario

TITLE: THE COMPARATIVE PHYSIOLOGY OF FATHEAD MINNOWS AND RAINBOW TROUT: INSIGHTS INTO THE TOLERANCE OF THE TWO SPECIES TO TOXICANTS

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NUMBER OF PAGES: xiv, 195

Abstract

In this thesis the physiological systems of two unrelated freshwater fish with different tolerances to many toxicants were compared. Comparisons were made between fathead minnow (FHM) and rainbow trout (RBT) that were approximately 1.5 g. The approach was to examine and compare physiological systems in FHM and RBT that would be affected by these toxicants. Established methodologies such as the monitoring of whole body concentrations, transepithelial fluxes, unidirectional fluxes, and enzyme activity rates were used. In addition, swimming as a performance measure was also compared in the two species.

Differences between the two species were found in gill function and morphology, detoxification, and cellular resistance. The greater sensitivity of FHM than RBT to H^+ ions was related to the fact that FHM appeared to regulate ions with less precision than RBT. There were significant linear relationships between whole body Na^+ (r^2 0.24) and Cl^- (r^2 0.33) and temperature as well as with Na^+ uptake (r^2 0.70) and temperature in FHM after up to 2 months acclimation. In contrast there were no significant relationships between these parameters and temperature in acclimated RBT. In addition, FHM did not up-regulate Na^+ uptake even after they had lost up to 31% of whole body Na^+ whereas there were 2-fold increases in RBT after whole body Na^+ losses of 22%. Furthermore, a variety of challenges such as epinephrine injection, saline injection, osmotic shock and acute exposure to low external Ca^{2+} caused significantly greater Na^+ losses in FHM than RBT. As well acute exposure to low pH and copper provoked significantly greater Na^+ losses in FHM than RBT when combined with low external Ca^{2+}. Fathead minnows also had significantly greater clearance rates of intraperitoneally injected mannitoi and PEG.

The greater tolerance of FHM than RBT to ammonia and organic toxicants was partially attributed to lower gill uptake of these compounds. Two models showed that FHM took up less ammonia than RBT over the range of 0 to 1 mM external ammonia. In addition, RBT had 2-fold higher ethanol uptake than FHM. These differences in uptake may be partially attributed to 2-fold higher lamellar surface area in RBT than FHM. In addition, FHM had 2- to 3-fold greater gill mucus content than RBT.

The activity rates of the ammonia detoxifying enzyme, glutamine synthetase were 1.7-fold higher in FHM than RBT. Fathead minnows also had 1.7-fold higher activity rates of the phase II biotransformation enzyme, glutathione-s-transferase, than RBT.

Fathead minnows also tolerated higher tissue concentrations of ammonia, and monochlorobenzene than RBT. At rest, whole body ammonia concentrations were 2- fold higher in FHM and mortality in RBT was associated with whole body ammonia concentrations of 3.7 ± 0.5 μmol g^{-1} whereas there was no mortality in FHM when whole body ammonia concentrations were 10.1 ± 1.2 μmol g^{-1}. Fathead minnows were also far more tolerant of intraperitoneally injected ammonia and monochlorobenzene than RBT.

Finally, the sprint test was a useful measure of swim performance in both species. The rank order of individual sprint performance was reproducible in FHM (Spearman's rank coefficient (r_s 0.95)) and RBT (r_s 0.92), and sprint performance was impaired in FHM and RBT with acute exposure to ammonia. However, in FHM sprint performance declined when sprints were repeated at 24 h intervals whereas it was unaffected by this treatment in RBT. In addition, swimming performance scaled with body size in RBT (30% of variation) but not in FHM (5% of variation). Fathead minnows also expended significantly more anaerobic energy than RBT after a Ucrit test, but not after a sprint test.

Acknowledgments

There are a number of people who have been very important and helpful in the completion of this thesis to whom I would like to express my gratitude and thanks. To begin, the cohort at the back of 216, Wendy McFarlane, Lisa Taylor, and Jeff Richards who have listened and shared. To Vandy Thomas, whose guidance was always helpful. To Dr. Sue Clearwater and Dr. Greg Pyle for editorial comments and sharing their thoughts and experiences. To additional fish folk Christina Miasaszek, Erin Fitzgerald, Danielle McDonald, Shawn Baskin, Emma Postlethwaite, Jacqui Dockray, Tyler Linton, Leela D'Cruz, Sheryl Edwards, Julie Lee, and many important others. I would like to thank Shaheen Zia, Dr. John Lott and Dr. Louise Barber for their help with the L.M. work and Vanessa Lougheed for her help with the microscope and the statistics. I would also like to extend thanks to my committee Dr. Chris Wood, and Dr. Pat Chow-Fraser for their comments and support and to my supervisor Dr. Gord McDonald who had lots of advice and was always willing to talk.

I would like to thank, Barb Crosbie, Dave Olmsted, Andrea Kirkwood, Nancy Donovan, and C.L. and Rob Van Bremen , for their support, pep talks, and belief that this would get done.

The support of my family has also been invaluable and sustaining. Thanks to Jen Croke, John Croke and Kate Aitken, Carolyn Croke, and the Curtis'. Also to Kathy and Marc Bell, and Glen and Jobyna Bell.

Thank you to my parents Joy and Fred Croke, who have provided unlimited support - from emotional and up. It's finally done!

Finally, I owe a tremendous amount to my husband Rob Bell whose steadfastness, unyielding support, and patience gave me the resolution to complete this thesis. I love you.

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Chapter 1
General Introduction

General Introduction

The external environment of temperate freshwater fish requires them to maintain ion balance in a hypotonic environment, dispose of excess water as well as endogenously produced ammonia, tolerate temperature changes in a thermally labile environment, and tolerate periodic or chronic exposure to naturally occurring (i.e., ammonia, anoxia) or anthropogenically occurring (i.e., toxicants, low pH, metals) pollutants. The physiological, biochemical, and behavioural responses of a fish are designed to cope with these requirements. In this thesis I compared the way two unrelated freshwater fish species met some of these requirements, particularly the way in which the physiological systems in each species responded to pollutants.

Comparative physiology can be used to elucidate mechanisms. Often these mechanisms become clearer when one compares the way in which different species meet their functional requirements. Moreover, these types of comparisons can eventually lead to broad biological generalizations arising from the use of different species as one experimental variable (Prosser, 1950). This type of approach may be particularly useful to toxicology.

Traditionally, toxicology focused upon the concentration required to cause 50% lethality in a population within a set time period. However, because of the overwhelming number of untested chemicals, models were developed that could predict toxicity based upon some physiochemical feature of the chemical (i.e., QSAR's; Blum and Speece, 1990). More recently toxicologists have developed models that predict the toxicity of a chemical by the response of an animal or the amount of chemical uptake in an animal. Examples of these models include lethal body burden (Lassiter and Hallam, 1990; McCarty and MacKay, 1993), physiologically based toxicokinetics (McKim and Nichols, 1994), and fish acute toxicity syndromes (McKim and Bradbury, 1987). Thus, current models

have focused upon explaining toxicity differences amongst chemicals. Comparatively little effort has focused upon explaining differences in species sensitivity to toxicants. In this thesis the objectives were to establish the physiological differences between two unrelated freshwater fish species and to assess these differences based upon how they could contribute to differences in tolerance to toxicants.

Choice of Species

The two freshwater fish species chosen for this study were the cyprinid, fathead minnow (FHM; *Pimephales promelas*) and the salmonid, rainbow trout (RBT; *Oncorhynchus mykiss*). These two unrelated species are separated by approximately 100 million years of evolution (Young, 1962) and differ in their life histories and habitat. However, both species are widely used in toxicological testing, so the relative sensitivities of each to a wide variety of compounds are known. There are also known differences in the tolerance of FHM and RBT to a variety of toxicants. For example, FHM are approximately one pH unit less tolerant of low pH in acute exposures (Hickie et al., 1993), and in the natural environment FHM are not found in soft water lakes with a pH below 5.9 (Matuszek et al., 1988) while RBT can be found at a pH as low as 4.8 (Harvey, 1979). Furthermore, FHM are roughly 3-fold more tolerant of ammonia (Thurston et al., 1983; Thurston and Russo, 1983) and many organic toxicants (Vittozzi and De Angelis, 1991) than RBT. In addition, the proven methods for culturing and handing FHM and RBT (EPS, 1992), as well as the easy availability and abundance of the two species also make them good study species.

The differences in life history and habitat preferences between the two species presented several choices for a comparative study. First, FHM are much shorter-lived, smaller fish, with a life span of 2^{+}- to 3^{+}-years, an adult size of 5 to 7 cm, and the onset of sexual maturity occurs at approximately 5 months (Scott and Crossman, 1973; EPS,

1992). In contrast, the life span of RBT is around 6^+- to 8^+-years, the adult size is 50 to 76 cm, and they are sexually mature at 3^+- to 5^+-years (Scott and Crossman, 1973). In this thesis I chose to compare similarly-sized RBT and FHM (i.e., around 1 to 2 g, 4.5 cm), rather than fish of the same relative age. Thus, all of the comparisons in this thesis are between adult FHM and juvenile RBT. This choice meant that the influence of scaling effects were less of a concern, and similar experimental set-ups could be used for the two species. There were, however, some difficulties with this decision. First, comparing fish of different relative ages meant that FHM were sexually mature whereas RBT were not. One way to minimize this difference was to avoid using FHM that were obviously in breeding condition. This was possible because male FHM in breeding condition develop two blackened vertical bars near the front of the body and tubercles on their heads and females develop an ovipositor (EPS, 1992). In addition, the difference in the relative age of the two species could influence metabolism because young rapidly growing fish (i.e., juvenile RBT) may have higher metabolic rates than older more slowly growing fish (i.e., adult FHM). However, the literature suggests that oxygen consumption rates are only slightly higher in 1- 2 g RBT than FHM. For example, standard and active oxygen consumption rates of 2.5 mg kg^{-1} min^{-1} and 13 mg kg^{-1} min^{-1} respectively, were reported for 2 g FHM held at 18 °C (MacLeod and Smith, 1966), whereas standard and active oxygen consumption rates of 3-5 mg kg^{-1} min^{-1} and 16 mg kg^{-1} min^{-1} respectively, were reported for 0.5 g RBT held at 20 °C (Lucas and Priede, 1992). This suggests that differences in metabolism between the two species in this thesis play only a small role in differences between the physiological systems of FHM and RBT.

Secondly, there are differences in the thermal preferences of the two species. Fathead minnows prefer warm still water, and their thermal optimum is between 23 and 25 °C (Brungs, 1971). In contrast, juvenile RBT prefer cool, fast moving water and their thermal optima is between 11 to 14°C (Scott and Crossman, 1973). In this thesis, most

comparisons between the two fish were done at temperatures between the thermal optimum of the two species (i.e., 14 to 19 °C). The critical thermal maxima of RBT is between 26 and 30 °C, whereas FHM have higher critical thermal maxima, between 33 and 40 °C (see review: Beitinger et al., 2000). Thus, temperatures closer to the thermal optima of FHM could not be used for RBT. In addition, the use of the same temperature for both species in most experiments simplified the design of many of the experiments, and reduced the complication of separating temperature effect differences from species differences.

Finally the diet of FHM in the wild is more herbivorous than that of RBT (Scott and Crossman, 1973). In this thesis FHM were fed a diet of nutrafin flakes and frozen brine shrimp, whereas RBT were fed trout chow. This choice was made in order to keep fish healthy. The crude protein content of the trout chow was 51%, the Nutrafin flakes were 46% crude protein, and brine shrimp were 62% crude protein. These differences were taken into consideration in the ammonia study (chapter 3) but were ignored in other studies.

Organization of Chapters

Four studies were conceived to examine the relationship between physiology and the different responses of FHM and RBT to several toxicants. The first study (chapter 2) was based upon the fact that FHM are more sensitive to low pH than RBT (Harvey, 1979; Matuszek et al., 1988; Hickie et al., 1993). The primary mechanism of toxic action of H^+ ions is the disruption of ionoregulation at the gill surface (Wood and McDonald, 1982). Therefore, I hypothesized that differences in ion regulation of the two species was key to understanding the physiological basis of the differential tolerance of these two species to H^+ ions, or other toxicants that disrupt ionoregulation at the gill surface. My approach was to document ion regulation of the two species under routine (i.e., resting) and challenge (i.e., perturbations such as stress, toxicant exposure, and osmotic shock) conditions using standard techniques to measure whole body Na^+ and Cl^- balance, Na^+ fluxes, and Na^+ and

Cl^- uptake. Differences in ion regulation between the two species were then assessed for how they could have contributed to the tolerance of either species to low pH or other toxicants which affect ion regulation at the gill surface of fish.

The second study (chapter 3) was based upon the fact that FHM are 3-times more tolerant to ammonia compared with RBT (Thurston et al., 1983; Thurston and Russo, 1983). The primary mechanism of toxic action of ammonia is less clear than that of low pH (Tomasso, 1994). Ammonia is known to disrupt nervous tissue function in the brain of mammals (Schneker et al., 1969; Minana et al., 1996) and it is speculated that it has similar effects in fish (Smart, 1978). In addition, it has been shown to affect the neuromuscular junction (Heald, 1975; Beaumont et al., 2000). Therefore I hypothesized that differences in ammonia tolerance between the two species were due to differences in either ammonia uptake, excretion, detoxification, cellular resistance to ammonia, or a combination of these factors. My approach was to measure ammonia and urea excretion, whole body ammonia concentrations, as well as the activity of an ammonia detoxifying enzyme, glutamate synthetase at rest and during elevated ammonia exposure in both species. Any differences between the two species were then assessed for how they could contribute to the greater tolerance of FHM to ammonia.

In the third study (chapter 4) was based upon the generally greater tolerance of organic chemicals in FHM compared with RBT (Mayer and Ellersieck, 1989; Vittozzi and De Angelis, 1991; Ramamoorthy and Baddaloo, 1995). The difference in tolerance between the two species ranges from 2- to 4-fold greater in FHM for non-specifically acting toxicants such as narcotics, up to more than 100-fold greater for specifically acting toxicants such as pesticides that inhibit acetylcholinesterase. The focus of this study will be differences between the two species for non-specifically acting toxicants. Using the same criteria as the ammonia study (Chapter 3), I hypothesized that differences in tolerance to organic toxicants between the two species were due to differences in either toxicant uptake,

excretion, detoxification, cellular resistance to a toxicant, or a combination of these factors. Accordingly, my approach was to measure non-electrolyte uptake, clearance rates of non-metabolizable markers, activity rates of a detoxifying enzyme, and differences in tolerance to an anaesthetic and a narcotic chemical in both species.

The fourth study (chapter 5) investigated the use of a sprint test to assess performance in the two species. Swim performance is often used in chronic toxicological studies as a means of determining whether or not fish are negatively impacted by toxicant exposure (Wedemeyer and McLeay, 1981; Beitinger and McCauley, 1990). In this study, I examined and compared factors that might affect the interpretation of sprint performance in two disparate species. I hypothesized that differences in the response of sprint performance between the two species to factors such as scaling, velocity, fuel use, repeated sprint trials, reproducibility of individual performance, acute and seasonal temperature change, and toxicant exposure might lead to either the inappropriate use of the sprint test, or erroneous conclusions based upon sprint test results, particularly when comparing fish species with different habitat preferences (i.e., current *vs.* non-current, RBT *vs.* FHM respectively).

The highlights of the physiological differences between FHM and RBT in each chapter were then combined in Chapter 6 to create a summary on how physiological differences could contribute to tolerance to toxicants.

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Chapter 2

Ionoregulation in rainbow trout and fathead minnow under routine and challenge conditions: Implications for sensitivity to gill surface active toxicants

Introduction

It is now well established that freshwater fish species differ in their tolerance to toxicants that act on the gill (McDonald et. al, 1989). It is also clear that there are substantial differences among freshwater fish species in gill morphology (e.g., tight junction depth, McDonald et. al, 1991a), and physiology (e.g., Na^+ uptake kinetics, Ca^{2+} binding affinity, Freda and McDonald, 1988), as well as in response to various challenges to ion balance (e.g., stress, McDonald et. al, 1991b; McDonald and Robinson, 1993; McDonald et. al, 1993; exercise, Gonzalez and McDonald, 1994). However, it is still not well established what specific features of the gill underlay differences in tolerance. Nonetheless, studies that compare gill morphology and physiology in two or more species provide useful insights into both mechanisms of toxicity and the physiological basis for differences in tolerance (Freda and McDonald, 1988; McDonald et al., 1991a, 1991b). Therefore, the overall objective of the present study is to further develop an understanding of the relationship between tolerance to toxicants that act at the gill and the physiology of two species that are differentially sensitive to such toxicants.

The approach adopted was to choose two species with a well established difference in tolerance to low pH (a toxicant that acts specifically on gill ion regulation; Wood and McDonald, 1982) and to compare in detail various features of their ionoregulatory mechanisms. The two species chosen were a cyprinid, fathead minnow (FHM; *Pimephales promelas*), and a salmonid, rainbow trout (RBT; *Oncorhynchus mykiss*). Fathead minnows are approximately 10 times more sensitive to low pH exposure in soft water than RBT (144-h LC50: pH 5.2 in FHM *vs.* pH 4.3 in RBT; Hickie et al., 1993). Ion regulation was studied by examining and comparing whole body Na^+ and Cl^- concentrations and transepithelial Na^+ and Cl^- fluxes under routine environmental conditions and in response to various challenges to ion balance. The routine studies focused on whether or not ion regulation measurements made on fish held under routine

conditions (i.e., normoxia, resting fish, seasonal fluctuations in temperature) could predict differences in the response of two species to perturbations to ion regulation. Therefore, emphasis was placed on identifying and comparing values for resting, undisturbed fish between the two species. This approach assumes that a normal value or range for an ion measurement can be determined. Towards this end, the seasonal effect of temperature on Na^+ balance and whole body Cl^- concentrations, Na^+ and Cl^- uptake kinetics, and transepithelial Na^+ fluxes under routine conditions were measured. A variation of this approach is the one advocated by Grippo and Dunson (1991; 1996) which recommended whole body Na^+ concentrations as a biomarker of acid and metal toxicity.

In the challenge studies, challenges that should perturb ion balance were chosen with the view that they might amplify species differences and inform on the ionoregulatory strategies of FHM and RBT. Unlike other studies, this study compared the responses of FHM and RBT to a number of ion regulatory challenges. Two types of challenges were used: 1) challenges that were not toxic to the fish, but were known to affect ion regulation either by externally or internally mediated means, and 2) exposure to toxicants that are thought to act on the gill. The former type of challenge included exposure to high osmolarity (Lahlou, 1969; Gonzalez and McDonald, 1992), confinement stress and intraperitoneal injections of saline or epinephrine (McDonald and Robinson, 1993; Postlethwaite and McDonald, 1995), and the acute removal of external Ca^{2+} (McDonald and Rogano, 1986).The latter type of challenge included exposure to low pH (Freda and McDonald, 1988) and toxic copper concentrations (Lauren and McDonald, 1985).

Materials and Methods

Experimental Animals

Juvenile rainbow trout (0.5 - 9 g) were obtained from Rainbow Springs Fish Hatchery in Thamesford, ON. Fathead minnows (0.5 - 4 g) were obtained from Steel City Bait in Hamilton, ON or Rainbow Springs Fish Hatchery in Thamesford, ON. All fish were held in tanks supplied with dechlorinated, Hamilton, ON municipal tap water (1 mmol L^{-1} Ca^{2+}, 0.6 mmol L^{-1} Na^{+}, 0.3 mmol L^{-1} Mg^{+1}, 0.8 mmol L^{-1} Cl^{-}) at ambient temperature (seasonal range of 4 to 22 °C) for at least three weeks prior to use. Both species were fed to satiation once daily with either trout chow (RBT) or a mixture of Nutrafin flakes and frozen brine shrimp (FHM).

Experimental Approach

Ionoregulatory strategies in the two species were evaluated by measuring transepithelial Na^{+} fluxes, unidirectional Na^{+} and Cl^{-} fluxes, and whole body Na^{+} and Cl^{-} concentrations. The protocols used for each of these measurements are outlined below.

Transepithelial Fluxes

The contribution of the kidney and urine to transepithelial ion fluxes were assumed to be negligible relative to the contribution of the gills. This assumption was based on Wood (1988) and McDonald and Wood (1981), who found that the renal contribution to Na^{+} efflux was less than 20% of the combined total gill and kidney Na^{+} efflux. All transepithelial fluxes were carried out in either covered cylindrical plastic containers (8 cm high X 11 cm diameter) or black plastic bags (12.5 cm high X 15 cm across X 5 cm wide) suspended in 5 L black perspex boxes. All containers were individually aerated with airlines made of polyethylene tubing (PE 50). Each container was filled with a known volume of water (range 200 - 300 ml). Two to three fish were added to give a biomass-to-volume ratio of approximately 1 g : 50 ml. The duration of the measurement period ranged from 1 - 4 h depending on the experiment. Water temperature

was maintained by bathing the outside of containers with water of the desired temperature. Water samples (5 - 10 ml depending on the experiment) were taken from containers at known time intervals (e.g., 0, 1, 2 and 4 h). Water samples were preserved by adding 10 µl of concentrated nitric acid and typically analyzed for ion content within 48 h. Fish were removed from containers and weighed at the end of the measurement period. Ion fluxes were calculated with equation 1 in the calculations section.

Unidirectional Fluxes

Unidirectional fluxes were measured on fish held in either darkened glass aquaria (10 to 30 L) or covered cylindrical plastic containers (8 cm high X 11 cm diameter). Resting unidirectional fluxes were determined on fish that had a minimum 24 h adjustment period in the glass aquaria. For fish held statically in glass aquaria for longer than 24 h, one half of the aquaria water was replaced every other day (replacement water was adjusted to the appropriate temperature and water quality conditions). Aquaria were aerated with glass airstones and the cylindrical plastic containers were aerated with airlines made out of polyethylene tubing (PE 50). The uptake of Na^+, Cl^- or Cu ($J_{in}{}^{ion}$) was measured by absorption of isotope into the fish. A stock solution radiolabelled with isotope (2 to 7 µCi ml^{-1} of ^{24}Na, ^{36}Cl, or ^{64}Cu) was added to the water. After a five minute mixing period, an initial water sample was taken (10 ml). At the end of the measurement period (2 - 5 h depending on the experiment) a final water sample was taken. These water samples were analyzed for radioactivity (within 1 - 4 h) and for the total content of the ion of interest. At the end of the measurement period fish were euthanized with a lethal dose of anaesthesia (tricaine methanesulphate, MS-222, 0.1 g L^{-1} buffered to pH 7.0 with $NaHCO_3$). Fish were then rinsed in deionized water, blotted dry, weighed, and then analyzed for isotope content. Ion uptake was calculated with equation 2 in the calculations section.

Whole Body Na^+ and Cl- concentrations

Fish were euthanized with a lethal dose of anaesthesia and then rinsed in deionized water, blotted dry, and weighed. Carcasses were transferred to screw-cap plastic 15 ml tubes and two to three volumes (to weight) 1N H_2SO_4 was added. Carcasses were then digested in an 80°C oven for 24 h. Samples were assessed for complete digestion by visual examination which determined that all of the tissue was broken down. Next samples were shaken and then centrifuged at 5125 G for 4 min in an clinical centrifuge (IEC, Damon). The supernatant was then decanted into 1.5 ml microcentrifuge tubes and analyzed for ion content.

Experimental Series

Ion Regulation under routine conditions

1. The effect of chronic temperature change on whole body Na^+ and Cl^- concentration and Na^+ uptake

The effect of chronic temperature change on whole body Na^+ and Cl^- concentrations and Na^+ uptake (J_{in}^{Na+}) in FHM and RBT was investigated. Two methods were used to change temperature. In the first method seasonal variations in holding temperature were exploited by sampling fish from the holding tanks throughout the year. This method was used for whole body Na^+ and Cl^- concentrations in FHM (N = 45) at 4, 15, and 18°C and in RBT (N = 48) at 4, 15, 19, and 21°C, as well as J_{in}^{Na+} in FHM (N = 42) at 16, 19 and 21°C and in RBT (N = 27) at 16, 19 and 21°C.

In the second method fish were held at a constant temperature for a period of 3 weeks to 2 months. Fish that had been held in hardwater holding tanks at 15 °C for a period of 2-3 weeks were used. Fish were transferred to one of several 30 L darkened glass aquaria. Next water temperature in the glass aquaria was raised or lowered to the desired temperature (i.e., there were glass aquaria held at temperatures ranging from 5 to

25 °C) at a rate that did not exceed 2 °C per day. Fish were fed ad libidum every 2 days. In the first trial FHM (N = 64; 1.75 ± 0.06 g) were held at 6, 12, 18 and 24 °C for up to 2 months and RBT (N = 47; 7.8 ± 0.3 g; J_{in}^{Na+} only) were held at 6, 10, and 19 °C for up to 1 month. In the second trial FHM (N = 37; 1.24 ± 0.07 g) were held at 10, 15, 20 and 25 °C and RBT (N = 27; 1.49 ± 0.06 g) were held at 10, 15 and 20 °C for 3 weeks. Whole body Na^+ and Cl^- concentrations, and J_{in}^{Na+} were measured at the end of the acclimation period.

2. Sodium Uptake Kinetics

The effect of external Na^+ concentration on J_{in}^{Na+} in FHM and RBT was examined twice (see Table 2.1 for water conditions). In the first trial FHM (1.22 ± 0.10 g) and RBT (0.81 ± 0.05 g) were exposed in 4 L black perspex boxes (N = 6 per species per concentration, 13 °C). A radioactive $^{24}Na^+$ stock solution (4 µCi ml^{-1} $NaCO_3$) was added within 10 min. of the fish being transferred to the boxes. Sodium uptake was measured after 2 h of isotope exposure. In the second trial FHM (0.72 ± 0.04 g) and RBT (0.88 ± 0.06 g) were removed from holding tanks and placed together in glass aquaria (filled to 4 L; N = 5 per species per concentration, 20 °C). A radioactive ^{24}Na stock solution (2 µCi ml^{-1} $NaCO_3$) was added to the aquaria within 10 min of the fish being added. Sodium uptake was measured after 4 h of isotope exposure.

3. Sodium loss rates

Transepithelial Na^+ fluxes were measured in two different water conditions (see Table 2.1). For both water conditions the measurement period was 4 h. In the first trial net Na^+ fluxes were measured (N = 6 per species) on FHM (1.24 ± 0.11 g) and RBT (3.67 ± 1.27 g) in dechlorinated Hamilton water. In the second trial the water was nominally Na^+-free. Therefore, Na^+ efflux in FHM (2.02 ± 0.18 g) and RBT (3.51 ± 0.79 g) was measured (N = 7 per species). Sodium-free water was made by adding $CaCl_2$ to de-ionized water.

4. Chloride uptake

Chloride uptake was compared in resting FHM and RBT (see Table 2.1 for conditions) held in dechlorinated Hamilton tap water. Fathead minnow (1.20 ± 0.08 g; N = 12) and RBT (1.64 ± 0.07 g; N = 12) were placed together in a 30 L glass aquaria and given 24 h adjustment period. Next a radioisotope stock solution (^{36}Cl; 4.8 μCi ml^{-1}) was added. The duration of the measurement period was 5 h.

5. Chloride uptake kinetics

The effect of external Cl^- concentration on $J_{in}^{Cl^-}$ in FHM (3.01 ± 0.13 g) and RBT (1.95 ± 0.10 g) was examined (see Table 2.1 for water conditions). This was done in two different trials. Fish (N = 3 per concentration per species) were placed in separate plastic tubs and the measurement period was 2 h in each trial. A radioactive stock solution of ^{36}Cl (trial 1: 4 μCi ml^{-1}, 0.25 meq L^{-1} NaCl; trial 2: 4 μCi ml^{-1}, 1.25 meq L^{-1} NaCl) was used to adjust the Cl^- concentration.

6. Total CO_2

The amount of white muscle total CO_2 was determined in FHM (1.04 ± 0.07 g, N=5) and RBT (3.08 ± 0.2 g, N=6). Fish were removed from hardwater holding tanks and euthanized with a lethal dose of anaesthesia. Fish were then freeze-clamped between two liquid nitrogen cooled aluminum plates. White muscle was removed and kept in liquid nitrogen until it could be analyzed for total CO_2.

Challenges to Ion Regulation

7. The effect of acute Ca^{2+} removal

The effect of acute Ca^{2+} removal on Na^+ efflux in FHM (1.8 ± 0.1 g) and RBT (7.2 ± 0.4 g) was examined. Both species (N = 4 per species per concentration) were placed in separate bags with deionized water and the external Ca^{2+} concentration was

adjusted with a $CaCl_2$ stock solution to a particular concentration (see Table 2.1 for water conditions). The duration of the measurement period was 4 h.

8. The effect of 2 weeks of holding in low Ca^{2+} water

Fathead minnows (N = 36; 0.90 ± 0.10 g) and RBT (N = 30; 2.9 ± 0.3 g) were exposed to low external Ca^{2+} (see Table 2.1 for water conditions) for two weeks in 30 L glass aquaria to look at the effect of low external Ca^{2+} on whole body Na^+ and Cl^- concentrations. Fish were fed ad libidum every 2 days. After 2 weeks fish were killed and whole body Na^+ and Cl^- concentrations were measured .

9 . The effect of acute exposure to high external NaCl

The effect of acute exposure to 260 mM NaCl on mortality and whole body Na^+ and Cl^- concentrations in FHM and RBT was examined. This NaCl concentration was chosen because it was approximately 2-fold greater than plasma Na^+ concentrations in FHM and RBT (unpublished data). External NaCl concentration was elevated by adding NaCl to dechlorinated Hamilton tap water. In the first experiment FHM (2.79 ± 0.20 g; N = 15) and RBT (2.51 ± 0.33 g; N = 15) were statically exposed to 260 mM NaCl in 30 L glass aquaria for 24 h. Fish mortality was recorded at 0, 0.5, 1, 2, 4, 8, 16 and 24 h. The estimated time to 50 % mortality was obtained by log probit analysis. In the second experiment FHM (0.91 ± 0.06 g; N = 20) and RBT(1.26 ± 0.11 g; N = 20) were held in separate 5 L black perspex boxes supplied with water by a 280 L recirculating system adjusted to 260 mM NaCl. Fish were removed at time 0, 2, 8, 12 and 24 h, and whole body Na^+ and Cl^- concentrations were measured. In addition, fish mortality was recorded at 0.5, 1, 2, 4, 5, 6, 7, 8, 12, 16, 20 and 24 h.

10. Effect of Epinephrine and Saline Injections

The effect of injected epinephrine bitartrate dissolved in 0.6% NaCl and 0.6% NaCl alone on Na^+ efflux was examined in FHM and RBT (see Table 2.1 for water

conditions). Fathead minnows (2.0 ± 0.2 g) and RBT (1.4 ± 0.2 g) were intraperitoneally (IP) injected with either 1.9 μmol of epinephrine dissolved in 30 μl of 0.6% NaCl or 30 μl 0.6% NaCl alone (N =4 per species per treatment) using a 26.5 gauge needle (Becton Dickinson) attached to a 100 μl Hamilton syringe. Fish were transferred to black plastic bags. Fish were held in the bags for a minimum of 30 sec before the first, time 0 water sample was taken. This approach ensured that any rapid leak of the saline from the injection site would not be included in the calculation of net Na^+ fluxes. A rapid leak would be more likely than a slow leak from the injection site given the small gauge of needle that was used and the fact that needles were changed every 2 to 3 fish. The measurement period was 1 h, and water samples were taken at 0, 10, 30 and 60 min. Water samples were then analyzed for Na^+ content.

11. Effect of confinement stress

The response and recovery of whole body Na^+ and Cl^- concentrations and J_{in}^{Na+} in FHM and RBT to a stress protocol that consisted of a combination of injection and confinement was examined. Fish were IP injected with 30 μl of 0.6% NaCl and then placed in groups of 12 (6 FHM and 6 RBT) in a net (10.5 cm long X 7.5 cm wide X 11 cm deep). A plastic grid (10 cm long X 7 cm wide) was inserted in the net to prevent escape. Then the nets were suspended in water over a gently bubbling air stone on a wet table continuously supplied with dechlorinated Hamilton tap water. After 4 h in the nets, fish were released into 4 L black perspex boxes (one box for each sample time period) that were continuously supplied with dechlorinated Hamilton tap water (see Table 2.1). In the first experiment whole body Na^+ and Cl^- concentrations were sampled in FHM (1.98 ± 0.07 g) and RBT (1.94 ± 0.09 g) at 2, 24 and 48 h. Control fish were sampled directly from the holding tanks.

In the second experiment J_{in}^{Na+} in control fish and fish recovering from the stress protocol was examined. Control fish were transferred directly from the holding tanks to a

4 L black perspex box and recovering fish were placed in boxes at the end of the stress protocol. The continuous dechlorinated water supply to the 4 L black perspex boxes (one for each time period per species) was shut off and a radiolabelled Na^+ stock solution (^{24}Na; 2 $\mu Ci\ ml^{-1}$) was added. Sodium uptake was measured on FHM (N = 42; 2.23 ± 0.10 g) and RBT (N = 36; 2.66 ± 0.12 g) in a series of 2 h flux periods; 3 to 5 h, 11 to 13 h, 23 to 25 h, and 47 to 49 h post-confinement.

<u>Toxicants that Act on the Gill</u>

12. Effect of acute low pH exposure

To look at the effect of acute low pH exposure on transepithelial Na^+ fluxes FHM (1.4 ± 0.27 g) and RBT (2.14 ± 0.36 g; N = 12 per species) were placed in black plastic bags (see Table 2.1 for water conditions). The pH was adjusted to 4.3 by titration with H_2SO_4. The pH was monitored throughout the 1 h exposure with an PHM 82 standard pH meter (Radiometer) and pH electrode (Cole Parmer) that was placed in each bag every 10 min. Water samples were taken at 0, 20 and 60 min.

In the second trial, FHM (1.03 ± 0.11 g) and RBT (2.81 ± 0.23 g) were first acclimated to soft water for two weeks (see Table 2.1 for water conditions). Next fish (N = 40 per species) were placed in PVC tubes (12.5 cm high X 7 cm diameter) with a rubber stopper in the bottom. Each PVC tube was continuously supplied with softwater from a 280 L recirculating system. After a 24 h adjustment period the pH of the recirculating system was lowered to pH 5.2 with the addition of H_2SO_4. The pH was maintained at pH 5.2 by addition of H_2SO_4 using a pH meter (PHM 82, Radiometer) and a titrator (TTT80, Radiometer).

13. Effect of Acute Cationic surfactant exposure

The effect of acute exposure to one of two cationic surfactants on net Na^+ fluxes in FHM and RBT was examined (see Table 2.1 for water conditions). Fish were exposed

to either 1.15 mg L^{-1} benzalkonium chloride (BC; which represented the 96-h LC50 of RBT; Vittozzi and De Angelis, 1991) or 5 mg L^{-1} Dimethyldioadecyl-Ammonium-Bromide (DTAB; the concentration 5 mg L^{-1} was determined by a range finding experiment on RBT). Fish were removed from holding and placed in plastic tubs (N = 4 tubs of 2 fish per tub per species per treatment). Next a stock solution was added to the tubs to give the desired concentration of BC (FHM; 0.34 ± 0.03 g; RBT; 1.91 ± 0.14 g) or DTAB (FHM; 1.02 ± 0.07 g; RBT; 2.45 ± 0.17 g). The duration of the measurement period was 4 h and water was sampled at 0, 1, 2 and 4 h.

14 Effect of acute copper exposure

The effect of acute Cu exposure on transepithelial Na^+ fluxes and J_{in}^{Cu} in FHM (0.87 ± 0.07 g) and RBT (0.84 ± 0.05 g) was examined. Both species (N = 6 tubs of 2 fish per species per treatment) were placed in plastic tubs (see Table 2.1 for water conditions). Next a radiolabelled stock solution of ^{64}Cu (1.5 µCi ml^{-1}, 200 µg ml^{-1} $CuSO_4$) was added to containers to bring the Cu concentration to 400 µg L^{-1} (approximately 4-fold larger than the 96h LC50 for RBT in hardwater; Taylor et al., 2000). This concentration was chosen because Lauren and McDonald (1985) showed that it had a large effect on net Na^+ losses in RBT.

Experimental Analysis

Whole body Na^+ and Cl^- content

To measure Na^+ content the supernatant from whole body acid digests was diluted 1:1000 in distilled water (in triplicate) and then read on an atomic absorption spectrometer (Varian AA-1275). Chloride content of the undiluted supernatant was measured with a Chloridometer (CMT-10, Radiometer). Whole body Na^+ and Cl^- concentrations were expressed as µeq g^{-1}.

Water Na^+ and Cl^- content

Water Na^+ content was measured on an atomic absorption spectrometer (Varian AA - 1275). If necessary water samples were first diluted so that Na^+ content fell in the range of 50 to 150 μeq L^{-1} (typically 4-fold dilution for dechlorinated Hamilton tap water). Dilutions were done in triplicate. Chloride content of the water was measured (in triplicate) with the colorimetric assay of Zall et al. (1956) and read on a spectrophotometer (4054, LKB Biochrom) at 480 nm.

Ion Uptake

Ion uptake was measured by absorption of radioactivity into the fish. Specific activity (CPM neq^{-1}) was determined by measuring the radioactivity of the water samples (CPM ml^{-1}) and dividing this by the total concentration (neq ml^{-1}) of the ion of interest (Na, Cl or Cu) in the water. Water samples with radioisotopes that emitted gamma radiation (^{24}Na, ^{64}Cu) were counted on a gamma counter (Minaxi auto gamma 5000 series, Canberra Packard) and decay-corrected to the beginning of the measurement period to account for the half-life of ^{24}Na and ^{64}Cu (15.3 and 12.6 h respectively). Water samples with radioisotopes that emitted beta radiation (^{36}Cl) were mixed with Aqueous Counting Scintillant (Amersham) according to manufacturer's recommendations and counted on a beta counter (RackBeta 1217 liquid scintillation counter, LKB Wallac). Fish exposed to gamma emitters (^{24}Na, ^{64}Cu) were placed in 18 ml plastic scintillation vials (Fisher) and counted on a gamma counter. Fish exposed to beta emitters (^{36}Cl) required more tissue preparation. Fish were freeze-clamped between liquid nitrogen cooled aluminum plates and then ground to a fine powder under liquid nitrogen. Three aliquots of ground powder per fish were measured (approximately 100 mg aliquots) into tared 18 ml glass scintillation vials, and 1 ml of soluene (Packard) was added. These samples were placed in a 50 °C oven for 12 h to digest, and then Hionic-fluor (Packard) was added according to

manufacturer's recommendations. Samples were counted on a beta counter. The amount of quenching of ^{36}Cl was small in both FHM and RBT so the effects of quenching were not considered.

Total CO_2

Total CO_2 in white muscle was determined using the methods outline in Portner et al. (1990). Briefly white muscle tissue samples were removed from cold storage and placed in a motar and pestle with liquid nitrogen. Then the tissue was ground behind a plexiglass shield to prevent CO_2 contamination. Once ground, approximately 200 mg was removed and added to an ice cold metabolic inhibitor cocktail (150 mM 2H20 KF, 6 mM Na_2 nitrilotriacetic acid) in a previously tared centrifuge tube. This was quickly mixed, more cocktail was added and then the cocktail and tissue slurry was vortexed for 5 seconds. Next the slurry was centrifuged for 15 sec at 12 400 rpm (135A, Fisher). The supernatant was analyzed on a Total CO_2 analyzer (Corning, TCO_2).

Calculations

The Na^+ fluxes were calculated using the following equation:

$$J_{net}^{Na+} \text{ (or } J_{out}^{Na+}) = ([Na_i] - [Na_f]) \text{ X vol / (t X M)} \qquad (1)$$

Where "$[Na_i]$" and "$[Na_f]$" represent the initial and final concentration of Na^+ for a given time period in $\mu eq\ L^{-1}$, "vol" represents the average volume of water in the fluxing container for a given time period in L, "t" represents the time period in hours and "M" represents the mass of fish in the flux container in g.

J_{in}^{ion} (Na, Cl, Cu) was calculated according to the following equation:

$$J_{in} = Q_t / (SA \text{ X } t \text{ X } M) \qquad (2)$$

where "Q_t" is the number of counts in the fish in cpm, "SA" is the specific activity of the water in cpm neq^{-1}, "t" is the time in hours and "M" is the mass of the fish in g.

<u>Statistics</u>

Means ± one standard error of the mean (SEM) are reported throughout. Comparisons among species and treatments were made by an analysis of variance ($p< 0.05$). Tukey's multiple range test was used to resolve differences between species. In time series within species, a Dunnett's t test was used to compare treatment effects with control. An F statistic (Zar, 1996) was used to compare coefficients of variation for whole body ions in the two species. Seasonal whole body ion and ion uptake data was tested for normality using a Shapiro-Wilk test. Data that was not normally distributed was assessed with a Kruschel-Wallis test. The descriptive coefficients (K_m, and J_{max}) for uptake kinetics were calculated using a Michealis Menton model in SAS Jmp (version 2.0.5).

Results

<u>Ion Regulation under routine conditions</u>

Sodium balance

The regulation of Na^+ balance in FHM and RBT seems to be broadly similar. For example, at 15° C, whole body Na^+ concentrations were almost identical between FHM and RBT of comparative size (49.7 ± 0.71 *vs.* 50.4 ± 0.73 μeq g^{-1}, 1.82 *vs.* 2.65 g, N of 27 and 20, respectively; Fig. 2.1). However, the rates of Na^+ uptake (J_{in}^{Na+}) at 15 °C were 18% higher in FHM than RBT (374 ± 20 *vs.* 307 ± 16 neq g^{-1} h^{-1}, N of 30 and 33 respectively, Fig. 2.2). As a result, daily Na^+ turnover rates at 15 °C were slightly higher in FHM than RBT (18% *vs.* 15% whole body Na^+ day^{-1}).

Seasonal changes in temperature had a major effect on Na^+ homeostasis in FHM (Fig. 2.1A and 2.2A) but not in RBT (Fig. 2.1B and 2.2B). In acclimated FHM whole body Na^+ concentrations increased linearly with temperature over the range of 4 to 25 °C and there was also an effect of temperature on J_{in}^{Na+} (whole body Na^+ increased from 45 to 58 μeq g^{-1} and J_{in}^{Na+} from 100 to 590 neq g^{-1} h^{-1}). In contrast, there were no statistically significant effects of temperature (4 to 21 °C) on whole body Na^+ (Fig. 2.1A) or J_{in}^{Na+} (Fig. 2.2B) in RBT. In fact, the average whole body Na^+ concentration and J_{in}^{Na+} for all temperatures were 49.7 ± 0.7 μeq g^{-1} (N = 75) and 321 ± 15 neq g^{-1} h^{-1}(N = 115) in RBT, and were not significantly different from the 15 °C values.

Sodium uptake kinetics

Sodium uptake kinetics differed in FHM and RBT. In the first trial at 13 °C, J_{in}^{Na+} were too variable in both species to determine the kinetic constants ($K_{m,}$ J_{max}) in either species (Fig. 2.3A). In the second trial at 21 °C Na^+ uptake kinetics were determined in both species (external Na^+ concentration range 0.05 - 1.0 meq L^{-1}; Fig. 2.3B). Fathead minnows had much lower Na^+ affinity (i.e., higher K_m, 567 ± 145 *vs.* 95 ± 30 μeq L^{-1},

FHM *vs.* RBT respectively) and showed a trend towards a higher J_{max} than RBT (937 ± 122 *vs.* 630 ± 56 neq g^{-1} h^{-1}, FHM *vs.* RBT respectively).

Routine sodium losses

Transepithelial Na^+ fluxes were not significantly different between FHM and RBT when measured as net Na^+ fluxes and as Na^+ efflux. Sodium efflux was 1.6% and 1.5% of whole body Na^+ h^{-1} in RBT and FHM respectively (Fig. 2.4). Net Na^+ fluxes were 1.5% of whole body Na^+ h^{-1} in both species. However, actual rates of Na^+ efflux in fish exposed to 0.6 meq L^{-1} Na^+ were higher. If Na^+ influx of 300 to 400 neq g^{-1} h^{-1} are assumed for either species (Fig. 2.2) then Na^+ efflux was 2 - 2.5% h^{-1}. Also, net Na^+ fluxes were more variable than Na^+ effluxes (Fig. 2.4) because it was difficult to resolve small changes in Na^+ concentration against the large Na^+ background (0.6 meq L^{-1} Na^+ *vs.* nominally Na^+-free)

Chloride balance

Whole body Cl^- concentrations were consistently lower than Na^+ concentrations in both species, but the difference between Na^+ and Cl^- was much greater in FHM than RBT; 20 μeq g^{-1} difference in FHM and 10 μeq g^{-1} difference in RBT (Fig. 2.1). Elevated HCO_3^- concentrations may have contributed to the larger anion gap in FHM (white muscle total CO_2 was 7.9 ± 0.5 *vs.* 5.2 ± 0.2 μmol g^{-1} in FHM and RBT, respectively, N = 5). In addition, there was a seasonal temperature effect on Cl^- homeostasis in FHM but not in RBT (Fig. 2.1). Whole body Cl^- concentrations increased linearly with temperature from 4 to 25° C in FHM (22 to 35 μeq g^{-1}). Even after correcting for the effect of temperature (correction to 15°C using equation in Fig. 2.1A), there was significantly more variation among individual whole body Cl^- concentrations in FHM than RBT (coefficient of variation; 22.8% vs. 9.0% FHM and RBT respectively, $p < 0.001$).

Chloride uptake

Resting chloride uptake rate at 19°C was similar in the two species (227 ± 25 *vs.* 249 ± 22 neq g^{-1} h^{-1}, in FHM and RBT respectively, N =12) and was significantly lower than J_{in}^{Na+} at the same temperature in each species (361 ± 30 *vs.* 288 ± 34 neq g^{-1} h^{-1} in FHM and RBT, respectively; Fig. 2.2). There was a 3% difference between the two species in the daily Cl^- turnover rate (19% *vs.* 16% in FHM and RBT respectively).

Chloride uptake kinetics

Chloride uptake kinetics were similar in FHM and RBT (Fig. 2.5). In both species J_{max} was approximately 360 neq g^{-1} h^{-1} (320 ± 40 *vs.* 400 ± 36 neq g^{-1} h^{-1} in FHM and RBT, respectively) and the K_m was approximately 130 µeq L^{-1} (80 ± 50 vs. 150 ± 70 µeq L^{-1} in FHM and RBT, respectively). However, there was a great deal of individual variability J_{in}^{Cl-} at each concentration in both species.

Challenges to Ion Regulation

Low Calcium Exposure

Acute reduction of external Ca^{2+} had a significantly greater effect on Na^+ efflux in FHM than RBT(Fig. 2.6). Sodium efflux in FHM was 2.4-fold greater than RBT Na^+ efflux at 0.25 meq L^{-1} Ca^{2+} and 2.8-fold greater at 0.05 meq L^{-1} Ca^{2+}. The two species had similar Na^+ efflux when external Ca^{2+} concentrations were high (2-3% whole body Na h^{-1} at 1.65 meq L^{-1} Ca^{2+}).

Both FHM and RBT seemed to adapt to low external Ca^{2+}. For example, Na^+ efflux was approximately 2-fold larger in the first hour (0 to 1 h, Fig. 2.6A) than from 2 to 4 h in both species (Fig. 2.6B). Furthermore, both species were able to maintain whole body ion balance with long term exposure to low external Ca^{2+}. After two weeks exposure to 0.05 meq L^{-1} Ca^{2+}, whole body Na^+ and Cl^- concentrations in RBT and FHM were not significantly different from fish held in hard water (i.e., whole body Na^+ 47.8 ± 1.8 vs.

49.7 ± 0.7 μeq g^{-1} in RBT, and 47.4 ± 1.7 vs. 50.4 ± 0.7 μeq g^{-1} in FHM; Table 2.2 and Fig. 2.1).

Osmotic Shock

Acute exposure to saline solution (260 mM NaCl) caused mortality in both species. However, the impact was much greater on FHM than RBT. The time to 50% mortality (ET50) in FHM was 2.89 ± 0.49 h. In contrast, the same exposure did not produce enough mortality in RBT to estimate an ET50 (15% mortality over 24 h).

The much higher mortality of FHM was associated with rapid and massive increases in whole body ion concentrations. Fathead minnow whole body Na^+ concentrations increased 2.5-fold and Cl^- concentrations increased 3.5-fold, between 1 - 2 h of exposure (Fig. 2.7). In contrast, RBT whole body Na^+ and Cl^- concentrations increased 1.5-fold over a 2 h period. Thereafter, there were no additional increases whole body ion concentrations in RBT over 24 h of saline exposure. Rainbow trout that did not survive the saline exposure (15% of the total) had whole body Na^+ and Cl^- concentrations 2.5-fold greater than controls.

Stress Tests

There were no significant differences between net Na^+ loss after intraperitoneal injection of either epinephrine or epinephrine delivery vehicle alone (30 μl 0.6% NaCl, no epinephrine) within either species (Table 2.3). However, injection caused substantially greater net Na^+ losses in FHM than RBT. After epinephrine was injected, FHM lost approximately 13% of their whole body Na^+ in 1 h compared to 2% in RBT. Furthermore, injection of the epinephrine delivery vehicle alone (30 μl 0.6% NaCl, no epinephrine) caused FHM to lose 17% of whole body Na^+ in 1 h compared to 2% whole body Na^+ in RBT.

Because injection of the epinephrine delivery vehicle (i.e., 0.6% NaCl, no epinephrine) alone provoked a similar response to epinephrine, it was used to cause a stress response in subsequent experiments. Injection plus 4 h confinement caused a significant decrease in whole body Na^+ and Cl^- concentrations in both species (Fig. 2.8). At 2 h post-confinement, Cl^- losses in FHM were significantly greater than in RBT (44% *vs*. 28%, respectively) while whole body Na^+ losses were not significantly different between FHM and RBT (31% *vs*. 22% respectively). The stress protocol also caused mortality in both species although the pattern and amount differed. In RBT, mortality was only 5%, and it all occurred within 8 h post-confinement. In contrast, FHM had greater mortality (14%) that continued to occur up to 48 h post-confinement. These differences in mortality paralleled the rate of whole body Na^+ and Cl^- recovery in the two species. Fathead minnow had not recovered whole body Cl^- by 48 h post-confinement and recovered whole body Na^+ between 24 - 48 h post-confinement. In contrast, RBT whole body Na^+ and Cl^- recovered between 2 - 24 h post-confinement (Fig. 2.8).

Finally, the difference in recovery rates of whole body Na^+ in the two species corresponded to differences in J_{in}^{Na+} post-confinement. Sodium uptake increased 1.7-fold in RBT at 4 h post-confinement (Fig. 2.9). In contrast, J_{in}^{Na+} did not change significantly in FHM between 3 - 48 h post-confinement.

Exposure to Gill Surface Active Agents

Low pH

Acute exposure to pH 4.3 in hardwater (1.65 meq L^{-1} Ca^{2+}) caused similar net Na^+ losses in both species that were only slightly larger than those seen at circumneutral pH (2% vs. 1.5% whole body Na^+ h^{-1}; Table 2.4 and Fig. 2.4). However, acute exposure to pH 4.3 combined with acute softwater exposure had a 3-fold greater effect on net Na^+ fluxes in FHM than RBT (Table 2.4). Furthermore, prior acclimation to softwater (0.05 meq L^{-1} Ca^{2+} for 2 weeks) did not prevent FHM from being more sensitive to low

pH exposure than RBT. Fathead minnows lost approximately 3-fold more Na^+ than RBT with exposure to pH 5.2 (842 ± 118 *vs.* 232 ± 44 neq g^{-1} h^{-1}, N = 40 per species, 1.03 ± 0.11 g *vs.* 2.81 ± 0.23 g, FHM and RBT respectively).

Cationic Surfactants

Neither of the two cationic surfactants used in this study (1.15 mg L^{-1} BC, and 5 mg L^{-1} DTAB), caused Na^+ losses greater than Na^+ fluxes done in hardwater and acute softwater (Table 2.5, Fig 2.6).

Copper

Acute exposure to 400 μg l^{-1} Cu in high external Ca` (1.8 meq L^{-1}) caused larger net Na^+ fluxes in FHM than in RBT (Fig. 2.10A). Initial Na^+ losses in FHM (0 to 1 h) were approximately 3-fold higher than RBT (15 *vs.* 6% of whole body Na^+). After 4 h Cu exposure, FHM had lost 35% of their whole body Na^+ and RBT had lost 25% (Fig. 2.10A). Acute exposure to low Ca^{2+} (0.05 meq L^{-1}) combined with Cu exposure exacerbated the effect of Cu in both species (Fig. 2.10B). Initial Na^+ losses were much greater in FHM than RBT (22% *vs.* 10% of whole body Na^+ at the end of 1 h, Fig. 2.10B). After 4 h Cu exposure the two species had similar Na^+ losses (37% *vs.* 31% of whole body Na^+ in FHM and RBT respectively) but FHM had higher levels of mortality than RBT during this time period (75% *vs.* 50% respectively).

Greater Na^+ losses caused by Cu exposure in FHM than RBT paralleled greater J_{in}^{Cu} in FHM. In hard water, J_{in}^{Cu} was 1.7- fold larger in FHM than RBT (Tabie 2.6). Acute soft water amplified J_{in}^{Cu} in both species by about 3-fold, but the difference between the species was maintained (Table 2.6).

Discussion

Ion Regulation Under Routine Conditions

This study showed that there were at least four important differences in ion regulation between FHM and RBT. First, there were significant effects of chronic temperature change on J_{in}^{Na+} and whole body Na^+ and Cl^- concentrations in FHM, but none in RBT. Secondly, there was greater variation in whole body Cl^- concentrations among individuals in FHM than RBT. Thirdly, there were differences in Na^+ uptake kinetics in the two species. Lastly, FHM had lower whole body Cl^- and a larger anion gap between whole body Na^+ and Cl^- compared with RBT.

The general conclusion from the differences in the effects of chronic temperature change between the two species is that FHM regulate Na^+ and Cl^- with less precision than RBT. This could reflect a reduced capacity of FHM to regulate ion uptake and loss, or that a larger change (i.e., reduction or increase in ion concentrations) is required before regulation will occur in FHM compared to RBT. For example, the effects of seasonal temperature changes on ion balance in FHM suggest they are less capable of regulating both the diffusive efflux of ions, and active ion uptake than RBT (Figs 2.1 and 2.2). The study of Gonzalez and McDonald (2000) supports this view. They showed that RBT not only adjusted ion uptake to compensate for acute temperature effects, they also changed the rates of ion loss. As a result they found that there was no chronic effect of temperature on ion balance in RBT. In contrast, the same study showed that common shiners (*Notropis cornutus*) - a cyprinid, did not adjust ion uptake or loss with acute temperature change and thus there was a chronic temperature effect on ion balance in this species (Gonzalez and McDonald, 2000). This supports the view that FHM, and other cyprinid species are less capable of adjusting ion uptake and loss than RBT. However, it does not eliminate the possibility that a greater magnitude of ion reduction or gain might elicit ion regulation in these species.

It is not clear why FHM have lower whole body Cl^- concentrations than RBT (Fig. 2.1). Large differences between plasma Na^+ and plasma Cl^- concentrations have been associated with very low rates of J_{in}^{Cl-} in the freshwater eel (*Anguilla anguilla*; Bornancin et. al, 1977) and the freshwater killifish (*Fundulus heteroclitus*; Wood and Marshall, 1994). However, this was not the case for FHM as the rates of J_{in}^{Cl-} were similar in FHM and RBT.

I expected to find differences in Cl^- uptake kinetics between FHM and RBT because of the differences in Na^+ uptake kinetics between the two species. However, there were no significant differences in Cl^- uptake kinetics between the two species (Fig. 2.5). This may have been due to the large amount of variation in Cl^- influx at each Cl^- concentration in both species. It is possible that the large amount of variation in Cl^- influx is normal. Mackay (1974) noted that rates of J_{in}^{Na+} and J_{in}^{Cl-} in goldfish (*Carassius auratus*) were highly variable over a 12 h period and therefore suggested that ion uptake was intermittent rather than continuous. This idea has received little attention, but intermittent Cl^- uptake could explain the high amount of variation in both species. For these experiments J_{in}^{Cl-} was measured by the uptake of radioactivity in the fish over a 2 h period. Moreover, for the Na^+ uptake kinetic experiments J_{in}^{Na+} showed greater variability when it was measured over 2 h (Fig 2.3A) than over 4 h (Fig 2.3B). If most fish maintain similar daily rates of ion uptake but the J_{in}^{ion} is intermittent over a 24 h time period then extending the duration of the exposure period to radioactivity will decrease differences between individuals.

It is possible that the differences in ion regulation under routine conditions between FHM and RBT shown in this study may reflect a general cyprinid model of ion regulation *vs.* an RBT model of ion regulation. In a direct comparison there was a chronic temperature effect on ion regulation in common shiners but not in RBT (Gonzalez and

McDonald, 2000). In addition, separate studies have shown temperature effects on ion uptake in goldfish chronically exposed to a different temperature (Mackay, 1974; Maetz, 1972) but not in RBT (McCarty and Houston, 1977). Secondly, the cyprinid species, common shiners and goldfish, also have larger differences between whole body (or plasma) Na^+ and Cl^- than RBT which cannot be explained by low rates of $J_{in}^{Cl^-}$ (Freda and McDonald, 1988; MacKay, 1974). Finally, cyprinids consistently had higher J_{max} and K_m in comparisons between common shiners and RBT (Freda and McDonald, 1988), goldfish and RBT (Maetz, 1972; Postlethwaite and McDonald, 1995), and FHM and RBT (Fig. 2.3). These findings suggest that groups of fish may have similar ionoregulatory strategies and thus, the implications of each ionoregulatory strategy to tolerance to toxicants would be shared by these groups.

Ion Regulation under Challenge Conditions

The differences in the responses of FHM and RBT to various challenges to ion regulation shown in this study support the conclusion that FHM regulate ion balance with less precision than RBT. Furthermore, in challenges that perturbed ion balance, FHM typically lost more Na^+ than RBT. In each case, these greater Na^+ losses appeared to be related to weaker, or more easily perturbed tight junctions between adjacent gill cells in FHM than RBT. The responses are discussed separately for each type of challenge used.

Effects of the Removal of External Ca^{2+}

Fathead minnows showed a greater dependence upon external Ca^{2+} for protection against Na^+ loss than RBT (Figs. 2.6 and 2.10, Table 2.3). External Ca^{2+} is thought to reduce gill electrolyte losses by stabilizing gill membranes and tight junctions between adjacent cells (Hunn, 1985). Moreover, high external Ca^{2+} is thought to protect against metal (i.e., Cu, Zn, Cd) and H^+ (i.e., low pH) toxicity by competing with charged cations for binding sites on the gill (McDonald et al., 1980; Graham and Wood, 1982; Playle et. al, 1992; Erickson et al 1996). The exact nature of the greater dependence of FHM on Ca^{2+} to

reduce electrolyte losses is not clear. It may be that FHM have a lower binding affinity for Ca^{2+} or that Ca^{2+} has a greater role in stabilizing tight junctions in this species.

The greater dependence on the protective effect of Ca^{2+} for FHM does not mean they cannot live in softwater (Matuszek et. al, 1988). In this study FHM and RBT recovered whole body Na^+ and Cl^- concentrations after prolonged exposure to low external Ca^{2+} (Table 2.2). In FHM and RBT acclimation to softwater causes an increase in chloride cell density in the primary and secondary lamellae of the gills (Laurent et. al, 1985; Leino et. al, 1987). These changes are believed to increase the active transport of Na^+ and Cl^- (Perry et. al, 1992). However, these changes may have a greater bioenergetic cost for FHM. The Canadian EPS guidelines (1992) for conducting toxicity tests with FHM state that up to two-fifths lower growth rates are expected in control fish held in softwater as compared to hardwater. In contrast, growth rates of RBT held in hardwater or softwater were similar (Taylor et. al, 2000).

Effects of Acute Exposure to High Salinity

Neither the juvenile RBT nor the adult FHM used in this study are capable of living in full-strength sea water. It is not until RBT smolt (i.e., 1 year older and approx. 50 g heavier than the RBT in this study) that they become anadromous and FHM are strictly freshwater fish (Scott and Crossman, 1973). However, the FHM in this study experienced more rapid increases in whole body Na^+ and Cl^- as well as greater mortality with exposure to 260 mM NaCl than RBT (Fig. 2.7). In addition, RBT survived at this concentration for at least 24 h, despite the relative stenohalinity of juvenile RBT (as compared to adult RBT). Gonzalez and McDonald (1992) suggested that cell shrinkage associated with exposure to high salinity would stretch tight junctions and increase paracellular ion flux. Perhaps FHM were more sensitive to high salinity because they were less able to reduce cell shrinkage or had weaker tight junctions. However, it may be that

RBT were more capable of increasing Na^+ and Cl^- efflux, or decreasing water loss than FHM.

Effects of Internally Mediated Stress

Fathead minnows had much greater electrolyte losses after internally mediated stress (i.e., increased catecholamines) than RBT. There was a striking 12-fold greater Na^+ loss rate in epinephrine- and saline-injected FHM than RBT (Table 2.3). Moreover, when both species were subjected to the confinement stress protocol FHM had greater whole body Na^+ losses and significantly greater whole body Cl^- losses than RBT (Fig. 2.8). It is not clear why the effect of injected saline was as great as that of epinephrine within species. It is possible that part of the increase in Na^+ loss rate was due to the salt in the injected saline. In FHM, approximately 35 to 50% of the Na^+ lost could be due injected salt. However, in RBT a 3-fold increase in Na^+ loss rate would be necessary to excrete all of the injected Na^+. Regardless of the source of Na^+, ion losses probably occurred because of the release of internal catecholamines in response to stress. Increases in plasma epinephrine have been shown to increase Na^+ losses by up to 40-fold (McDonald and Robinson, 1993; Gonzalez and McDonald, 1994; McDonald and Milligan, 1996). One of the more likely explanations for the link between increased plasma epinephrine and increased Na^+ losses is that epinephrine increases the transmural, and intralamellar, pressure of the gills. As a result, paracellular tight junctions distort and stretch thereby increasing the amount of electrolyte flux through paracellular channels (Kirschner, 1980; McDonald and Rogano, 1986; Gonzalez and McDonald, 1994; McDonald and Milligan, 1996). If this is the case, differences between FHM and RBT in the amount of Na^+ loss associated with internally mediated stress may be due to differences in the strength of the paracellular tight junctions in FHM and RBT. However, differences in the amount of epinephrine release, the response to epinephrine, or the ability to down-regulate epinephrine receptors could also contribute to the greater response of FHM.

The results of this study are consistent with those of Postlethwaite and McDonald (1995), as both showed that RBT up-regulated J_{in}^{Na+} during recovery from confinement stress and recovered whole body Na^+ and Cl^- losses within 24 h (Figs 2.8 and 2.9). In contrast, FHM did not up-regulate J_{in}^{Na+} and took more than 2-times longer than RBT to recover whole body Na^+ and Cl^- concentrations (Figs. 2.8 and 2.9). This supports the conclusion that FHM are less capable of J_{in}^{Na+} up-regulation than RBT. Fathead minnows did not increase J_{in}^{Na+} despite the fact that they had higher Na^+ J_{max} than RBT under unchallenged conditions (Fig. 2.3). Further research is required to determine whether or not FHM are capable of up-regulating J_{in}^{Na+} under these conditions despite the fact that they do not. Regardless of the reason why, the lack of increased ion uptake in FHM means that they had lower whole body ion concentrations for a longer period than RBT. Moreover, this strategy was detrimental to FHM as they experienced greater mortality over a more prolonged period after the confinement stress protocol than RBT.

Effects of exposure to toxicants

The results of this study are consistent with earlier studies that showed that both low pH and copper disturb electrolyte balance (i.e., Wood and McDonald, 1982; Lauren and McDonald, 1985). In contrast, cationic surfactants did not promote Na^+ loss in either species (Table 2.5). This suggests that cationic surfactants do not affect ion balance in fish (see review Wood, 1992).

However all of these results support the view that FHM are more dependent upon the protective effects of external Ca^{2+} than RBT (Tables 2.4 and 2.5 and Fig. 2.10). In all of the exposures, the combination of acute exposure to low external Ca^{2+} and a toxicant caused FHM to lose significantly more Na^+ than RBT. Furthermore, there were also significant differences in Na^+ loss between FHM and RBT with acute low pH exposure after they had been acclimated to low external Ca^{2+}. The greater protective effect of Ca^{2+} against electrolyte loss from toxicant exposure may be a cyprinid trait. The results of this

study agreed with a study of Freda and McDonald (1988) which showed that common shiners required greater external Ca^{2+} concentrations to protect against Na^+ loss at low pH than RBT. In addition, Ca^{2+} (i.e., hardness) has a greater modifying effect in cyprinid species than salmonid species on the acute toxicity of Cu and Zn (Spear and Pierce, 1979; Spear, 1979).

Ionoregulatory Strategies and Tolerance

The differences in the ionoregulatory strategies of FHM and RBT stem from the fact that FHM regulate ions with less precision that RBT. This view is supported by the differences in ion balance between FHM and RBT under both routine and challenge conditions. First, FHM did not up-regulate J_{in}^{Na+} after stress provoked large losses in whole body Na^+ (Figs. 2.8 and 2.9). Fathead minnows also appeared to be less able to change J_{in}^{ion} after chronic exposure to changes in temperature (Fig. 2.2). Secondly, FHM experienced greater changes in Na^+ in most of the challenges, and were more dependent upon the protective effects of external Ca^{2+}. Both of these responses are probably due to more easily disrupted tight junctions between adjacent gill cells in FHM than RBT leading to leakier paracellular pathways in FHM. This would contribute to the greater sensitivity of FHM to low pH, particularly in soft water. Furthermore, some of the traits shown in this study have been linked to a higher sensitivity to low pH in other species. For example, Freda and McDonald (1988) showed that a greater dependence on external Ca^{2+}, a lower affinity for Na^+, and higher J_{max} all correlated with greater sensitivity to low pH among three freshwater fish species. The implications of the greater dependence of FHM on external Ca^{2+} and their less precise ionoregulatory strategy for other gill surface active toxicants are less clear.

The findings of this study suggest that FHM should be more sensitive to gill active metals than RBT, particularly in soft water. In a study that directly compared FHM and RBT, Bury et al. (1999) showed that FHM were more sensitive to bound and unbound

silver than RBT when water chloride or dissolved organic carbon (DOC) were high. In contrast, in this study there were no significant differences in silver toxicity between the two species when Ca^{2+}, chloride, and DOC were low. This could be linked to the larger difference between whole body Na^{+} and Cl^{-} in FHM but it appears to contradict the findings of this study. However, the toxic mechanism of silver differs from other species. For example, Ca^{2+} does not appear to protect against silver (Bury et al., 1999). In contrast, cyprinids are generally more sensitive to copper and zinc in soft water than salmonids (i.e., copper; Spear, 1979; zinc; Spear and Pierce, 1979). However, few studies directly compare metal toxicity in FHM and RBT. Even separate studies on the two species that examine the toxicity of the same metal rarely use the same hardness, pH, temperature, and alkalinity. This makes it difficult to compare the metal tolerances of the two species. Nonetheless, it is likely that the less precise ionoregulatory strategy of FHM will affect their tolerance to many gill active toxicants.

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Table 2.1: Water conditions for experiments.

Experiment	Trial	Temperature (°C)	Water Conditions: Ca^{2+} (meq L^{-1})	Water Conditions: Na^{+} (meq L^{-1})
1. Seasonal effect of temperature	1	see text	1.65	0.6
	2	see text	1.65	0.6
2. Na^{+} uptake kinetics	1	13 ± 1	1.65 ($CaCl_2$)	0.07, 0.09, 0.50, 0.60 (NaCl)
	2	21 ± 1	1.65 ($CaCl_2$)	0.05, 0.1, 0.25, 0.5, 1 (NaCl)
3. Na^{+} loss	1	15 ± 2	1.65 ($CaCl_2$)	Na^{+}-free
	2	15 ± 2	1.65	0.6
4. Cl^{-} uptake	1	19 ± 1	1.65	0.6
5. Cl^{-} uptake kinetics	1	17 ± 1	1.65 ($CaC0_3$)	0.05, 0.1, 0.2, 1.0, 1.5 (NaCl)
	2	17.± 1	1.65 ($CaC0_3$)	0.3, 0.6, 1.0, 1.5, 2.5 (NaCl)
7. Low external Ca^{2+}	1	17 ± 1	0.05, 0.25, 0.45, 0.85, 1.65 ($CaCl_2$)	Na^{+}-free
8. Two weeks low external Ca^{2+}	1	15 ± 2	0.05	0.1
9. Osmotic Shock	1	16 ± 1	1.65	260 (NaCl)
	2	16 ± 1	1.65	260 (NaCl)
10. Epi and Saline injection	1	11 ± 1	1.65 ($CaCl_2$)	Na^{+}-free

11. Recovery from	1	16 ± 1	1.65	0.6
stress protocol	2	16 ± 1	1.65	0.6
12. Low pH	1	15 ± 1	1.65 ($CaCl_2$)	Na^+-free
Na+ loss	2	15 ± 1	0.05 ($CaCl_2$)	Na^+-free
	3	15 ± 2	0.05	0.1
13. Cationic toxicant	1	15 ± 2	1.65	0.6
	2	15 ± 2	0.05	0.1
14. Copper	1	16 ± 1	1.65	0.6
	2	16 ± 1	0.05	0.1

Table 2.2: Whole body Na^+ and Cl^- levels in FHM (N = 36; 0.9 ± 0.1 g) and RBT (N = 30; 2.9 ± 0.3 g) after at least two weeks acclimation to 0.05 meq L^{-1} Ca^{2+}.

Species	whole body Na^+ ($\mu eq\ g^{-1}$)	whole body Cl^- ($\mu eq\ g^{-1}$)
fathead minnow	47.4 ± 1.68	29.9 ± 1.5†
rainbow trout	47.8 ± 1.8	40.9 ± 1.0

Values are means ± SEM. Differences between species are indicated by a dagger, ($p < 0.05$). These values are not significantly different from whole body Na^+ and Cl^- concentrations in fish held in hardwater (Fig 1).

Table 2.3: The effect of intraperitoneally injected epinephrine or saline on Net Na^+ flux in RBT (N = 4 per treatment; 1.4 ± 0.2 g) and FHM (N = 4 per treatment; 2.0 ± 0.2 g).

Species	Net Na^+ flux (neq $g^{-1}h^{-1}$)	
	epinephrine injected	saline injected
fathead minnow	-6317 ± 985†	- 8364 ± 1084†
rainbow trout	- 1037 ± 234	- 1102 ± 233

Values are means ± SEM, and significant differences between species are indicated by a dagger, ($p < 0.05$).

Table 2.4: The effect of acute exposure to pH 4.3 on Na^+ efflux in FHM (1.4 ± 0.27 g) and RBT (2.14 ± 0.36 g; N = 6 per species per treatment).

Species	Na^+ efflux (neq g^{-1} h^{-1})	
	1.65 meq L^{-1} Ca^{2+}	0.05 meq L^{-1} Ca^{2+}
fathead minnow	-789 ± 211	-2133 ± 192*†
rainbow trout	- 1007 ± 264	-1424 ± 164

Values are means ± SEM. Significant differences between species in the same treatment are indicated by a dagger and significant differences within species are indicated by an asterisk ($p < 0.05$).

Table 2.5: The effect of acute BC or DTAB exposure on Net Na^+ fluxes in FHM and RBT (N = 4 per species per treatment).

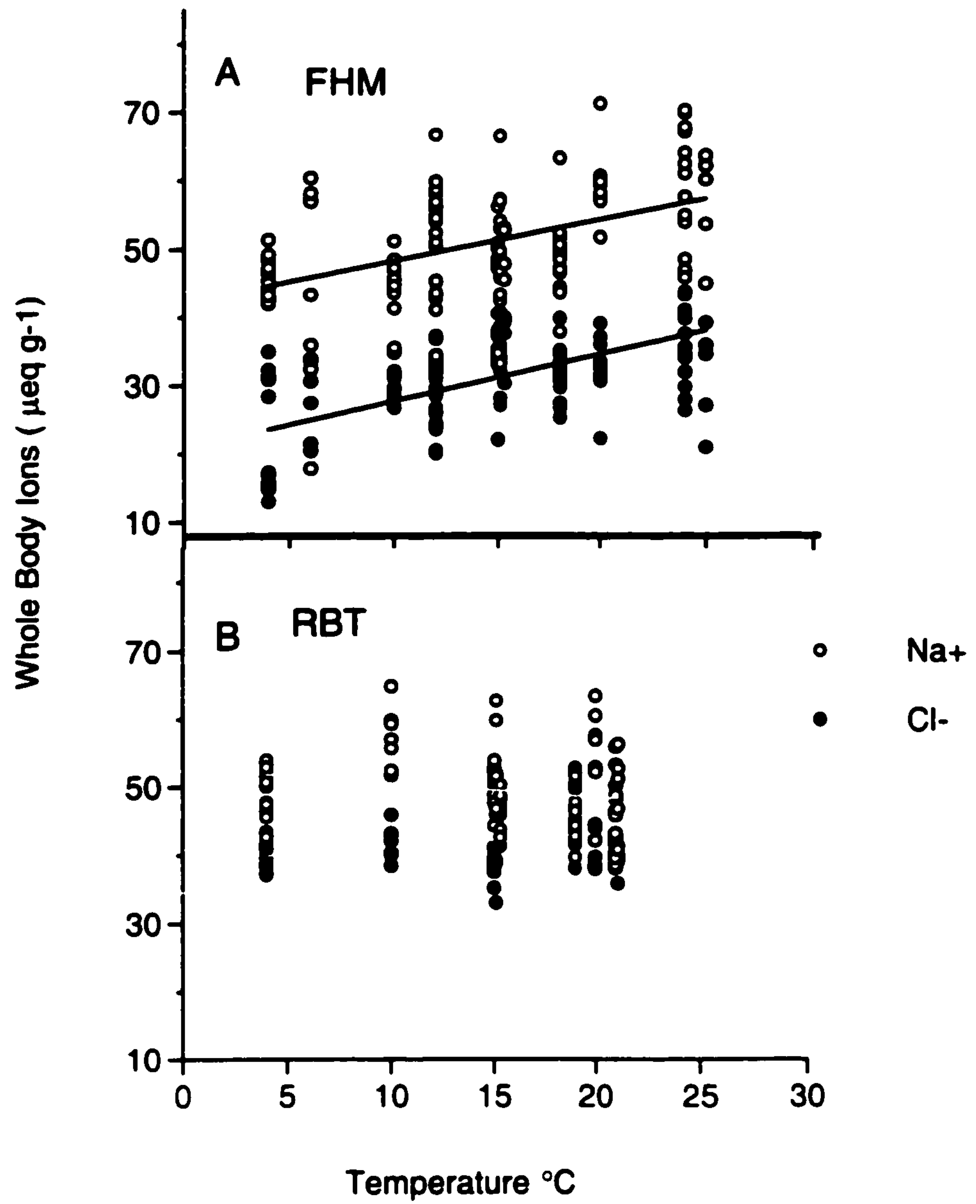
Species	Treatment	4 h Net Na^+ Fluxes (neq g^{-1} h^{-1})	
		1.8 meq L^{-1} Ca^{2+}	0.05 meq L^{-1} Ca^{2+}
fathead minnow	1.15 mg L^{-1} BC	-818 ± 194	-1019 ± 252†
rainbow trout		-510 ± 82	-308 ± 73
fathead minnow	5 mg L^{-1} DTAB	-558 ± 135	-1182 ± 120†
rainbow trout		-410 ± 204	-667 ± 127

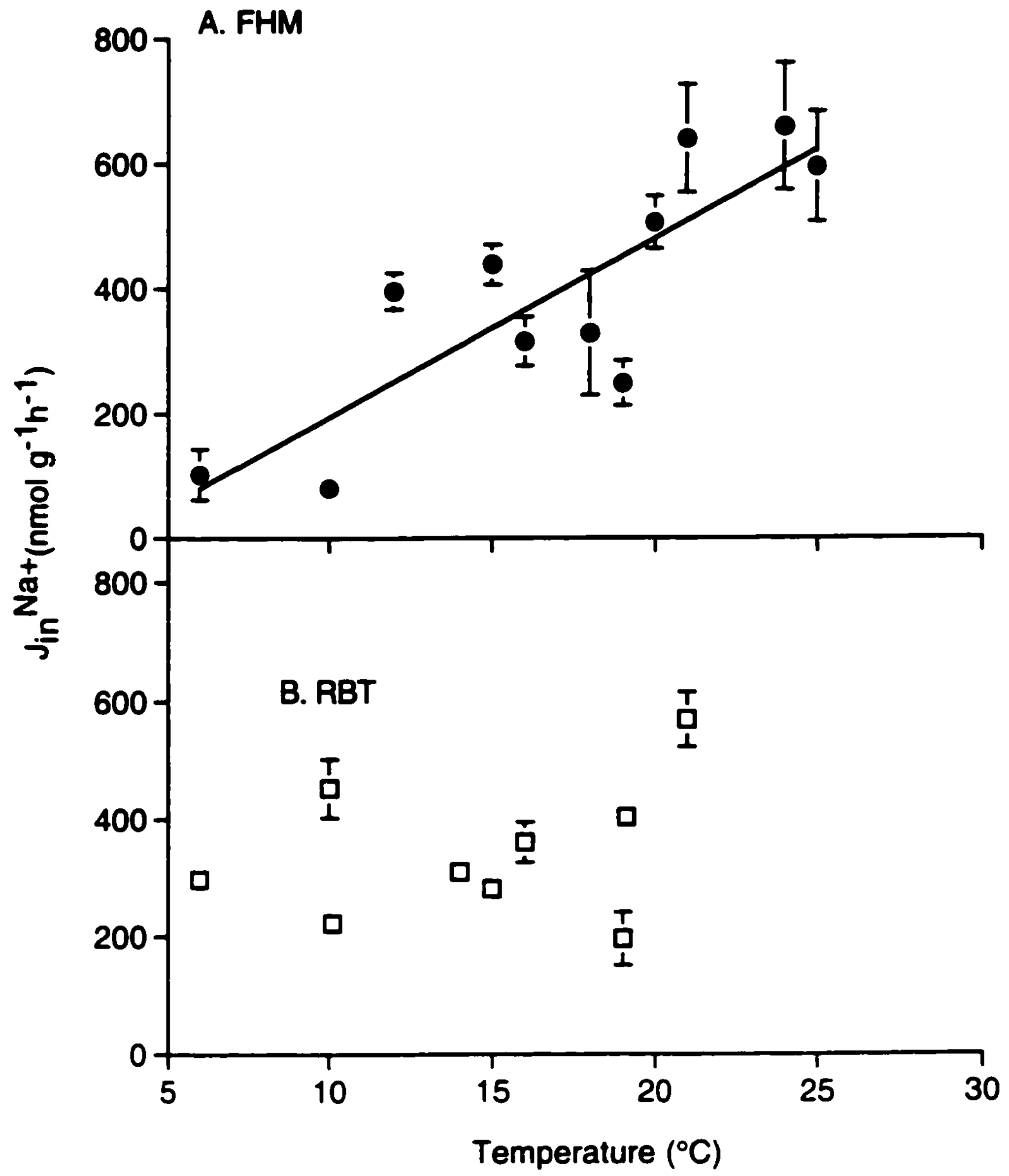
Values are means ± SEM. Significant differences between species are indicated by a dagger ($p < 0.05$).

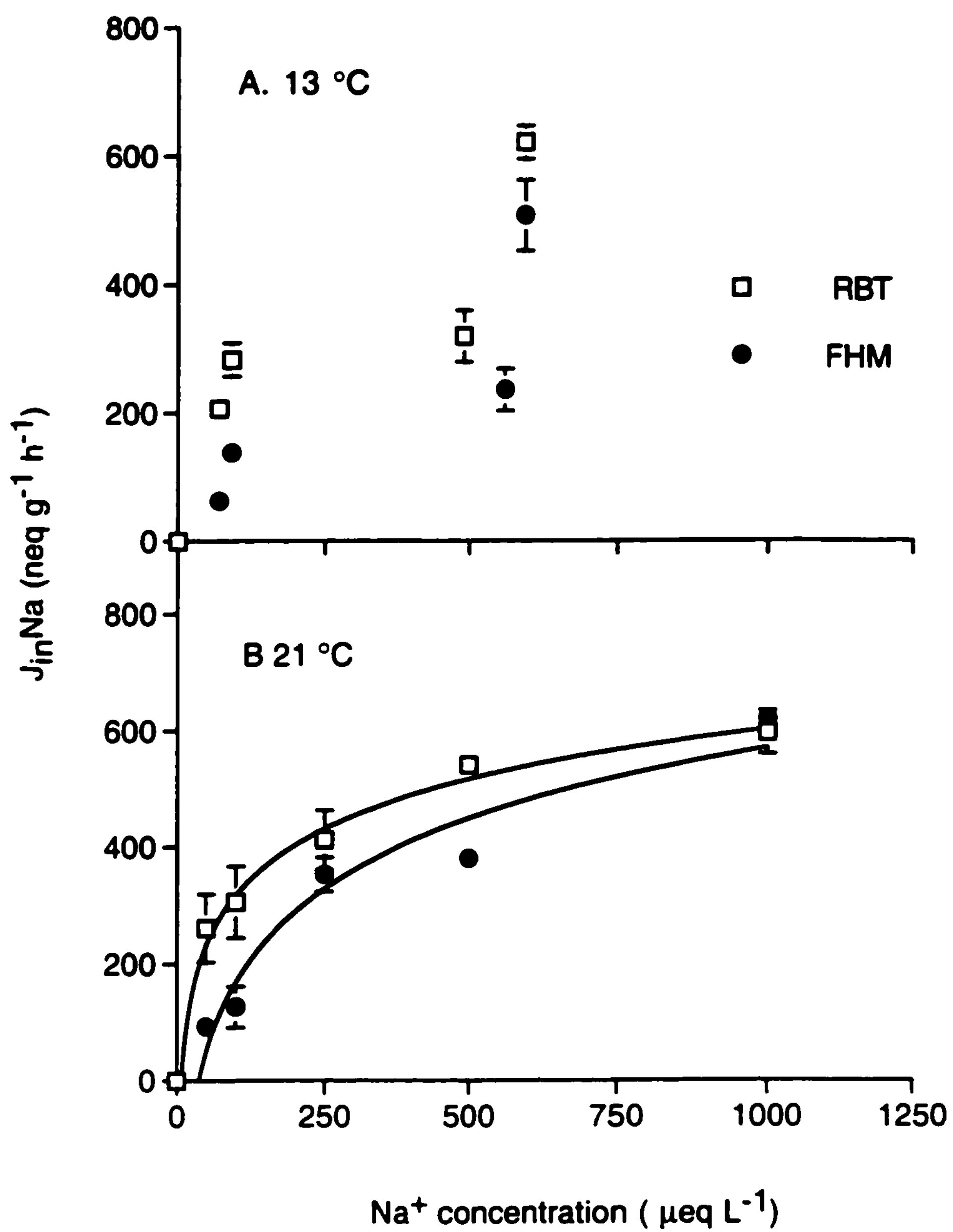
Table 2.6: Copper uptake in FHM (0.87 ± 0.07) and RBT (0.84 ± 0.05) after 4 h exposure to 400 μg L^{-1} Cu (N = 12 per species per treatment). Fish were not acclimated to softwater exposures. Exposures were done at 16 ± 1 °C.

Species	Cu uptake (ng g^{-1})	
	1.65 meq L^{-1} Ca^{2+}	0.05 meq L^{-1} Ca^{2+}
rainbow trout	0.42 ± 0.08	1.40 ± 0.27
fathead minnow	0.73 ± 0.17*	2.14 ± 0.36†

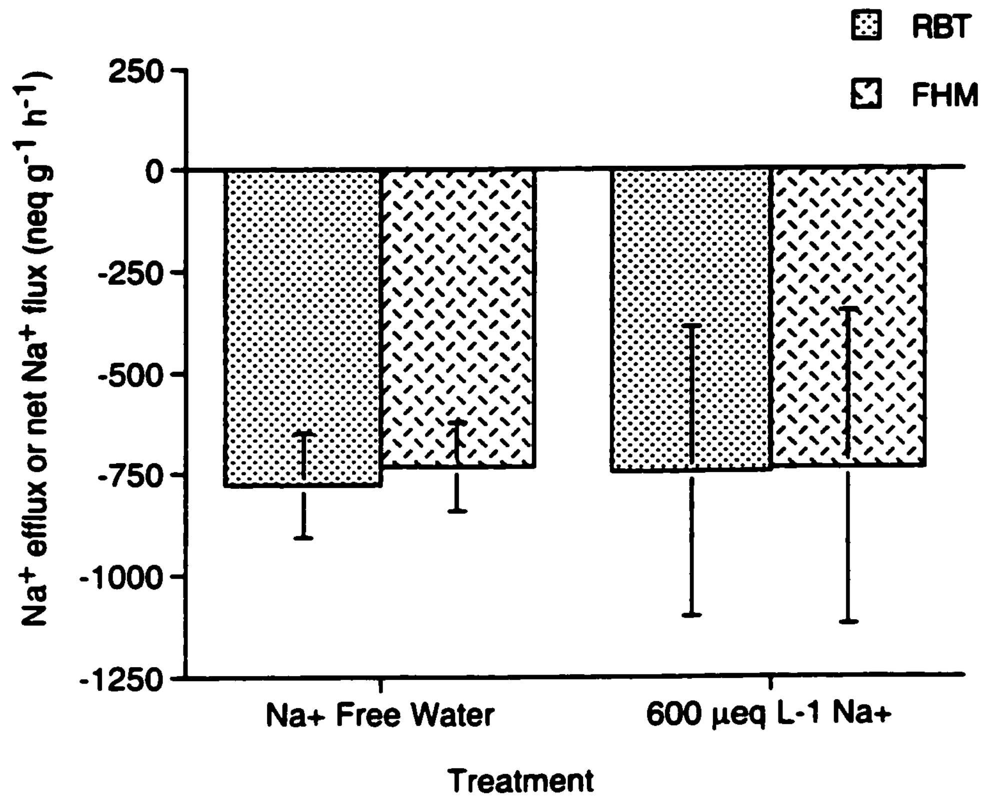
Values are means ± SEM. A dagger indicates a significant difference between species receiving the same treatment and an asterisk indicates a significant difference within species ($p<0.05$).

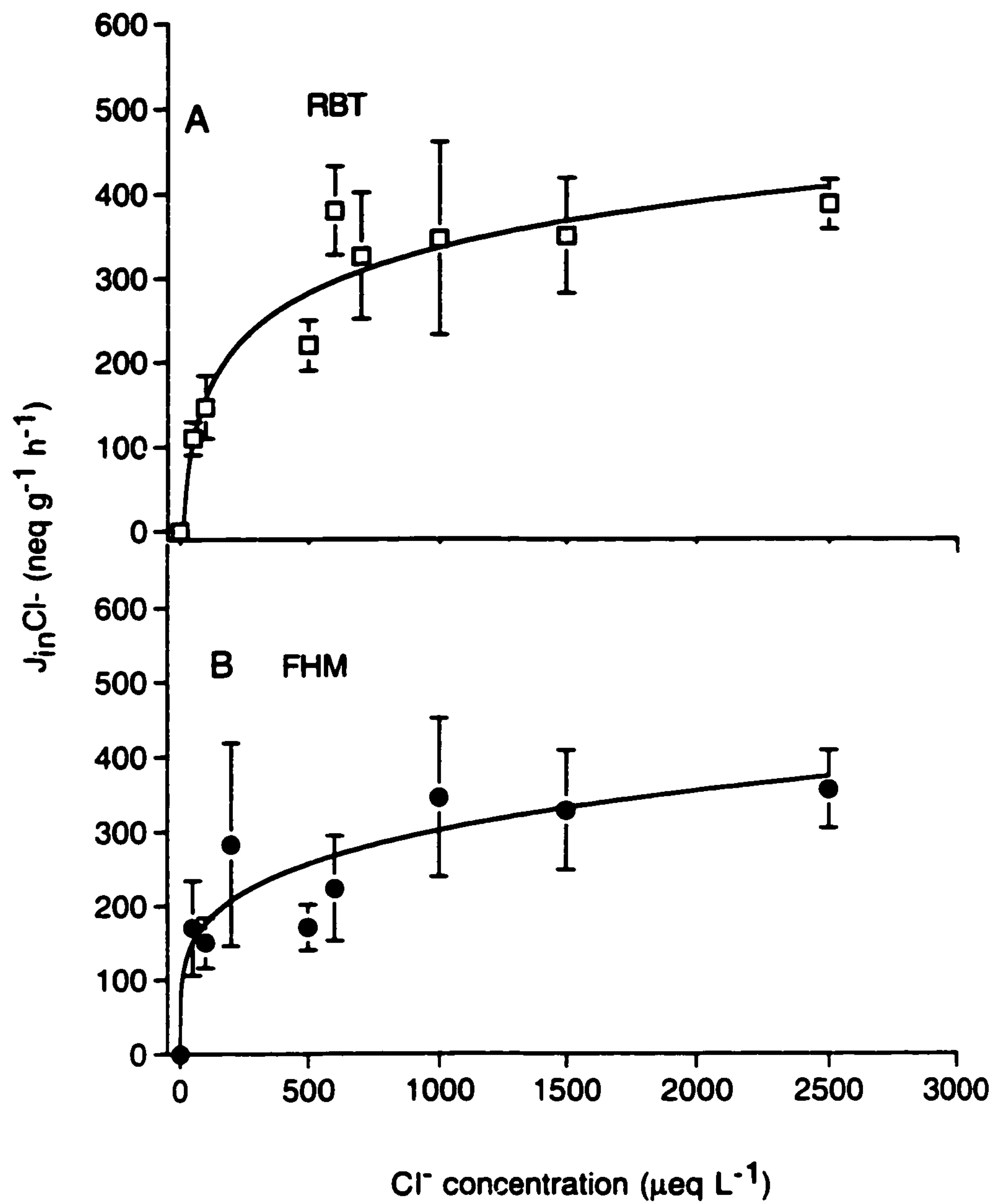

A FHM
B RBT
Whole Body Ions (µeq g-1)
70
50
30
10
0
5
10
15
20
25
30
Temperature °C
Na+
Cl-


A. FHM
B. RBT
J_{in}^{Na+}(nmol g^{-1}h^{-1})
800
600
400
200
0
5
10
15
20
25
30
Temperature (°C)

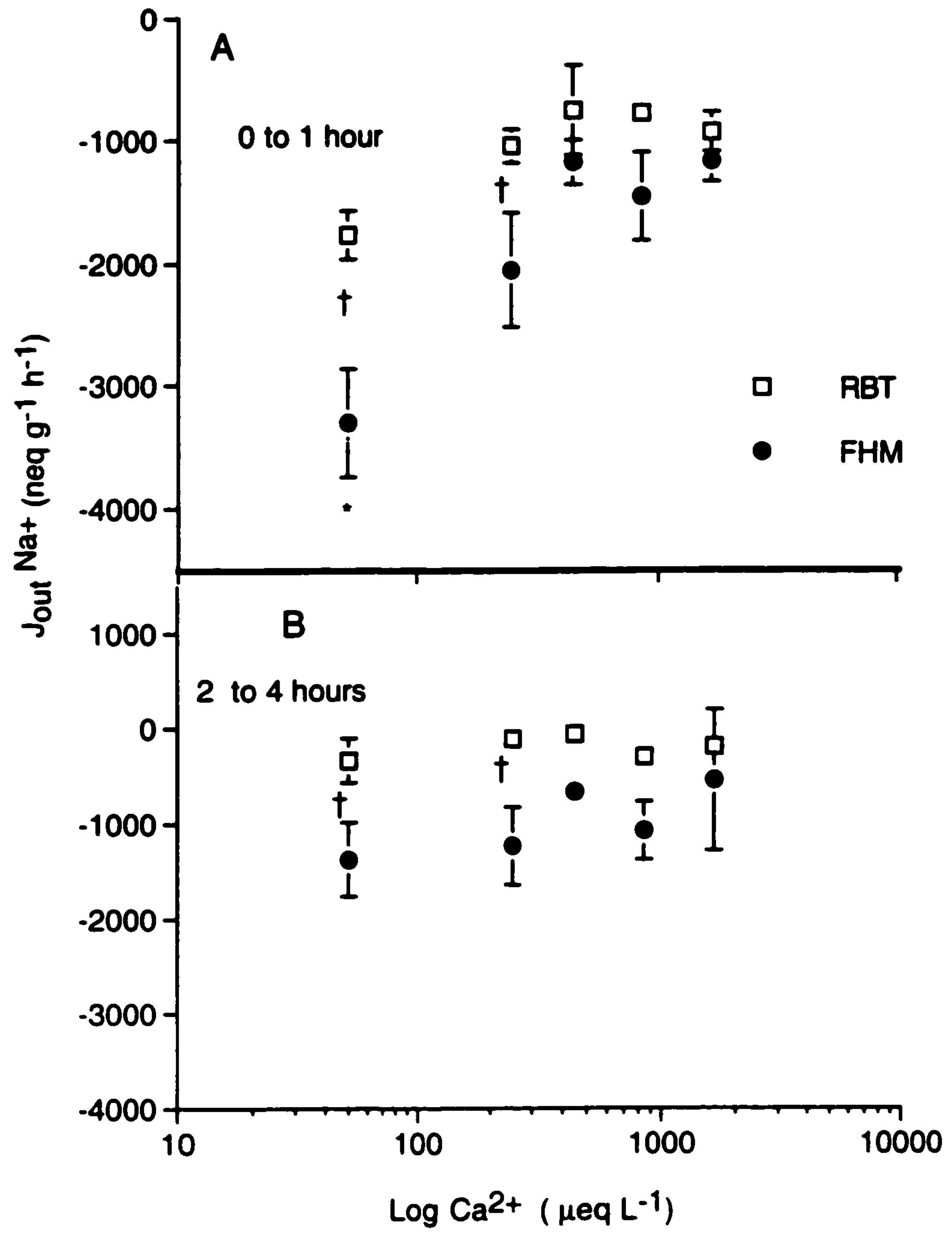


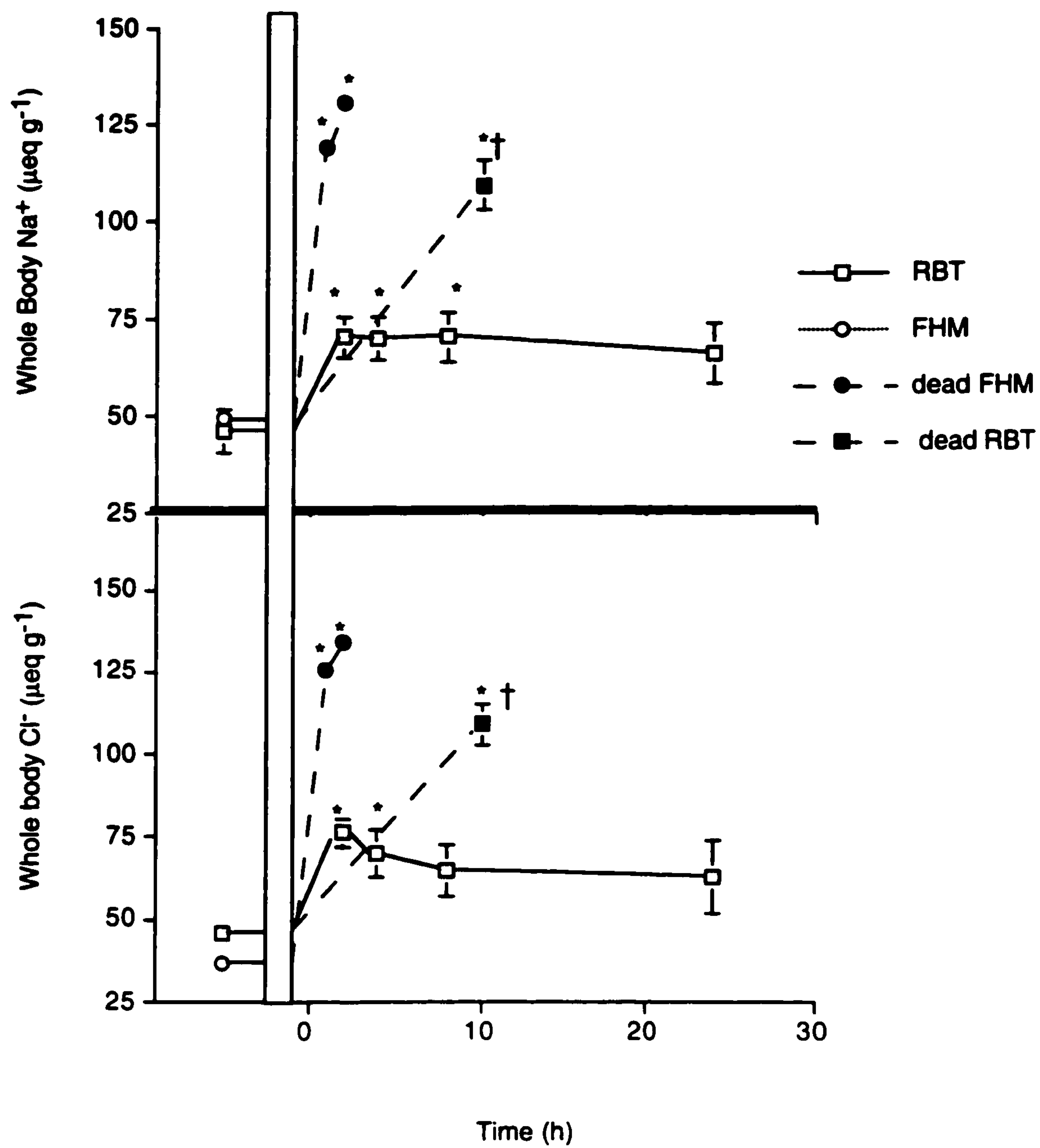
A. 13 °C
RBT
FHM
B 21 °C
$J_{in}Na$ (neq g^{-1} h^{-1})
Na$^+$ concentration (μeq L^{-1})
800
600
400
200
0
0
250
500
750
1000
1250


RBT
FHM
250
0
-250
-500
-750
-1000
-1250
Na+ efflux or net Na+ flux (neq g-1 h-1)
Na+ Free Water
600 µeq L-1 Na+
Treatment

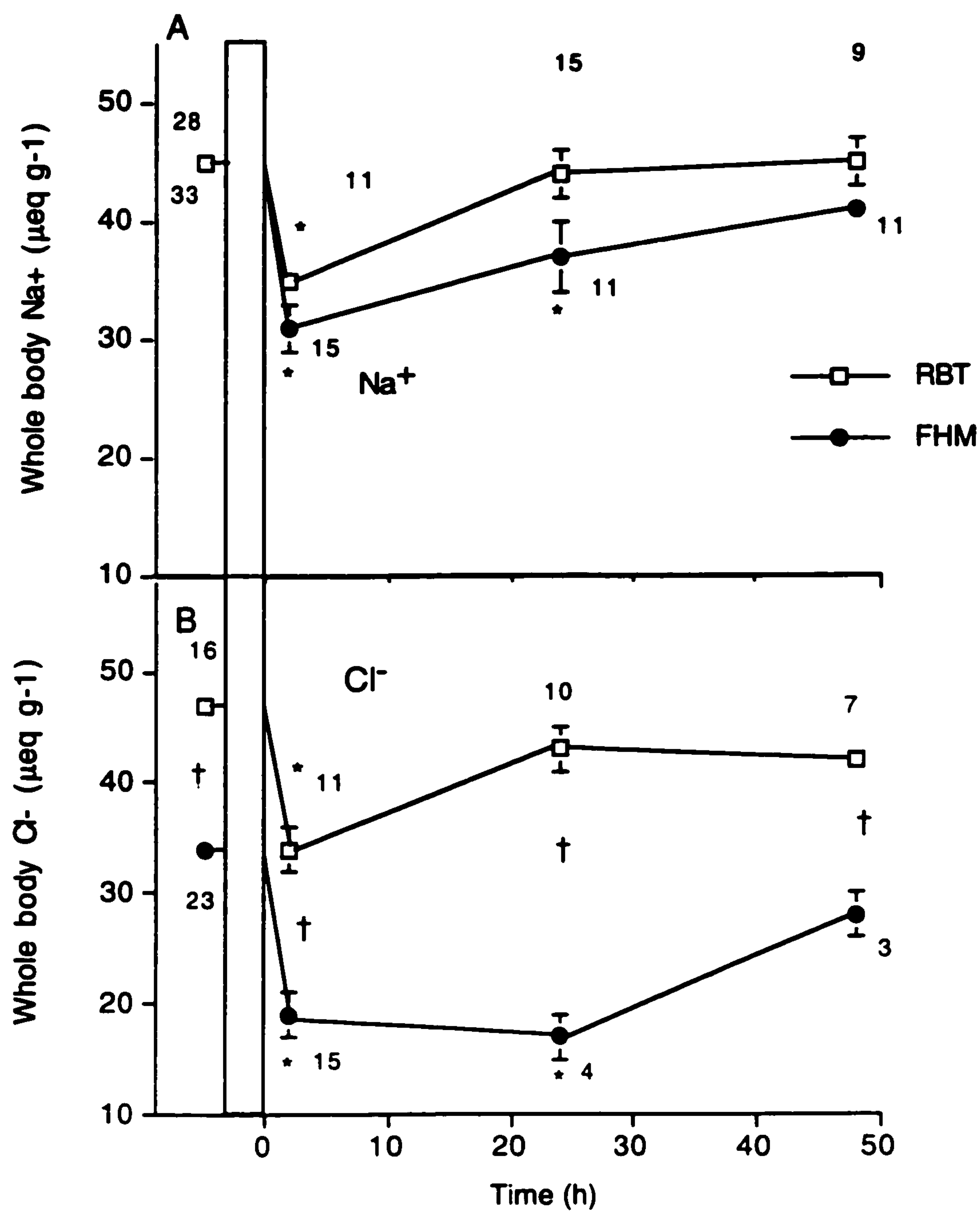


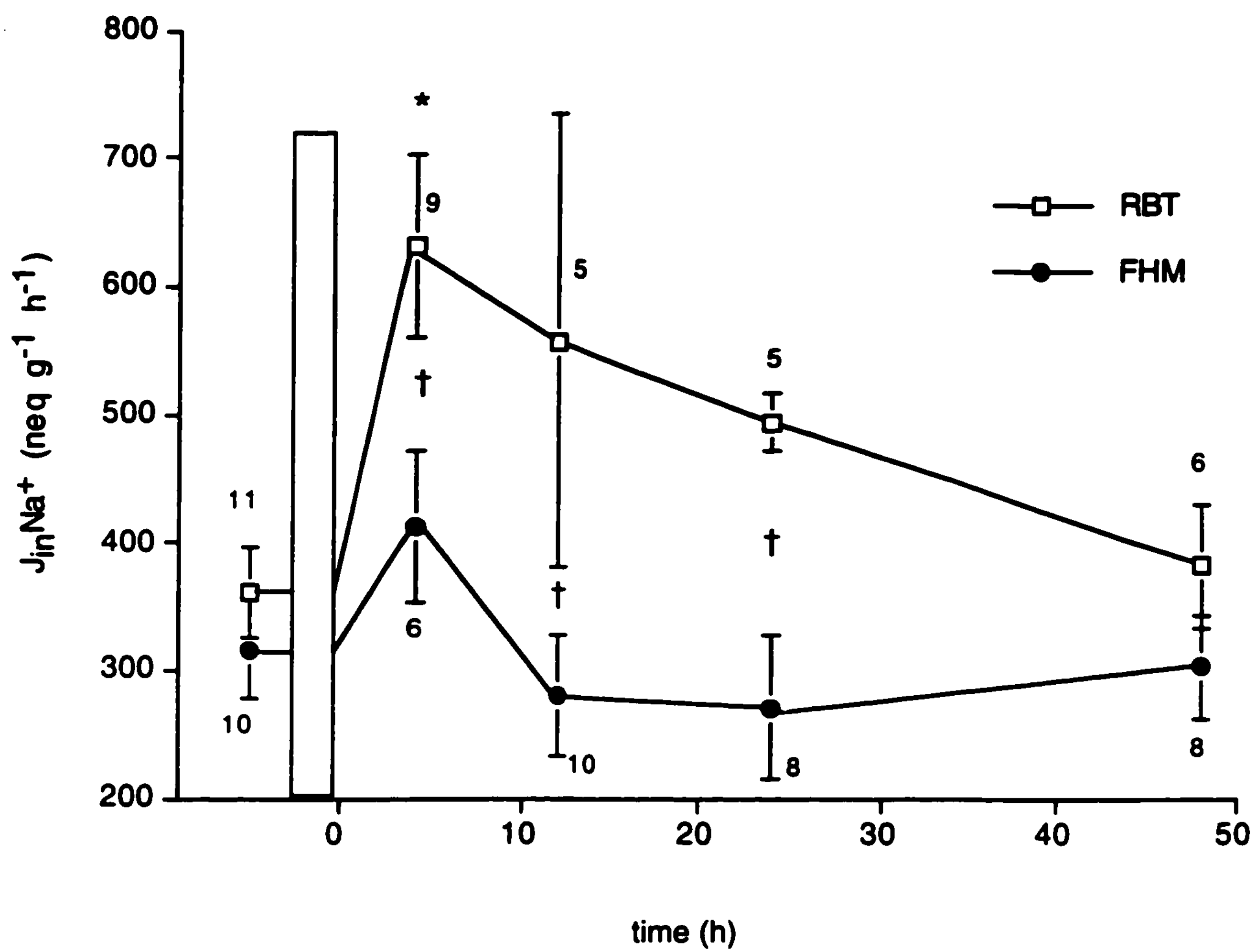
600
500
400
300
200
100
0
A
RBT
B
FHM
$J_{in}Cl^-$ (neq g^{-1} h^{-1})
0
500
1000
1500
2000
2500
3000
Cl^- concentration (μeq L^{-1})


A
0 to 1 hour
B
2 to 4 hours
RBT
FHM
J_{out} Na+ (neq g^{-1} h^{-1})
Log Ca2+ (µeq L-1)
0
-1000
-2000
-3000
-4000
1000
10
100
1000
10000

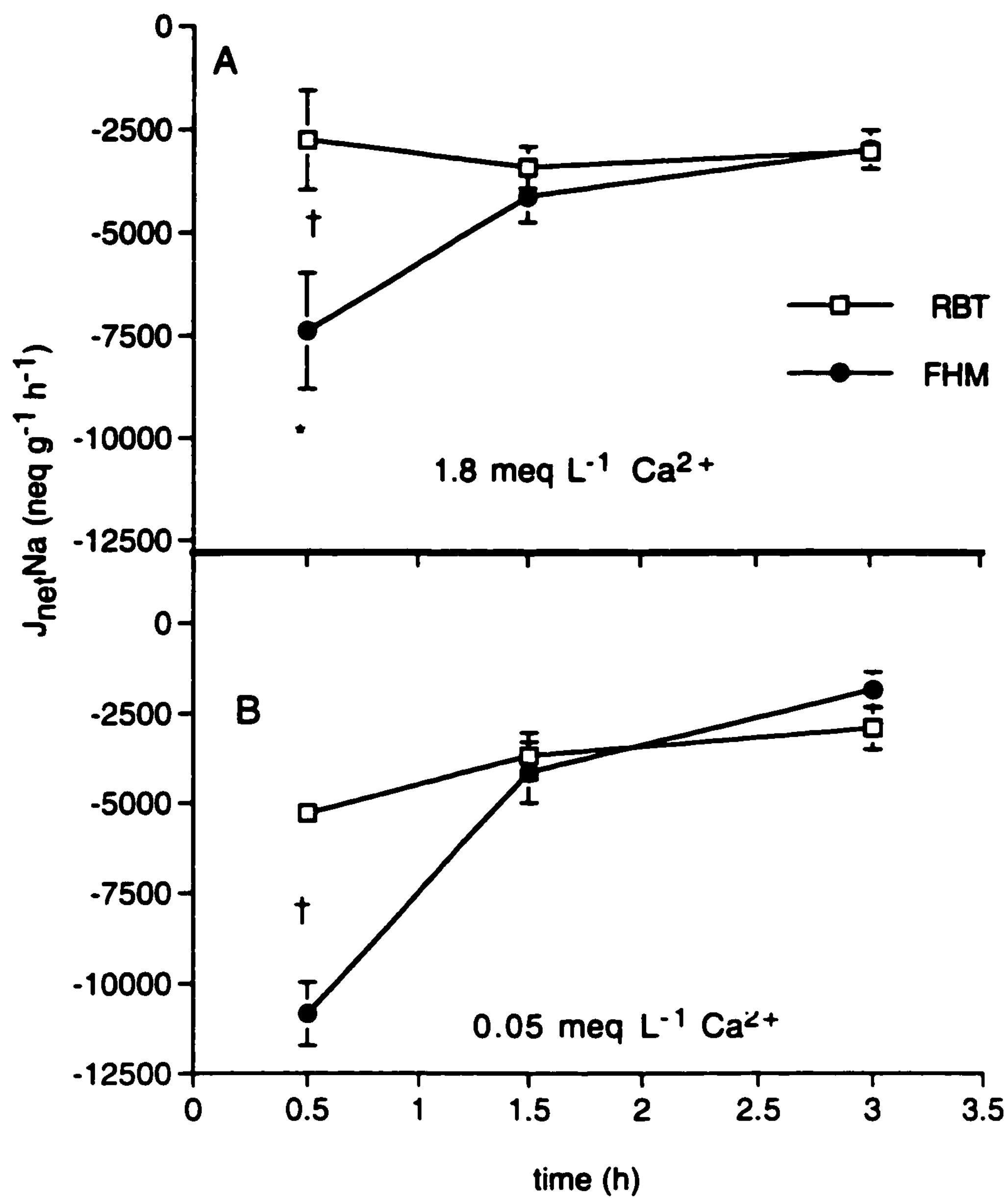


Whole Body Na+ (μeq g-1)
Whole body Cl- (μeq g-1)
150
125
100
75
50
25
0
10
20
30
Time (h)
RBT
FHM
dead FHM
dead RBT


A
Na+
RBT
FHM
Whole body Na+ (µeq g-1)
B
Cl-
Whole body Cl- (µeq g-1)
Time (h)
0
10
20
30
40
50
28
33
11
15
9
11
16
23
10
7
3
4



800
700
600
500
400
300
200
$J_{in}Na^+$ (neq g^{-1} h^{-1})
0
10
20
30
40
50
time (h)
RBT
FHM
*
†
11
10
9
6
5
10
5
8
6
8



A
B
0
-2500
-5000
-7500
-10000
-12500
$J_{net}Na$ (neq g^{-1} h^{-1})
RBT
FHM
†
*
1.8 meq L^{-1} Ca^{2+}
0.05 meq L^{-1} Ca^{2+}
0
0.5
1
1.5
2
2.5
3
3.5
time (h)

Chapter 3

Physiological mechanisms of higher ammonia tolerance in fathead minnow (*Pimephales promelas*) as compared to rainbow trout (*Oncorhyncus mykiss*)

Introduction

Ammonia is an ubiquitous aquatic pollutant that originates from industrial, agricultural, and aquacultural activities. It is estimated that industrial processes make more molecules of ammonia than any other chemical (Atkins, 1987). As a result of the many inputs of ammonia to the environment, the acute toxicity of ammonia to many fish species has been documented (Alabaster and Lloyd, 1980; Haywood, 1983; Tomasso, 1994). For example, salmonids are generally 1.6 to 12 times more sensitive to ammonia than cyprinids (Haywood, 1983). However, differences between studies in water pH and reporting styles make it difficult to compare toxicity data between studies and species. This is due to the fact that the unionized form of ammonia (NH_3) is 300 to 400 fold more toxic than the ionized form (NH_4^+; Thurston et al., 1981). Therefore, because the pK_a for the ionization of ammonia is approximately 9.6, ammonia is more toxic at high pH where more NH_3 is present (Cameron and Heisler, 1983). Many studies only report the toxicity of ammonia based upon the unionized form, although at neutral or acidic pH there is often enough ionized ammonia present that its toxicity should not be discounted (Thurston et al., 1981). Nonetheless, in two separate studies on acute toxicity (96h LC50) of ammonia in rainbow trout (RBT) and fathead minnow (FHM) that reported pH, total, and unionized ammonia, RBT were 3-fold more sensitive to ammonia than FHM (Thurston et al., 1983; Thurston and Russo, 1983).

Despite the large number of studies on ammonia toxicity the mechanism of toxic action remains unclear (Tomasso, 1994). Nonetheless it appears that in both fish and mammals the brain and nervous tissues are one of the main areas where ammonia exerts its toxic effects. Ammonia disrupts brain and nervous tissue function by lowering energy stores in the brain (i.e., by enzyme inhibition and disrupting the TCA cycle; Schneker, 1969; Alabaster and Lloyd, 1980, Minana et al., 1996), and it can cause membrane depolarization (Heald, 1975; Beaumont et al., 2000). This suggests that differences

between species may be due to differences in the ability of species to detoxify ammonia, in the amount of ammonia present in the organism, or the overall cellular tolerance in an organism to ammonia rather than differences in the toxic mechanism.

The overall purpose of this study was to determine whether or not there could be a physiological basis for the greater ammonia tolerance in FHM than RBT. Tolerance could arise by differences in gill uptake of ammonia, greater ammonia detoxification, greater ammonia excretion, and greater cellular tolerance. Thus the specific objective of this study was to determine which explanation is the most important. This problem was approached by examining the following differences between the two species: 1) ammonia tolerance by administering ammonia by waterborne and injection routes; 2) differences in ammonia excretion and urea excretion; 3) differences in ammonia uptake; and 4) differences in glutamine synthetase activity.

Materials and Methods

Experimental Animals

Juvenile rainbow trout (0.5 - 5 g) were obtained from Rainbow Springs Fish Hatchery in Thamesford, ON. Fathead minnows (0.5 - 4 g) were obtained from Steel City Bait in Hamilton, ON or Rainbow Springs Fish Hatchery in Thamesford, ON. All fish were held in tanks supplied with dechlorinated, Hamilton, ON municipal tap water (1 mmol L^{-1} Ca^{2+}, 0.6 mmol L^{-1} Na^{+}, 0.3 mmol L^{-1} Mg^{+1}, 0.8 mmol L^{-1} Cl^{-}, pH 7.8) at 16 ± 2 °C for at least three weeks prior to use (unless stated otherwise). Both species were fed once daily (approx. 1% day^{-1}) either trout chow (RBT) or a mix of Nutrafin flakes, and frozen brine shrimp FHM).

Experimental Approach

Ammonia effects were evaluated by measuring net transepithelial ammonia and urea fluxes, whole body ammonia and urea concentrations, and glutamine synthetase activity. The protocols used for each of these measurements are outlined below.

Fluxes

All fluxes were carried out in covered, cylindrical plastic containers (8 cm high X 11 cm diameter) individually aerated with airlines made of polyethylene tubing (PE 50). Each container was filled with a known volume of water (range 200 - 300 mls). Two to three fish were added to give a mass to volume ratio of approximately 1 g : 50 ml. Temperature was maintained by holding containers on a wet table supplied with water at 16 ± 2 °C, unless stated otherwise. Fluxes were 4 h in duration with water samples (5 or 10 ml) taken at 0, 1, 2 and 4 h (ammonia) or 0 and 4 h (urea). Fish were removed and weighed at the end of the 4 h measurement period. Water samples for ammonia analysis were preserved with 10 µl of concentrated nitric acid and typically analyzed within 24 h. Water samples for urea analysis were frozen at -20 °C until they could be analyzed.

Whole Body or Tissue Ammonia and Urea Concentrations, and Glutamine Synthetase Activity

Fish were euthanized with a lethal dose of anaesthesia (tricaine methanesulphate, MS222, 0.1 g l^{-1} buffered to pH 7.0 with $NaHCO_3$), blotted dry, and weighed. Next either individual tissues were removed by dissection and then rapidly frozen by freeze-clamping or the entire fish was freeze clamped. Tissues were freeze-clamped by holding the tissue in liquid nitrogen with a pair of forceps. Entire fish were freeze-clamped by compression of the whole body between two aluminum plates cooled with liquid nitrogen. Frozen samples were ground into a fine powder under liquid nitrogen and the powder was kept at - 80 °C until they could be analyzed for ammonia and urea concentrations, and glutamine synthetase activity.

Experimental Series

1. Nitrogen balance in fed and unfed resting animals

In this experimental series, resting values for ammonia excretion, urea excretion, and whole body ammonia and urea concentrations were measured in both FHM and RBT. Ammonia and urea excretion rates were measured in fish fed a daily satiation ration for at least one week prior to measurement (N = 19 per species for ammonia, N = 13 per species for urea; 16 ± 2 °C) and in fish that were starved for one week (N = 21 per species; 13 ± 1 °C; ammonia excretion only). After resting excretion rates were measured, whole body ammonia and urea concentrations (N = 8 per species; 16 ± 2 °C) were determined for a sub-sample of fed fish.

2. Acute Toxicity to waterborne ammonia

Both species (N = 15 per species) were placed together in an 80 L tank supplied with dechlorinated Hamilton tap water by a 350 L recirculating system maintained at 11 ± 1 °C. After fish had adjusted to the system for 2 h, the ammonia concentration was increased

to 2.7 mM using an ammonium sulphate stock solution. Fish mortality was recorded at 0, 0.37, 0.75, 1.5, 3, 6, 12 and 24 h. The estimated time to 50% mortality (ET50) was calculated using log probit analysis.

3. Acute Toxicity of injected ammonia

Both species (N = 10 per species per dose) were injected intraperitoneally (IP) with ammonium bicarbonate at doses of 15 μmol g^{-1} and 20 μmol g^{-1}. The test was terminated after 6 h. The estimated time to 50% mortality (ET50) was calculated using log probit analysis.

4. Acute Effects of waterborne ammonia

4a. Ammonia Excretion and Whole body Ammonia Concentrations

Both species were exposed to a range of external ammonia concentrations for 4 h to examine effects on whole body ammonia and ammonia excretion. Ammonia concentrations for RBT were 0, 0.25, 0.5. 0.8 and 1 mM and for FHM were 0, 0.25, 0.5, 1, 1.5 and 2 mM (N = 8 per species per concentration). Water was sampled at 0 and 4 h. Fish were sampled for whole body ammonia concentration after the fluxes were completed.

4b. Whole body ammonia, and urea concentrations and glutamine synthetase activity

Both species were exposed to 1 mM ammonia for up to 24 h to examine the effects of a longer duration ammonia exposure than described in 4a. All fish (N = 20 per species) were first exposed to 0 mM ammonia in black perspex boxes (4 L) supplied with dechlorinated Hamilton tap water by a partial-replacement-recirculating system. After 4 h, control fish (N = 5 per species) were euthanized. Ammonia concentration in the partial-replacement-recirculating system was then increased to a nominal concentration of 1 mM (actual 1.1 mM) using an ammonium sulphate stock solution. Ammonia concentration in the partial-replacement-recirculating system was maintained with a meter pump that delivered a dose of ammonium sulphate solution that increased the ammonia concentration

of the replacement water (rate of replacement, 1.72 ml min^{-1}). Water total ammonia concentration was monitored throughout the exposure. Both species (N = 5) were sampled after 4 and 8 h of ammonia exposure; however, because of mortality between 8 and 24 h there were no RBT available for the 24 h time period. All samples were analyzed for whole body ammonia and urea content and control and 8 h samples were also analyzed for whole body glutamine synthetase activity.

4c. Urea Excretion and Brain Glutamine Synthetase Activity

Both species were exposed to 0.85 mM ammonia for up to 48 h to examine the effects of prolonged ammonia exposure on urea excretion and brain glutamine synthetase activity. Fish were placed in aerated glass aquaria (20 L) with 0 mM ammonia (controls) or 0.85 mM ammonia. For the latter treatment, a stock ammonium sulphate solution was used to increase ammonia to a nominal concentration of 0.85 mM (actual 0.87 ± 0.02 mM) using ammonium sulphate. Half of the water in the aquaria was replaced daily with water adjusted to the appropriate ammonia concentration. Over the 48 h exposure period, urea fluxes were determined at 0 - 4 h, 20 - 24 h, and 44 - 48 h. Fluxes were determined on fish removed from the aquaria and placed in individual containers adjusted to 0 or 0.85 mM ammonia. Brain glutamine synthetase activity was determined on fish sampled directly from the tanks at 4, 24 and 48 h. For both urea fluxes and brain glutamine synthetase activity a total of 8 control fish were sampled over 48 h, and 5 exposed fish were sampled at each of the 3 sampling times.

<u>Analytical Techniques</u>

Water ammonia and urea

Ammonia content of the water was measured using the colorimetric assay of Verdouw et al. (1978). Samples > 150 µM were diluted so that they fell in the range of 0 to 150 µM ammonia (dilution factors of 2 to 15 times). To measure urea in the water, water samples were first freeze-concentrated. This procedure consisted of freeze

evaporating 5 ml water samples (frozen at -20 °C) in a Labconco freeze drier. Samples were reconstituted with 1 ml of water, and analyzed for urea using the colorimetric assay of Price and Harrison (1987). Samples were read on a spectrophotometer (4054, LKB Biochrom).

Whole body ammonia and urea

Two aliquots of frozen ground powder per fish were measured (approximately 100 mg) into tared 5 ml test tubes and ice cold 8% perchloric acid was added. Samples were then homogenized for 30 seconds using a tissue homogenizer (Tissue Tearor, Fisher), decanted into a microcentrifuge tube, and stored on ice. They were then centrifuged (235A, Fisher) at 13 600 G for 1 min. Samples were then neutralized with 4 mM Tris (trishydroxymethylaminomethane), assayed for ammonia and urea concentration using the methods of Bergmeyer (1985) and read on a spectrophotometer (4054, LKB Biochrom) at 340 nm.

Glutamine Synthetase Activity

Aliquots (approx. 200 mg) of powdered frozen whole body or an entire frozen brain samples (approx. 5 mg) were weighed out into tared microcentrifuge tubes and a volume of 4-times sample weight of ice cold homogenization buffer was added (buffer; 20 mmol L^{-1} K_2HP0_4, 10 mmol L^{-1} HEPES, 0.5 mmol L^{-1} EDTA, 1 mmol L^{-1} dithiothreitol, 50% glyercol, pH = 7.5 adjusted with NaOH). These samples were homogenized on ice with a cordless motor pestle (Kontes, New Jersey) and centrifuged at 13 600 G for 1 min. The supernatant was stored on ice until it could be analyzed for glutamine synthetase activity using the ferric chloride method described in Barber and Walsh (1993). Whole body samples were incubated at room temperature for 2 h and brain samples were incubated for 0.5 h at room temperature. Samples were read at 540 nm on a spectrophotometer. Enzyme activity was expressed as μmol product formed g^{-1} wet tissue min^{-1} at 22 °C.

Calculations

Net ammonia or urea fluxes were calculated according to the following equation:

$$J_{net} \text{ Ammonia (or Urea-N)} = (([N_i] - [N_f]) \times \text{vol}) / (t \times M) \quad (1)$$

where "$[N_i]$" and "$[N_f]$" represent the initial and final concentrations of ammonia or urea-N (urea concentrations were multiplied by 2 to give urea-N) in the water in nM, "vol" represents the average volume of water in the fluxing container for a given time period in ml, "t" represents the duration in hours and "M" represents the mass of fish in the flux container in grams.

Statistics

Means ± one standard error of the mean (SEM) are reported throughout. Comparisons amongst species and treatments were made by analysis of variance ($p < 0.05$). Tukey's multiple range test was used to resolve differences between species. For either time or concentration series within species, Dunnett's t-test was used to compare treatment effects with control.

Results

Routine Conditions

Under most circumstances, ammonia excretion rates were lower in FHM than RBT. In fed animals (ration ~1% day^{-1}), ammonia excretion was 65% lower in FHM than RBT (Table 3.1). Furthermore, starvation for one week amplified the ammonia flux difference between the species. Ammonia excretion rates in starved fish declined significantly to 24% of the fed rate in RBT and to only 10% of the fed rate in FHM. As a result, the ammonia excretion rate in starved FHM was only 28% that of RBT. In addition, the lower excretion in fed FHM was accompanied by 2-fold higher whole body total ammonia (Table 3.2). As a result, the turnover of ammonia (i.e., time taken to excrete the whole body content) was substantially longer in FHM than in RBT (2.2 h *vs.* 30 min).

In fed animals, urea excretion was 62% lower in FHM than RBT (Table 3.3), although in both species urea excretion made up the same fraction of total nitrogen excreted (approx. 11%). However, whole body urea concentrations were not significantly different in the two species (Table 3.2).

Ammonia Toxicity

Exposures to elevated ammonia in the water confirmed known differences in ammonia tolerance between the two species. An acutely lethal ammonia concentration (2.5 mM total ammonia; pH 8.0) produced 100% mortality in RBT and 55% mortality in FHM. The ET_{50} for FHM was about six times higher than RBT (21.0 ± 14.2 h *vs.* 3.6 ± 1.3 h). Furthermore, exposure to lower total ammonia (1 mM) produced 80% mortality in RBT between 8 and 24 h but no mortality in FHM.

Exposures to elevated ammonia by IP injection of ammonium bicarbonate also confirmed the differences in ammonia tolerance between the two species. After an IP injection of 15 $\mu M\ g^{-1}$, RBT died within minutes (Table 3.4). In contrast, there were no mortalities in FHM after IP injections of 15 or 20 $\mu M\ g^{-1}$ ammonium bicarbonate over a 6 h

period (Table 3.4). The monitoring period was not extended beyond 6 h as the ammonium load would have been completely excreted by this time.

Sublethal effects of ammonia exposure

Ammonia Loading

When both species were exposed to sub-lethal concentrations of external ammonia for 4 h, FHM were better at maintaining whole body ammonia concentrations than RBT. Rainbow trout maintained whole body ammonia concentrations (at approximately 1.2 μM g^{-1}) until the external ammonia concentration was approximately 0.5 mM. Above this concentration RBT whole body ammonia concentrations increased rapidly with external concentration to a high of 3.7 ± 0.5 μM g^{-1} after 4 h exposure to 1 mM (Fig. 3.1). In contrast, FHM maintained whole body ammonia concentrations (at approximately 3.0 μmol g^{-1}) until the external total ammonia concentration was approximately 1.5 mM. However, at 2 mM external ammonia FHM whole body ammonia concentrations increased by approximately 5-fold to a high of 10.1 ± 1.2 μM g^{-1} (Fig. 3.1).

In both species, as whole body ammonia concentrations were being held steady (i.e., over the external ammonia concentration range of 0.25 - 0.5 mM in RBT and 0.25 - 1.5 mM in FHM), ammonia excretion rates decreased (Fig. 3.2). Ammonia excretion rates in RBT were significantly lower than controls at external ammonia concentrations of 0.5 mM and above. In FHM, ammonia excretion rates were significantly lower than controls at 1 mM external ammonia. In addition, control ammonia excretion rates were significantly lower in FHM than RBT and this trend continued at higher concentrations.

In both species, there were no additional significant increases in whole body ammonia concentrations when exposure to 1 mM total ammonia was extended from 4 h to 8 h (Fig. 3.3). In RBT and FHM whole body ammonia concentrations remained at about 4 μmol g^{-1} from 4 h to 8 h of 1 mM ammonia exposure (Fig. 3.3). However, as mentioned

above between 8 and 24 h hours all of the RBT except one died. In contrast, there were no mortalities in FHM during this time period.

Ammonia Detoxification

With up to 8 h exposure to 1 mM total external ammonia, there were no significant differences from controls in whole body urea concentrations in either species, nor were there any significant differences between the two species (Fig. 3.4). In addition, whole body glutamine synthetase activities were similar in the two species and did not change significantly from controls after 8 h exposure to 1 mM ammonia in either species (Table 3.5).

Neither species showed any sustained significant changes in urea excretion rates when they were exposed to 0.85 mM total external ammonia for up to 48 h (Fig. 3.5). However, urea excretion rates in FHM increased significantly at 4 h but by 24 h they were similar to controls. In addition, urea excretion rates in FHM were 43 to 65% lower than those in RBT throughout the 48 h exposure. Brain glutamine synthetase activity was significantly higher in FHM than RBT in unexposed and exposed fish (Table 3.6). Neither species increased brain glutamine synthetase activity with up to 48 h exposure to 0.85 mM total external ammonia.

Discussion

Most studies that have examined physiological effects of ammonia in fish have studied only a single species. Relatively few have compared ammonia effects on more than one species in the same study and those that have tend to be fairly narrowly focused on one or two effects. For example, species comparisons have been made on the tolerance of injected ammonia in three freshwater species (Wilson et al., 1969), on urea excretion with elevated ammonia exposure in two freshwater species (Olson and Fromm, 1971), and on ammonia tolerance and plasma ammonia levels in three seawater species (Person-Le Ruyet et al., 1995). The more broadly focused work on comparing the effects of elevated ammonia exposure on physiological responses in fish and ammonia tolerance has been done on seawater species (Wang and Walsh, 2000). This is the first study to specifically address the differences in ammonia tolerance between two freshwater fish species based on comparisons done in the same study. Our results suggest that the higher tolerance of FHM to ammonia is based upon more than one physiological mechanism and suggest which areas might be the most important source of higher tolerance in FHM.

<u>Ammonia Excretion</u>

One of the striking differences between the two species was the lower ammonia excretion rate in FHM whether fed or starved (Table 3.1). In fed fish the difference existed despite the fact that both species were fed a similar ration (~1% day^{-1}). As well, urea excretion did not make up the difference in nitrogen excretion between the two species (Table 3.3). It is possible that the difference in protein content between the two diets (46% for FHM *vs.* 51% for RBT) could have partially contributed to the differences in ammonia excretion rate, but based upon a calculation using the data of Beamish and Thomas (1984), a difference of this magnitude is likely to only affect J_{net}^{Am} by 9%. Furthermore, the relative difference between the species increased with 7 days of starvation. By this time ammonia excretion rates typically stabilize at minimum endogenous production rates

(Fromm 1963; Savitz, 1969; Wood, 1993). Therefore the most likely explanation for low ammonia excretion rates in FHM is a lower rate of protein turnover. This could be due to the difference in life stage of the two species. Fathead minnows were nearing the end of their potential for growth while RBT were at the beginning of their potential for growth (FHM were approx. 1/3 of adult size *vs.* RBT were approx. 1/1000 adult size).

Estimated Turnover Times for Whole Body Ammonia

In addition to lower rates of ammonia excretion in FHM there was also a substantially longer retention of ammonia compared to RBT, 2.2 h vs. 30 min (Table 3.7). The retention time estimates for other fish species calculated from tissue ammonia concentrations, and ammonia and urea excretion rates are also summarized in Table 3.7. Retention times calculated with data from Linton et al. (1997) on RBT are similar to those reported here, and RBT has the lowest retention times of all the species examined. Retention times much higher than FHM have also been reported (i.e., 14 h in midshipmen; Wang and Walsh, 2000). Interestingly, ammonia turnover time correlates with 96h LC50 values for total ammonia, (i.e., species with a longer ammonia turnover time tend to be more tolerant of ammonia). This is perhaps not surprising as a species that has a high lethal threshold to external ammonia might be expected to be more tolerant to endogenously produced ammonia. Nonetheless, ammonia turnover time may be a useful screening tool (or biomarker) for predicting a species sensitivity to ammonia.

Uptake of Ammonia From the Environment

A novel finding here was that both species maintained whole body ammonia levels constant in the face of increasing external ammonia up to a threshold concentration (>0.8 mM for RBT; >1.5 mM for FHM). Above the threshold there was an abrupt increase in whole body ammonia levels (Fig. 3.1). This finding has not been previously reported, primarily because most studies have examined effects of external ammonia on plasma rather than whole body ammonia (e.g., Wilson and Taylor, 1992; Wilson et al.,

1994; Rasmussen and Korsgaard, 1998). Others have examined the effects of external ammonia on whole body, brain or liver ammonia concentrations but only at a single high external ammonia concentration (Levi et al., 1974; Arillo et al., 1981; Schenone et al., 1982; Iwata, 1989).

Whole body ammonia concentrations stay constant either because net ammonia production decreases or ammonia permeability at the gill is reduced. The former could be achieved either directly by reductions in ammonia formation (i.e., reduction in protein catabolism) and/or by increases in the detoxification of ammonia to other end products of nitrogen metabolism. To decide the relative importance of each of these strategies, I have taken a modeling approach. The models for RBT and FHM are shown in Figs 3.6 and 3.7 respectively.

A model for RBT ammonia uptake from the environment

As a starting point, the model for RBT (Fig. 3.6) assumes ammonia permeability at the gill remains constant over a range of external ammonia concentrations (i.e., ammonia uptake increases linearly with external ammonia concentration). With this assumption the sole mechanism for keeping whole body ammonia concentrations constant would be a decrease in net ammonia production. The model can then be broken down into three separate relationships. The first (depicted by a solid line - curve 1), shows the measured whole body ammonia levels in RBT after 4 h exposure to each elevated ammonia concentration. The second (depicted by a dotted line - curve 2), shows the estimated net ammonia production. Finally, the third (depicted by a slashed line - curve 3), shows the predicted uptake of ammonia.

Estimating net ammonia production

Here I have assumed that ammonia excretion and production are equal to one another. Therefore, I used the reduction in ammonia excretion with increasing water ammonia (Fig. 3.2) to create the steep part of the curve 2. The starting value here is 5.2

μmol g^{-1} (ammonia production over 4 h at zero external ammonia). I have assumed that ammonia production does not decline below 2.5 μmol g^{-1} over a 4 h period because ammonia excretion rates appear to stabilize at higher external ammonia (Fig. 3.2).

Reduction in net ammonia production

Any decrease in net ammonia production would be the result of decreased ammonia formation and/or an increase in ammonia detoxification. The most likely end products of ammonia detoxification are urea and glutamine (Korsgaard et al., 1995). However, urea can be ruled out as there were no significant changes in either whole body urea concentrations in RBT or in urea excretion rates with exposure to 1 mM and 0.85 mM external ammonia, respectively (Figs. 3.4 and 3.5). While this study did not measure glutamate or glutamine, generous estimations derived from a study on goldfish exposed to elevated external ammonia suggest that glutamine formation would be no greater than 0.15 μmol g^{-1} h^{-1} (Levi et al., 1974). Here, I assumed that glutamine produced in 24 to 48h would be similar to that produced in 4 h, and that blood and muscle glutamine increases would be similar. The model predicts a decrease of 0.6 μmol g^{-1} h^{-1} net ammonia production (Fig. 3.6). Therefore, detoxification could account for no more than 30% of the decrease in net ammonia production. Consequently 70% must be due to a decrease in net ammonia formation. The only way this could occur is by a reduction in protein catabolism. The means for signaling and effecting this reduction is unclear. However, other studies have implicated changes in protein synthesis and degradation with nitrogen loading (toadfish; Walsh and Milligan, 1995) and prolonged exposure to sub-lethal concentrations of ammonia (RBT; Linton et al., 1997). This suggests that fish may have a facility for rapidly modifying protein catabolism when stressed.

Rate of ammonia uptake

The inward diffusion of ammonia can be calculated by taking the sum of the increase in whole body ammonia concentrations (curve 1) and the estimated decrease in net

ammonia production (curve 2) at each external ammonia concentration. The result (curve 3) is a linear relationship between uptake and ammonia concentration ($r^2 = 0.994$) with an intercept of zero. This finding can be taken as at least partial validation of the starting point of the model because it shows by calculation the same result as the initial assumption. Furthermore, this conclusion is supported by the finding that plasma ammonia concentration increase linearly with external ammonia (over the range of 0 to 0.9 mM total ammonia) in at least one species, the turbot (*Scophthalmus maximus*; Rasmussen and Korsgaard, 1998).

A model for FHM ammonia uptake from the environment

The FHM model (Fig. 3.7) begins with the same premise and the same three relationships as the RBT model. The first relationship, (depicted by a solid line - curve 1), shows the measured average whole body ammonia levels in FHM after 4 h exposure to each elevated ammonia concentration. The second, (depicted by a dotted line - curve 2), shows the estimated net ammonia production. Finally, the third, (depicted by a slashed line - curve 3), shows the predicted uptake of ammonia.

Estimating net ammonia production

Here, I have again assumed that ammonia excretion and production are equal. Like the RBT model I have therefore used the reduction in ammonia excretion with increasing water ammonia from Fig. 3.2 to create the steep part of curve 2 (starting value is 3.2 μmol g^{-1} over 4 h at zero external ammonia). Because ammonia excretion was lower in FHM compared to RBT, the net ammonia production was lower. I have assumed that in FHM net ammonia production does not decline below 1.3 μmol g^{-1} over a 4 h period using the same criteria as that used for RBT.

Reduction in net ammonia production

As in the RBT model, any decrease in net ammonia production for FHM would be achieved through a combination of decreases in ammonia formation and/or increases in

ammonia detoxification. In FHM, detoxification contributed more (approx. 46%) to reduced net ammonia production as compared to RBT. This is because the estimate of 0.15 μmol g^{-1} h^{-1} glutamine formation (Levi et al., 1974) was compared to the lower net ammonia production of 0.3 μmol g^{-1} h^{-1} in FHM. Furthermore, detoxification could be even greater in FHM than RBT. I used the same estimate of glutamine formation for the two species (as calculated from Levi et al., 1974), but my results suggest that FHM may have higher rates of glutamine formation because brain glutamine synthetase activity was higher (Table 3.6). In addition, although whole body glutamine synthetase activity was similar in the two species, rates in the brain are probably more relevant given the putative site of toxicity of ammonia.

Rate of ammonia uptake

As in the RBT model, the inward diffusion of ammonia can be calculated by taking the sum of the increase in whole body ammonia concentration (curve 1) and the estimated decrease in net ammonia production (curve 2) at each external ammonia concentration. The result (curve 3) was a more complicated relationship than that seen in the RBT model. In the FHM model, the inward diffusion of ammonia can be divided into three separate relationships. First, there was a linear relationship between uptake and external ammonia between concentrations of 0 and 1 mM ($r^2 = 0.993$). For this relationship, the rate of inward diffusion of ammonia was lower than that seen in RBT (5.51 *vs.* 3.7 μmol g^{-1} mM external ammonia^{-1}; RBT *vs.* FHM, Fig. 3.6 and Fig 3.7). Secondly, between 1 mM and 1.5 mM the inward diffusion of ammonia appears to cease. Finally, above 1.5 mM there is a sharp increase in ammonia uptake. The reasons for these variations in inward diffusion with external ammonia concentration are unclear. Nonetheless, the model suggests that FHM are capable of regulating ammonia permeability at the gill up to about 1.5 mM and above this concentration there is a catastrophic

breakthrough phenomenon. In any case, these findings suggest that FHM are less permeable to ammonia than RBT at low external ammonia concentrations (i.e., 0 to 1 mM).

Tolerance to Ammonia

The greater ammonia tolerance of FHM than RBT appears to be due to differences between the two species in cellular resistance, ammonia detoxification, and a combination of differences in ammonia uptake and formation listed in order of importance. Fathead minnows and RBT appear to have quite different cellular resistances to ammonia. First, a whole body ammonia concentration of approximately 4 $\mu mol\ g^{-1}$ was associated with mortality in RBT, but there was no mortality in FHM with whole body ammonia concentrations as high as 10 $\mu mol\ g^{-1}$ (Figs. 3.1 and 3.3). In addition, FHM tolerated higher injected doses of ammonia than RBT (Table 3.4). It is well known that ammoniotelic vertebrates are more tolerant of plasma ammonia than ureotelic vertebrates (i.e., 2000 µm plasma ammonia in fish *vs.* 50 µm in mammals; Walsh, 1997). It is possible that smaller differences also exist between more closely related species. Recent work has found that compounds containing a trimethylamine group protected against both ammonia toxicity and glutamate neurotoxicity in fish (Tremblay and Bradley, 1992) and mice (Minana et al., 1996). This could provide some insights on where to begin looking for sources of cellular resistance to ammonia in FHM *vs.* RBT.

This is not to say that cellular detoxification is unimportant in the greater tolerance of FHM to ammonia. There was higher brain glutamine synthetase activity in FHM than RBT (Table 3.6). A similar relationship of brain glutamine synthetase activity in fish and 96h LC_{50} for ammonia was noted by Wang and Walsh (2000) for two species of toadfish, *Opsanus beta* and *O. tau*, and for the plainfin midshipmen, *Porichthys notatus*. However, the lack of increased detoxification enzyme activity with 48 h ammonia exposure in this study argues against detoxification as the most important factor in FHM higher ammonia tolerance.

Finally, the models developed in this study (Figs. 3.6 and 3.7) to explain the maintenance of whole body ammonia concentration as external ammonia increased have several implications. First, they suggest that both species down-regulate ammonia formation in an effort to maintain whole body ammonia levels with elevated ammonia exposure. Secondly they suggest that over the range of 0 to 1 mM external ammonia, FHM have lower ammonia uptake than RBT (slope 3.7 *vs.* 5.3 μmol g^{-1} mM^{-1}, FHM vs. RBT, Figs 3.7 and 3.6). These findings suggest that differences in ammonia uptake and ammonia formation also play a role in the greater tolerance of FHM. Thus, the results of this study emphasize that the greater tolerance of FHM than RBT to ammonia has multiple origins.

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Table 3.1: Ammonia excretion in fed fish (approx. 1% body weight day^{-1}) and fish with no ration for one week. Fed RBT (N = 6; 5.5± 0.4 g) and FHM (N = 6; 1.7 ± 0.1 g) were held at 16 ± 1 °C and starved RBT (N = 21; 0.8 ± 0.1 g) and FHM (N = 21; 1.4 ± 0.1 g) were held at 13 ± 1 °C.

Species	J_{net} ammonia (nmol g^{-1} h^{-1})	
	fed	starved
Rainbow trout	-1426 ± 139	-328 ± 21
Fathead minnow	-1004 ± 64†	-92 ± 8†

Values are means ± SEM and significant differences between species are represented by a dagger, ($p < 0.05$).

Table 3.2: Whole body ammonia levels in fed RBT (N = 8; 1.28 ± 0.13 g) and FHM (N = 8; 1.53 ± 0.23 g) and whole body urea levels in fed RBT (N = 8; 1.57 ± 0.21 g) and FHM (N = 8; 1.27 ± 0.12 g).

Species	Whole body concentration ($\mu mol\ g^{-1}$)	
	ammonia	urea
Rainbow trout	0.79 ± 0.13	0.59 ± 0.13
Fathead minnow	2.16 ± 0.30†	0.63 ± 0.09†

Values are means ± SEM. Significant differences between species are represented by a dagger, ($p < 0.05$).

Table 3.3: Urea-N and ammonia excretion in fed (approx. 1% body weight day^{-1}) RBT (N = 13; 1.9 ± 0.3 g) and FHM (N = 13; 1.5 ± 0.2 g). Both species were held at 16 ± 1 °C.

Species	J_{net} (nmol g^{-1} h^{-1})	
	ammonia	urea-N
Rainbow trout	-1429 ± 90	-168 ± 18
Fathead minnow	-884 ± 144†	-104 ± 16†

Values are means ± SEM and significant differences between species are represented by a dagger, $p < 0.05$.

Table 3.4: The estimated time to 50% (ET_{50}) mortality for FHM (N = 10 per dose; 1.43 ± 0.24 g) and RBT (N= 10 per dose; 1.96 ± 0.10 g) intraperitoneally injected with 15 and 20 μmol g^{-1} ammonium bicarbonate.

Species	ET50 at each ammonium bicarbonate dose (min)	
	15 μM g^{-1}	20 μM g^{-1}
Rainbow trout	14 ± 10	too fast
Fathead minnow	no deaths	no deaths

Table 3.5: The effect of 8 h exposure to 1 mM total external ammonia on whole body glutamine synthetase activity in RBT (N =3 per treatment; 1.7 ± 0.3 g) and FHM (N = 3 per treatment; 1.3 ± 0.2 g).

Species	Glutamine Synthetase activity (μmol min^{-1} g^{-1})	
	Control	8 h 1 mM amm exposure
Rainbow trout	1.313 ± 0.004	1.174 ± 0.065
Fathead minnow	0.985 ± 0.108	0.953 ± 0.155

Values are means ± SEM.

Table 3.6: The effect of 48 h exposure to 0.85 mM total external ammonia on brain glutamine synthetase activity in RBT (N = 8 controls, N = 5 per time period; 1.5 ± 0.1 g) and FHM (N = 8 controls, N = 5 per time period; 1.2 ± 0.1 g) .

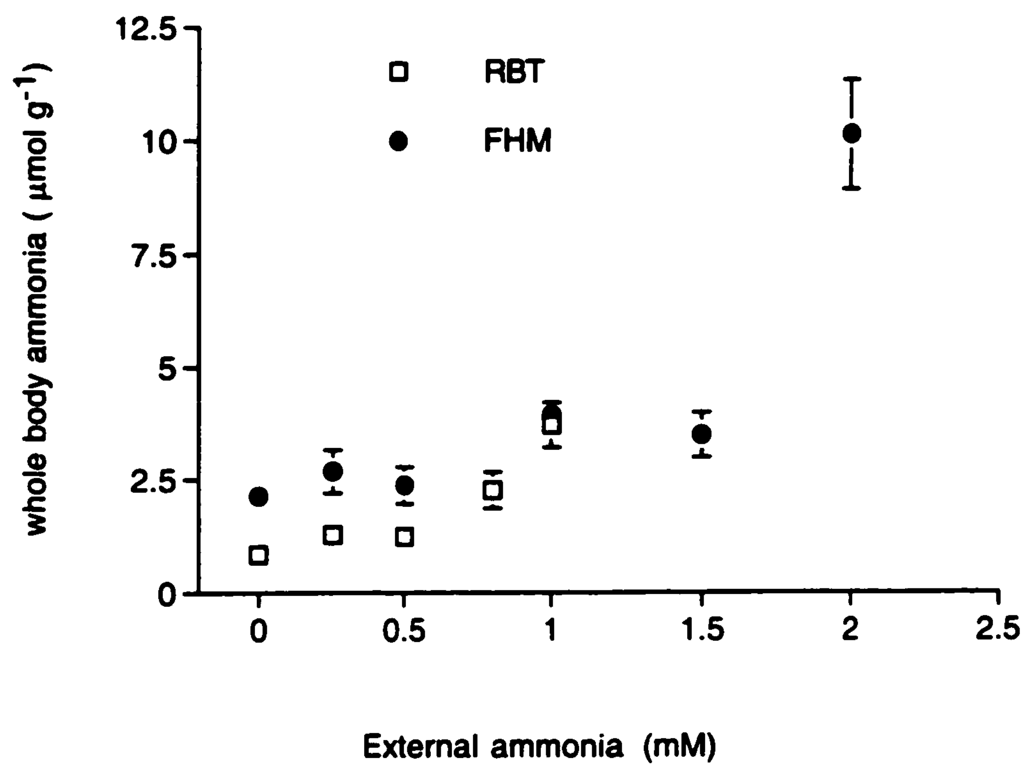
Species	Glutamine Synthetase activity (μmol min^{-1} g^{-1})			
	control	4 h	24 h	48 h
rainbow trout	13.6 ± 1.8	14.2 ± 1.2	14.9 ± 1.2	12.3 ± 0.7
fathead minnow	21.2 ± 1.7†	20.8 ± 1.8†	19.4 ± 2.0	22.2 ± 4.0†

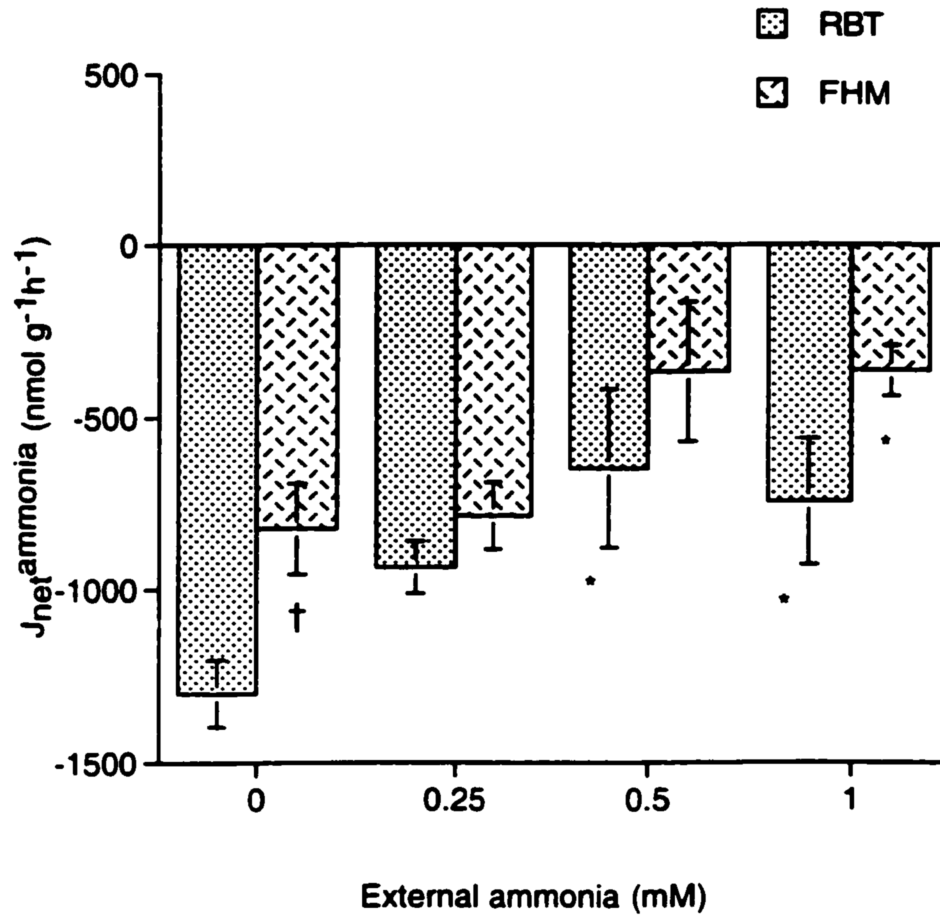
Values are means ± SEM. Significant differences between species at a time period are indicated by a dagger, $p < 0.05$.

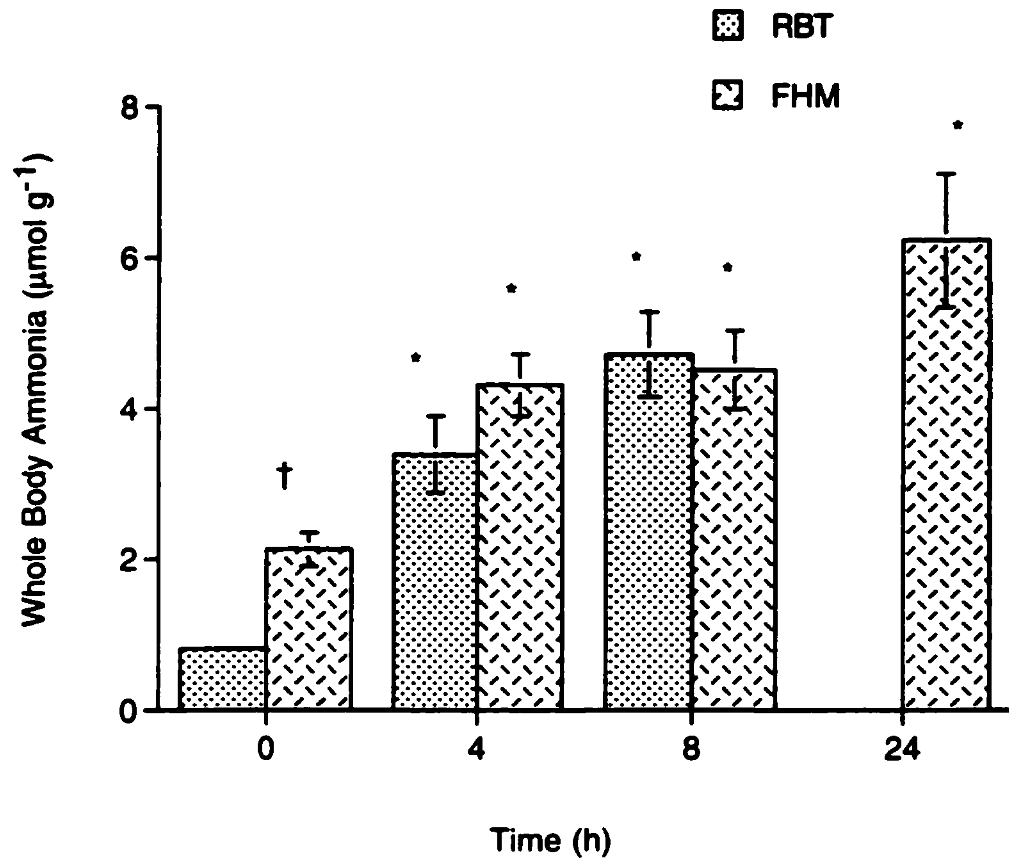
Table 3.7: Estimated ammonia turnover times derived from reported values for ammonia and urea excretion rates and whole body ammonia levels or estimated whole body ammonia.

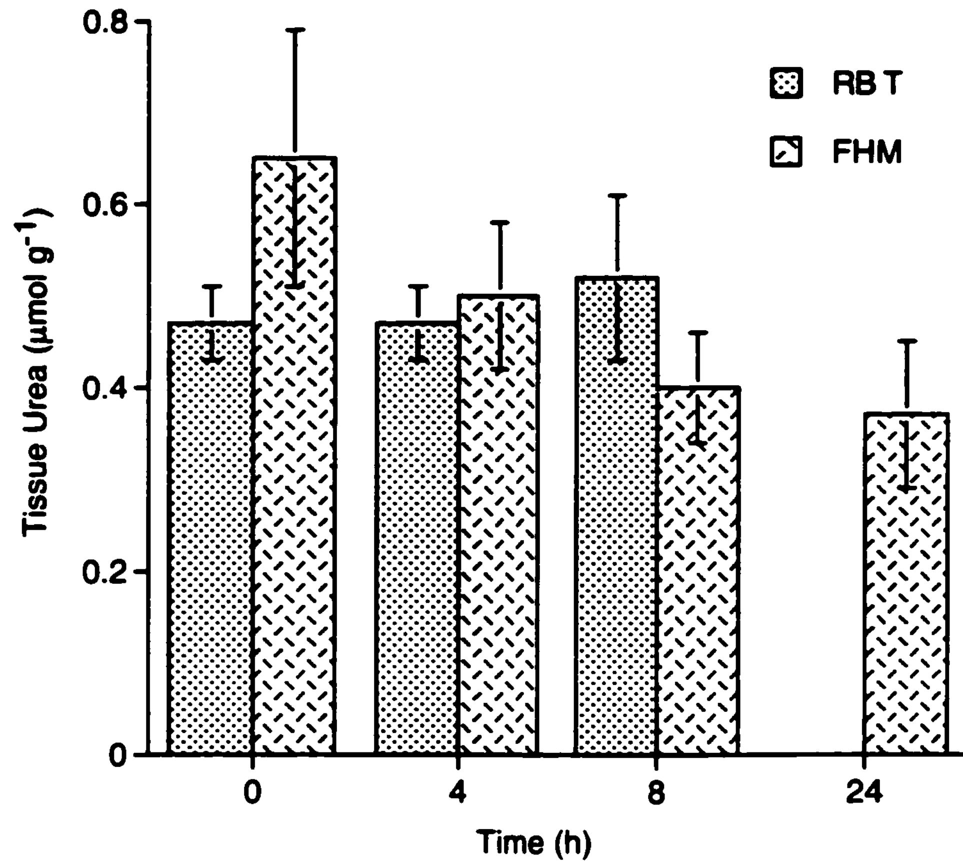
Species	ammonia toxicity	Estimated tissue ammonia	ammonia + urea excretion	Turnover time	Source
	96h LC_{50}	(µmol g^{-1})	(µmol g^{-1} h^{-1})	h	
fathead minnow	1.8 - 6 mM	2.2	0.988	2.2	present study
rainbow trout	0.6 - 2.6 mM	0.8	1.597	0.5	present study
rainbow trout	0.6 - 2.6 mM	1.05 - 1.3	0.850 - 1.2	0.92 to 1.5	Linton et al., 1997
mudskipper	> 15 mM	4	0.625	6.4	Iwata 1989
gulf toadfish	9.75 mM	1.1	0.230	4.8	Wang and Walsh, 2000
plainfin midshipmen	6 mM	3.2	0.231	13.8	Wang and Walsh, 2000
oyster toadfish	19.72 mM	1.2	0.100	12.0	Wang and Walsh, 2000

Whole body ammonia levels were estimated based upon literature values for muscle, liver, plasma, and brain levels. Not all studies reported values for each of these tissues. Fish were assumed to be 55% muscle, 2% liver, 19% extracellular fluid (where plasma ammonia = ECF ammonia) and 0.5% for brain (if data was available) and the remainder was treated as having no ammonia. This calculation does not account for the fact that tissue levels were based upon wet tissue (i.e., ECF included).

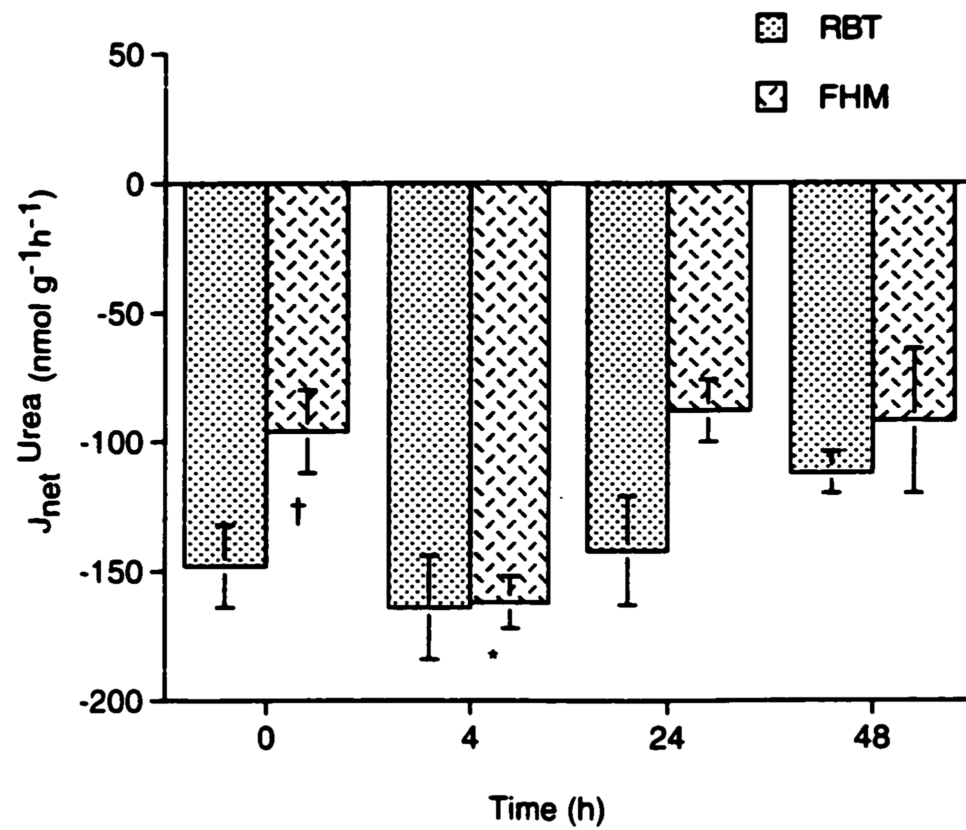

12.5
10
7.5
5
2.5
0
RBT
FHM
whole body ammonia (µmol g-1)
0
0.5
1
1.5
2
2.5
External ammonia (mM)


RBT
FHM
500
0
-500
-1000
-1500
J_{net}ammonia (nmol g^{-1}h^{-1})
†
*
*
*
0
0.25
0.5
1
External ammonia (mM)

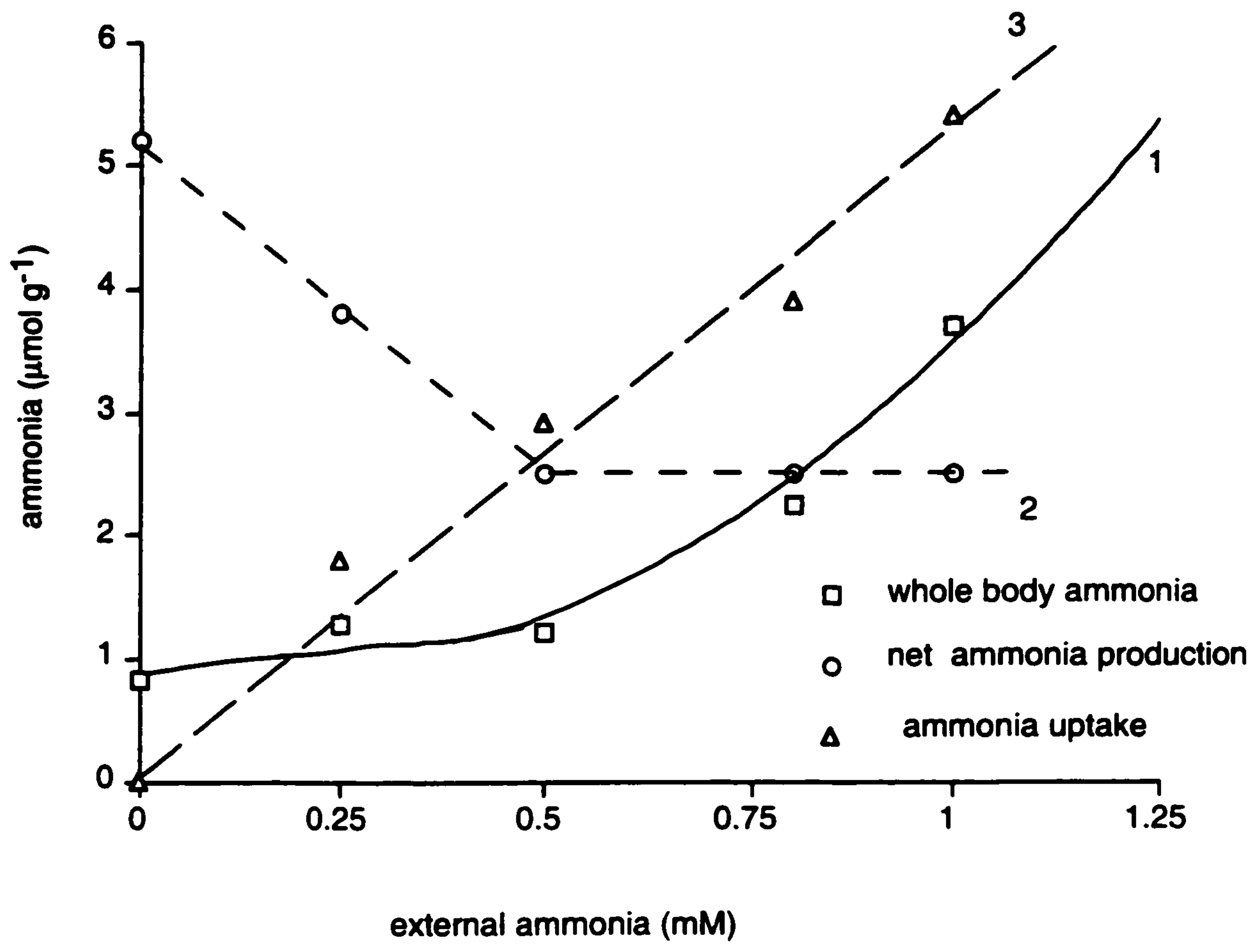

RBT
FHM
Whole Body Ammonia (µmol g-1)
0
2
4
6
8
†
*
0
4
8
24
Time (h)



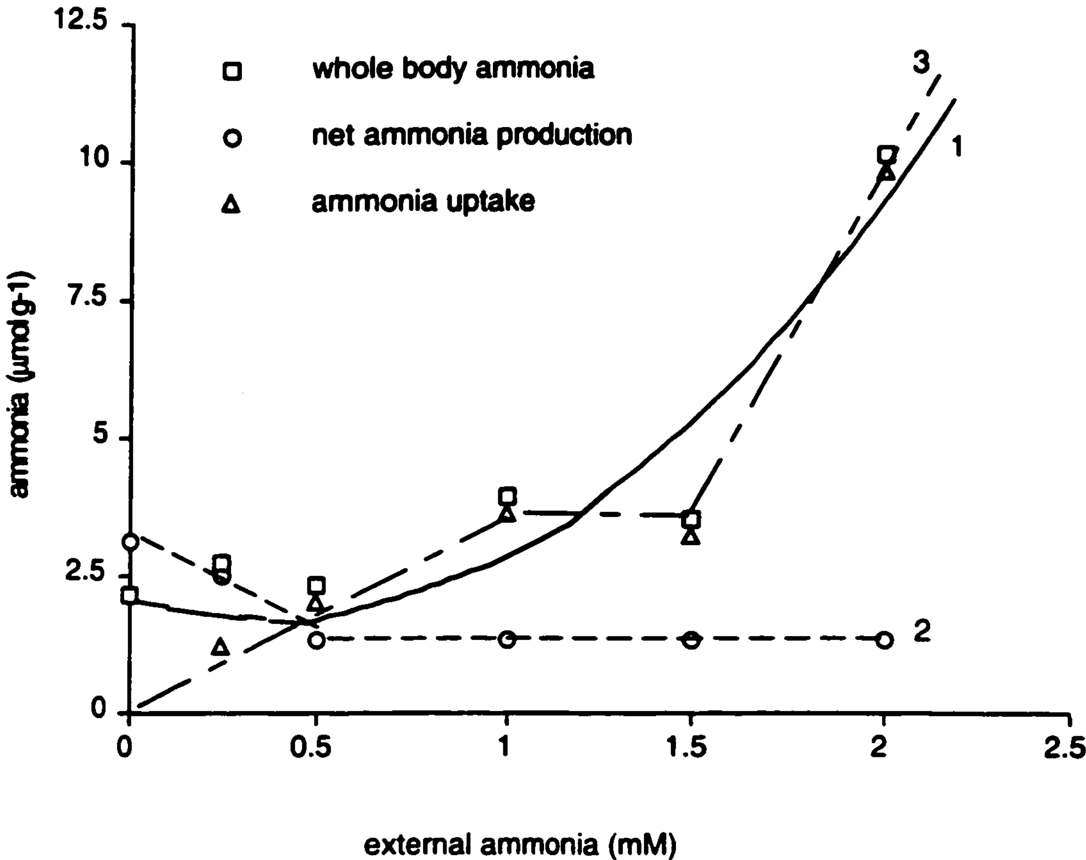
0.8
0.6
0.4
0.2
0
Tissue Urea (μmol g-1)
RB T
FHM
0
4
8
24
Time (h)



RBT
FHM
50
0
-50
-100
-150
-200
J_{net}^{Urea} (nmol g^{-1}h^{-1})
†
*
0
4
24
48
Time (h)



6
5
4
3
2
1
0
ammonia (μmol g-1)
0
0.25
0.5
0.75
1
1.25
external ammonia (mM)
3
1
2
whole body ammonia
net ammonia production
ammonia uptake



12.5
10
7.5
5
2.5
0
ammonia (µmol g-1)
whole body ammonia
net ammonia production
ammonia uptake
3
1
2
0
0.5
1
1.5
2
2.5
external ammonia (mM)

Chapter 4

A mechanistic study of the physiological basis of differences in tolerance to organic chemicals between fathead minnows and rainbow trout.

Introduction

I showed in Chapter 3 that fathead minnows (FHM) are 2- to 3-fold more tolerant to ammonia than rainbow trout (RBT) and speculated that this could have arisen from either lower uptake, greater rates of excretion or detoxification, or from greater tissue resistance. I concluded that the latter two were more likely but I was unable to rule out differences in ammonia uptake between the two species because ammonia is endogenously produced. The greater tolerance of FHM to ammonia, is, in fact, part of a general difference in tolerance to organic toxicants between the two species (Mayer and Ellersieck, 1989; Vittozzi and De Angelis, 1991).

Fathead minnows are about 2- to 4-fold more tolerant than RBT to a variety of chemicals such as o-xylene, pentachlorophenol, tetrachloroethanylene and proxpur, and up to 160-fold more tolerant to pesticides than RBT (Vittozzi and De Angelis, 1991; Ramamoorthy and Baddaloo, 1995). The physiological basis of differences in tolerance to organic toxicants between fish species has received little attention, with the exception of the pesticide literature. In these studies extremely large differences in sensitivity to pesticides between fish species were attributed to differences in brain enzyme activity. For example, the study of Johnson and Wallace (1987) showed that FHM brain acetylcholinesterase was more resistant to inhibition by maloxon than RBT acetylcholinesterase, and Jones et al. (1998) showed that FHM had less down-regulation of brain muscarinic cholinergic receptors than RBT when exposed to carbaryl or permethrin. Down-regulation of brain muscarinic cholinergic receptors occurs in pesticide exposure as a result of an increase in acetylcholine concentrations that arises from the inhibition of acetylcholinesterase (Jones et al., 1998).

Therefore, the purpose of this study was to explore the mechanistic basis for the general greater tolerance of FHM than RBT to organic non-electrolytes. The approach was to measure and compare non-electrolyte uptake, gill structure (including lamellar surface

area and mucus cell properties), clearance of two non-metabolizable compounds from the plasma, and differences in tolerance between FHM and RBT to tricaine methanesulphate and monochlorobenzene.

Differences in non-electrolyte uptake between the two species were examined with two compounds widely used in permeation studies (^{14}C urea, and ^{14}C ethanol). Urea and ethanol uptake were examined because they are a useful comparison as surrogates of hydrophilic and lipophilic substances respectively. In addition, previous studies have already shown species differences in urea uptake. McDonald et al. (1991a) showed greater urea uptake in RBT than small mouth bass (*Micropterus dolomieu*), under the circumstances where both were subject to confinement stress.

Gill structure was compared by examining the lamellar surface area, and gill mucus content in RBT and FHM. Gill surface area of RBT has been widely reported (for reviews see Hughes, 1966; Palzenberger and Pohla, 1992), but I am unaware of any studies on gill surface area in FHM. However, gill surface area in the salmonids RBT and brown trout (*Salmo trutta*) were approximately 2-fold higher than gill surface area in the cyprinid, carp (*Cyprinus carpio*; Palzenberger and Pohla, 1992). This suggests that gill surface area may differ in the two species used in this study. The thickness of the mucus layer on the gill surface is difficult to measure accurately (Shephard, 1981, 1992). Therefore, mucus on the gill surface was inferred from measures of gill mucus content which consisted of mucus cell volume and number, and gill sialic acid content. Earlier work showed that the cyprinid, common shiners (*Notropis cornutus*) had significantly more mucus cells and gill sialic acid than RBT (McDonald et al., 1991b). This suggests that there may be a difference in gill mucus content between RBT and other cyprinid species.

Elimination of compounds was compared between the two species by examining the clearance rates of two surrogates for xenobiotics, polyethylene glycol (PEG, m.w.

4000) and mannitol (m.w. 182). Polyethylene glycol is not metabolizable, although there may be some metabolization of mannitol (Munger et al., 1991). Thus, any differences between the two species in the clearance rates of either PEG or mannitol will be attributed to differences in excretion and not detoxification differences.

The remaining mechanisms, detoxification and tissue resistance were examined indirectly in a series of experiments. First, both species were exposed to a range of anaesthetic concentrations and time to stage II anaesthesia was compared. However, differences in FHM and RBT time to anaesthesia could be due to differences in uptake, detoxification, or tissue resistance. Therefore, in the next experiment, the two species were injected with a non-specifically acting organic chemical. The chemical monochlorobenzene was chosen because of the 6-fold greater tolerance of FHM to waterborne monochlorobenzene (FHM, 258 μmol L^{-1}; Pickering and Henderson, 1966; RBT, 42 μmol L^{-1}; Dalich et al., 1982). Fish were injected so that differences could be attributed to detoxification and tissue resistance rather than uptake. Finally, some tissue analysis was done. First, glutathione-S-transferase (GST) activity in crude liver homogenates of control fish was compared. Glutathione-S-transferase is an important phase II enzyme (i.e., conjugation enzymes that prepare the chemical for excretion) involved in the biotransformation of organic chemicals. A higher rate in control FHM than RBT would suggest that FHM are more prepared for detoxification than RBT. Secondly, lipid concentrations in the liver, white muscle, and whole bodies were compared. A much higher lipid concentration in FHM would suggest that they are better able to sequester and store organic chemicals (Lassiter and Hallam, 1990).

Materials and Methods

Experimental Animals

Juvenile rainbow trout (0.7 - 6 g) were obtained from Rainbow Springs Fish Hatchery in Thamesford, ON. Fathead minnows (0.7 - 5 g) were obtained from Steel City Bait in Hamilton, ON or Rainbow Springs Fish Hatchery in Thamesford, ON. All fish were held in tanks supplied with dechlorinated, Hamilton, ON municipal tap water (1 mmol L^{-1} Ca^{2+}, 0.6 mmol L^{-1} Na^{+}, 0.3 mmol L^{-1} Mg^{+1}, 0.8 mmol L^{-1} Cl^{-}) at ambient temperature for at least three weeks prior to use. Both species were fed once daily to satiation with either trout chow (RBT) or a mix of Nutrafin flakes and frozen brine shrimp (FHM).

Experimental Series

1. Ethanol and Urea Uptake

Approach

In these experiments FHM and RBT were exposed to radiolabelled markers in covered cylindrical plastic containers (8 cm high X 11 cm diameter). Each container was individually aerated with airlines made of polyethylene tubing (PE 50). The containers were filled with 250 mls dechlorinated tap water and two to three fish were added so that the biomass to fish ratio was approximately 1 g : 50 ml. After the fish had been in the containers for 30 min. the radiolabelled marker molecule was added using a stock solution. Water samples (10 ml) were taken 5 min. after the addition of the radiolabelled stock solution and at the end of the measurement period (4 h later). The water samples were then set aside until they could be analyzed for radioactivity. Fish were removed at the end of the measurement period, and euthanized with a lethal dose of anaesthesia (tricaine methanesulphate, MS-222, 0.1 g L^{-1} buffered to pH 7.0 with $NaHCO_3$). Next fish were rinsed in deionized water, blotted dry, and weighed. Fish were then freeze-clamped between two aluminum plates cooled in liquid nitrogen, and ground to a fine powder under

liquid nitrogen. Aliquots of this tissue were analyzed for radioactivity. Uptake was calculated using equation 1, and uptake coefficients were calculated using equation 2.

5A. Ethanol Uptake

Ethanol uptake in FHM (2.40 ± 0.25 g) and RBT (5.38 ± 0.52 g; N = 6 per species) was examined. A ^{14}C labeled ethanol stock solution (250 mmol L^{-1}, 3.3 mCi L^{-1}) was added to the containers to give an external ethanol concentration of 1 mM. This concentration of ethanol was approximately 0.2% of the FHM 96h LC50 for ethanol and 0.3% of the RBT 96h LC50 for ethanol (Mayer and Ellersieck, 1989). Experiments were done at 11 ± 1 °C.

5B Urea Uptake

Urea uptake in FHM (1.16 ± 0.10 g) and RBT (1.08 ± 0.10 g) was examined (N = 6 per species). A ^{14}C labeled urea solution (625 mmol L^{-1}, 0.5 mCi L^{-1}) was added to the flux containers to give an external urea concentration of 2.5 mM. Because fish excrete urea and this could affect the specific activity of the flux, a portion of the water samples (5 ml) were frozen at -20 °C until they could be analyzed for urea concentration. Experiments were done at 15 ± 1 °C.

2. Gill Structure

Gill surface Area

The gill surface area of the two species was examined using a protocol modified from McDonald et al. (1991b). Both species were removed from hardwater holding (N = 5 per species) and euthanized with a blow to the head. Next the gill basket was removed from FHM (3.38 ± 0.48 g) and RBT (2.03 ± 0.11 g) and fixed in neutral buffered formalin. One side of the four right arches of each fish were used. First, the arches for each fish were traced using a stereoscope (Carl Zeiss Jena, Technival with appropriate drawing tube attached) and the number of filaments and heights of filaments were recorded. Next, arch one and three of every fish were traced at higher power and the length of each

filament and the number of lamellae on each filament was recorded. These were compared and it was determined that the heights and number of lamellae did not differ significantly between arch one and three so arches two and four were not counted. The lamellae on filaments from arch one and three were then cut from the arch. An average of 6 filaments from each arch were traced at a higher power and the length of each lamellae was measured. It was necessary to use light microscopy to determine the widths of each lamellae and distance between lamellae for these fish because the gills were too small to accurately trace these measurements using the stereoscope. The heights of the lamellae were also compared to the tracings. Gill tissue from each fish was prepared for light microscopy using the methods outlined in the gill mucus cell section. Lamellar surface area was calculated using equation 3 in the calculations section.

Number of Gill Mucus Cells

Light microscopy was used to determine the number and size of mucus cells on the gills of the two species. Fish (N = 5 per species; 12 ± 1 °C) were removed from holding and euthanized with a blow to the head. Next, the gill basket of each FHM (1.13 ± 0.11 g) and RBT (0.84 ± 0.12 g) was removed and the first right arch was placed in neutral buffered formalin. After 24 h, these samples were washed for 2 h in tap water and then dehydrated in an ethanol series. The arches were then infiltrated with JB-4 methacrylate and embedded in JB-4 (Polysciences, Pennsylvania) according to manufacturer's directions. They were then sectioned on a Sorval Porter Blum JB-4 microtome. Next, sections were stained with periodic acid Schiff and hematoxylin according to the methods outlined in Clark (1981). Periodic acid Schiff staining was selective for mucus cells, and hematoxylin revealed the general cell structure and improved contrast. Mucus cells were counted over the full length of the filament and filaments were selected from the mid portion of the arches. Mucus cell heights and lengths were also recorded, and these were used to estimate volume.

Gill Sialic Acid Content

Sialic acid content of the gills was used as a measure of gill mucus content in FHM and RBT. Sialic acid is the principal component of epidermal mucus (Harris et. al, 1973). Fish were terminally sampled with a blow to the head and the gill basket was excised from FHM (1.39 ± 0.14 g) and RBT (2.40 ± 0.44 g; N = 3 per species). The entire gill basket was then frozen in liquid nitrogen, and ground to a fine powder under liquid nitrogen. Tissues were kept frozen at - 80°C until they could be analyzed for sialic acid concentration.

3. Plasma Clearance Rates of PEG and mannitol

The clearance rates of injected ^{14}C labeled polyethylene glycol (PEG) and ^{3}H labeled mannitol were examined in FHM (1.86 ± 0.14 g) and RBT (3.52 ± 0.22 g; N = 5 per species per treatment). Munger et al. (1991) showed that there is some binding of mannitol by the gills and the liver, suggesting that there is some metabolization of mannitol. However, the exposure period used in this study was kept shorter than that used by Munger et al. (1991; i.e., 4 h vs. 6 and 13 , respectively) in an effort to reduce any possible differences between the two species in mannitol metabolization. The second marker, PEG has been shown to be more resistant to metabolism by fish, and autoradiolysis than other similar markers such as inulin (Curtis and Wood, 1991). In this study, fish were first intraperitoneally (IP) injected with 30 μL of 0.6% NaCl that contained either 26.8 nmol PEG or 0.076 nmol mannitol and 0.1 μCi radioactivity. Fish were then placed in mesh cylindrical tubes with a PVC bottom (10 cm high X 9 cm diameter) that were set in plastic containers (8 cm high X 11 cm diameter) with 250 ml of Na^{+} free water (deionized water with 1.65 meq L^{-1} $CaCl_2$). After 5 min, the mesh tube and fish were rinsed in distilled water and placed in a second plastic container with 250 ml nominally Na^{+}-free water. Water samples (5 ml) were taken at 0, 0.5, 1, 2 and 4 h. No

attempt was made to distinguish between branchial and renal losses. After 4 h, fish were euthanized by a blow to the head and blood was collected by caudal severance. Blood samples were centrifuged for 1 min. at 13 600 G (235A microcentrifuge, Fisher). The plasma supernatant was drawn off, and the volume was recorded. These plasma samples were kept on ice until they could be processed for radioactivity analysis. Next, fish carcasses were weighed, and then freeze-clamped in liquid nitrogen cooled aluminum plates. Carcasses were then ground to a fine powder under liquid nitrogen and kept in liquid nitrogen until they could be processed for radioactivity analysis. Clearance rates for each marker were calculated according to equation 4 in the calculations section.

4. Detoxification and Tissue Resistance

Time to stage II anaesthesia

The time to stage II anaesthesia for a range of tricaine methanesulphate (MS-222) concentrations was examined in both species. Fathead minnows (1.56 ± 0.16 g) and RBT (1.15 ± 0.06 g) were exposed to 0.04, 0.08, 0.16, or 0.24 g L^{-1} MS-222 buffered to pH 7.1 with $NaHCO_3$ at 15 ± 1 °C (n = 5 per species per concentration). The time it took for each fish to reach stage II anesthesia was recorded, and then fish were removed, blotted dry, weighed and allowed to recover. The stages of anaesthesia (i.e., as described by Ryan, 1992) are shown in table 4.1.

Tolerance to injected monochlorobenzene

The tolerance of FHM (3.26 ± 0.21 g) and RBT (1.88 ± 0.15 g) to IP injected doses of monochlorobenzene was examined. Both species (n = 7 per species per dose) were injected with 98 or 196 nmol g^{-1} monochlorobenzene and the time to death was noted. The geometric mean of time to death was calculated using log transformed data, and standard error was expressed as the average of the positive and negative error.

Glutathione -S-Transferase activity

Glutathione-S-transferase activity of crude liver homogenates of RBT (1.33 ± 0.15 g) and FHM (1.75 ± 0.16 g) was examined (N = 11 per species). Fish were terminally sampled from 16 ± 1 °C hardwater holding with a blow to the head, and the livers were removed taking care not to contaminate the sample with bile. Samples were rinsed with cold homogenization buffer (50 mM HEPES, 150 mM KCl, 1 mM EDTA adjusted to pH 7.4), blotted dry, and then weighed in a tared microcentrifuge tube. Next, homogenization buffer was added and samples were stored on ice until their enzyme activity could be measured.

Lipid Concentrations

The amount of whole body, muscle, and liver lipid in FHM (2.80 ± 0.10 g) and RBT (2.58 ± 0.24 g) was compared. Fish (N = 10 per species) were terminally sampled from holding (12 ± 1 °C) with a lethal dose of anaesthesia. To measure the whole body lipid content fish were freeze clamped as described above, ground to a fine powder under liquid nitrogen, and then kept cold until lipid content could be analyzed. Liver and muscle tissues were sampled from a second set of N = 10 fish. Care was taken so that livers were not contaminated with bile and muscle was sampled from the right side of fish. Both types of tissue were immediately frozen in liquid nitrogen after removal and kept cold until they could be analyzed for lipid content.

Analytical Techniques

Radioactive Content of Fish and Water

Ethanol and urea uptake were measured by absorption of radioactivity into the fish. Mannitol and PEG clearance rates were measured by the appearance of radioactivity in the water as well as radioactivity of the plasma. Water samples were analyzed for

radioactivity by mixing with Aqueous Counting Scintillant (Amersham, Ontario) according to manufacturer's recommendations and counting them on a beta counter (LKB Wallac RackBeta Model 1217 scintillation counter). Radioactivity in the fish was measured on three approximately 100 mg aliquots frozen ground tissue per fish. Aliquots were mixed with 1 ml of soluene (Packard) and digested at 50°C for 12 h. Next Hionic-fluor (Packard) was added according to manufacturer's recommendations and samples were counted on a beta counter. For PEG and mannitol, radioactivity of the plasma was measured by mixing plasma samples with Hionic-fluor and counting them on a beta counter. There was similar quenching between the two species of both ^{14}C markers and ^{3}H markers so the effects of quenching were ignored when comparing the clearance rates of each marker between FHM and RBT. For all experiments a group of unexposed fathead minnow and rainbow trout were also processed and counted using the same approach and these values were used as background.

The specific activity (μM cpm^{-1}) for ethanol fluxes was calculated by dividing the known concentration of ethanol in the water (μmol ml^{-1}) by the radioactivity of the water (cpm ml^{-1}). Before calculating the specific activity of urea, water urea concentration (μmol ml^{-1}) was measured. Frozen water samples were freeze dried in a freeze drier (Labconco). Next water samples were reconstituted with 1 ml of water and urea concentration was measured using the colorimetric assay of Price and Harrison (1987). The average urea concentration during the measurement period was used to establish the specific activity for urea uptake in μM cpm^{-1}.

Gill Sialic Acid Content

An adaptation of the enzymatic neuramidase, NANA-adolase method of Bergmeyer (1985) was used to analyze N-acetyl neuraminic acid (NANA) in the gills of the two species. N-acetyl neuraminic acid is the principal sialic acid in fish mucus (Pickering, 1974). Aliquots of frozen powdered gill tissue (approx. 50 mg) were homogenized in a 50

mM, pH 7.5 tris(hydroxymethyl)-aminomethane (Tris) buffer. Samples were then centrifuged for 30 sec at 13 600 G (Fisher microcentrifuge 235A). The assay was done on 100 µl of supernatant. Briefly, 2.0 mls of reaction mixture (50 mM, pH 7.5 Tris, 15 µM NADH, 0.8 U ml^{-1} LDH) was added to 0.1 ml of each sample or NANA standard. Samples were then incubated at 37°C until the absorbance change became constant (i.e., 5 - 10 min). The absorbance of each sample was then read. Next, 30 µl of neuramidase (0.03 U ml^{-1}) and 30 µl of NANA-aldolase (0.3 U ml^{-1}) was added to each sample. Samples were then mixed and incubated at 37°C for 60 min. Sample absorbances were then read again. Absorbance was read on a luminescence spectrometer (LS 50, Perkin Elmer). The excitation wavelength was 340 nm, the emission wavelength was 460 nm, and the slit width was 10 nm. NANA content in the samples was calculated using the NANA standard curve.

Glutathione-S-Transferase Activity

Microcentrifuge tubes with homogenization buffer and livers were homogenized on ice with a cordless motor pestle (Kontes, New Jersey) for 30 sec. Next, samples were centrifuged at 13 600 G in a microcentrifuge for 5 min (235A, Fisher). Then the supernatant drawn off and held on ice until glutathione-S-transferase activity could be measured by the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB; Habig et al., 1974). Samples were read at 340 nm on a microplate reader (MRX, Dynatech Industries). Protein content was determined using the method of Bradford (1976).

Lipid Content

The sulphophosovanillin method of Barnes and Blackstock (1973) and Christie (1982) was used to analyze lipid concentration. Briefly aliquots (~ 50 mg) of either whole body or muscle frozen tissue were weighed out, or the livers were weighed. Next 3 mls of a chloroform : methanol solution were added and samples were stored on ice until all the

samples were ready to be assayed. After analysis, samples were read at 520 nm on a spectrophotometer.

Calculations

The uptake of ethanol and urea were calculated according to the following equation:

$$Jin = (Q * SA) / (M * t) \quad (1)$$

where "Q" is the radioactivity in the fish in cpm, "SA" is the specific activity in µM cpm-1, "M" is the mass of the fish (g) and "t" is the time period (h).

The uptake coefficients of ethanol, and urea were calculated according to the following equation:

$$\text{Uptake Coefficient} = J_{in} / C_w \quad (2)$$

where "J_{in}" is the amount of chemical a 1g fish will take up in 1 h, and "C_w" is the concentration of the chemical in the water.

The lamellar surface area of FHM and RBT was calculated using the following equation from Hughes (1966):

$$LSA = (2 L / d') * b * l \quad (3)$$

where "L" is the filament length, "d' " is the spacing of individual lamellae along the filament, "b" is the height of lamellae, and "l" is the base width of lamellae.

Clearance rates of PEG and mannitol were calculated by plotting total cpm per container versus time and determining the slope "S" for each individual container (cpm h^{-1}). Plasma sample radioactivity "P" was expressed as cpm μl^{-1}. The rates were then calculated according to the following equation:

$$\text{Clearance rate} = (S/M) / P \quad (4)$$

to give clearance rate ($\mu l\ g^{-1}\ h^{-1}$), where "M" is the mass of the fish (g) in the container.

Statistics

All values are reported as means ± one standard error of the mean (SEM). Comparisons among species and treatments were made by analysis of variance ($p < 0.05$). Tukey's multiple range tests and student "t" tests were used to resolve differences among treatments and between species.

Results

Ethanol and Urea Uptake

Ethanol uptake was significantly lower in FHM than RBT (Table 4.2). In contrast there were no significant differences in urea uptake between the two species (Table 4.2). The uptake coefficients, which account for concentration differences, showed that ethanol uptake was significantly greater than urea uptake in both species (Table 4.3). The uptake coefficient ratios of ethanol : urea were also similar in RBT and FHM (i.e., approximately 3:1 in both species).

Gill Structure

Gill Surface Area

The proportion of gill basket weight to total body weight was not significantly different between RBT and FHM (Table 4.4). However, lamellar surface area was significantly greater in RBT than FHM. This was largely attributable to differences in filament number between the two species. Rainbow trout had 2-fold more filaments per gram than FHM, but their filaments were approximately one-quarter shorter than FHM filaments. Despite the difference in filament length between the two species, RBT total filament length per gram body weight was 1.8-fold greater than FHM. There were no significant differences between the two species in lamellae per mm filament, or lamellar height.

Estimates of gill mucus indicated that FHM had more gill mucus than RBT. The gill filaments of FHM had larger and more numerous mucus cells than RBT (Table 4.4). Moreover, FHM had 3-fold greater gill sialic acid content than RBT (Table 4.4).

Plasma Clearance Rates

The clearance rates of injected PEG and mannitol were 1.6- and 4.6-fold greater, respectively, in FHM than RBT (Table 4.5).

Detoxification and Tissue Resistance

Anaesthesia

Fathead minnows took 17-fold longer to anaesthetize than RBT at the lowest concentration. However, as the concentration was increased to the range of 0.08-0.24 g L^{-1} FHM took 3.4-fold longer to anaesthetize (Fig. 4.1). An alternative way of looking at this is that FHM required a 3-fold greater concentration than RBT to anaesthetize to stage II in approximately 55 sec. (i.e., 0.24 *vs.* 0.80 g L^{-1}, FHM *vs.* RBT respectively). Finally, in both species there was a power relationship between buffered MS-222 concentration and time to stage II anaesthesia (Fig. 4.1; r^2 0.974 and 0.941, RBT and FHM, respectively).

Injected monochlorobenzene

Fathead minnows were significantly more tolerant of injected monochlorobenzene than RBT (Table 4.6). The difference in time to mortality increased with dose. Fathead minnows lived 2-fold longer than RBT when injected with 98 nmol g^{-1} monochlorobenzene and 2.7-fold longer when injected with 196 nmol g^{-1} monochlorobenzene.

Glutathione-S-Transferase

Glutathione-S-transferase activity was significantly higher in crude liver homogenates of FHM than RBT. The glutathione-S-transferase activity of FHM was 9.79 $\pm$ 0.68 nmol CDNB min^{-1} μg $protein^{-1}$ as compared to 5.22 $\pm$0.31 nmol CDNB min^{-1} μg $protein^{-1}$ in RBT.

Lipid

Fathead minnows had 1.7-fold higher lipid concentrations in the liver than RBT (Table 4.7). However, muscle and whole body lipid concentrations were not significantly different between FHM and RBT.

Discussion

Taken together the observations from the present study suggest that the higher tolerance of organic (i.e., non-electrolyte) toxicants in FHM than RBT could arise from one or more of the following: 1) lower branchial uptake in FHM, which is consistent with differences in gill structure between the two species; 2) increased clearance rates in FHM; and 3) increased detoxification, tissue resistance, or a combination of the two in FHM as compared to RBT.

Non-Electrolyte Uptake

Ethanol uptake was approximately 2-fold greater in RBT compared to FHM (Tables 4.2 and 4.3); a difference that is consistent with the approximately 2-fold greater lamellar surface area in RBT (Table 4.4). Isaia, in his 1984 review, argues that non-electrolytes may move across the gill either by diffusion through the lipid bilayer or by diffusion through aqueous channels. The fact that the greater uptake of ethanol is consistent with increased lamellar surface area supports the view that ethanol uptake occurs by diffusion through the lipid bilayer.

Diffusion through the lipid bilayer should be influenced by the lipid solubility of a compound, which is usually expressed by the log of the octanol-water (K_{OW}) partition coefficient (i.e., the ratio of the distribution of a chemical's concentration between octanol and water at equilibrium; MacKay et al., 1991). Several studies support the view that the uptake of a chemical is related to its lipid solubility. For example, in RBT uptake efficiency increased linearly for chemicals with a log K_{OW} from 1 to 3 (McKim et al., 1985). In addition, there is a clear negative relationship between LC50 and log K_{OW} for non specifically acting chemicals (i.e., narcotics) with a log K_{OW} below 4 in aquatic species (Veith et al., 1981; Di Toro et al., 2000). This, in combination with the results of this study, suggests that any compound of similar or greater lipid solubility than ethanol (log K_{OW} -0.30; MacKay et al., 1995) should pass through the gills predominately by diffusion

through the lipid bilayer. Furthermore, the results of this study suggest that differences in the area of the gills occupied by the lipid bilayer could have an important effect on species differences in chemical uptake and therefore toxicity.

An alternate explanation for the lower ethanol uptake in FHM is that their 2- to 3-fold higher mucus content may inhibit ethanol uptake (Table 4.4). This could occur by mucus increasing the thickness of the diffusion barrier (Shephard, 1992), mucus creating a gel matrix that slowed or trapped ethanol (Bansil, 1995), and/or by continual ethanol and mucus removal as it was sloughed off the gill surface (Mallat, 1985). However, it is difficult to conceive how increased mucus content could significantly decrease ethanol uptake in FHM as compared to RBT but not decrease urea uptake (Table 4.2). This suggests that either there are no differences in the thickness of the gill mucus layer between the two species, or that mucus has little or no effect on the uptake of small non-electrolytes.

Given all of the above it is not clear why there is no difference in urea uptake between the two species (Table 4.2). One possible explanation is that urea uptake at the gill is channel-mediated rather than by diffusion through the lipid bilayer. Recent studies have shown that there is facilitated bi-directional urea transport in the gills of gulf toadfish (*Opsanus beta*; Wood et al., 1998; McDonald et al., 2000). It is possible that this also occurs in other species such as RBT and FHM. In addition channel-mediated diffusion for urea is supported by the fact that the difference between ethanol and urea uptake (i.e., 3-fold greater ethanol based upon uptake coefficients, Table 5.3) is smaller than that predicted by the difference between the K_{OW} of ethanol and urea (16-fold difference, MacKay et al., 1995).

Plasma Clearance Rates of Model Non-Electrolytes

The significantly greater clearance rates of non-metabolizable non-electrolytes (PEG and mannitol) in FHM than RBT (Table 4.5), clearly suggest that FHM can eliminate xenobiotics more readily than RBT. Both mannitol (McDonald and Rogano, 1986) and

PEG (Curtis and Wood, 1991) are excreted by the gills and in the urine. In the case of mannitol most of the clearance is urinary in RBT, at least at rest (63%, G. McDonald unpublished results and McDonald and Rogano, 1986) but when stressed by epinephrine infusion mannitol clearance increases preferentially at the gills to 84% of the total. The authors attributed the latter to an increase in permeability of the paracellular junctions. At least 80% of PEG clearance is thought to occur in the urine of RBT at rest (Curtis and Wood, 1991). The effects of epinephrine on the distribution of branchial and renal PEG clearance in freshwater fish are, as far as I am aware, unknown.

In this study, IP injection of PEG or mannitol probably caused a stress response (i.e., epinephrine release) in both species. This is based upon the following two findings. First in chapter 2, IP injection of saline was shown to increase net Na^+ losses in FHM and RBT. These types of losses have been associated with increased epinephrine in previous studies (Gonzalez and McDonald, 1992; McDonald and Milligan, 1996). In these studies the authors attributed the increase in net Na^+ losses to an increase in the permeability of the paracellular junctions. Secondly, the combined branchial and urinary mannitol clearance rates in epinephrine infused RBT of 36 μl g-1 h-1 (McDonald and Rogano, 1986; McDonald, unpublished results) was similar to the RBT mannitol clearance rates reported in this study (Table 4.5).

If both species were stressed then the higher clearance rates of mannitol in FHM may be attributable to differences in branchial clearance. Specifically, they may be due to a greater leak through the paracellular pathways of the gill in FHM. This view is supported by the higher net Na^+ losses in stressed FHM than stressed RBT (chapter 2), which I attributed to differences in paracellular losses. This explanation is probably also true for the higher PEG clearance rates of FHM. It is reasonable to assume based upon the shift in mannitol clearance from largely urinary to largely branchial with epinephrine release that a greater proportion PEG clearance than 20% would occur via the gills under the

circumstances of this study and therefore differences in paracellular leak could contribute to the higher PEG clearance rates in FHM (McDonald and Rogano, 1986; Curtis and Wood, 1991). However, the possibility of more effective renal clearance cannot be eliminated.

Detoxification and Tissue Resistance

Anaesthesia

The time to stage II anaesthesia with MS-222 was 3- to 17-fold longer in FHM than RBT (Fig 4.1). These differences, particularly the 17-fold difference at lower concentrations, are unlikely attributable to a single explanation. A combination of two possible explanations for the longer anaesthetization time of FHM will be described. First, it is likely that FHM take up less MS-222 than RBT. MS-222 is approximately 50-times more lipid soluble than ethanol (Erickson and McKim, 1990; MacKay et al., 1995). This suggests that, like ethanol, MS-222 uptake is greater in RBT than FHM (see Table 4.2 for ethanol uptake). Secondly, the threshold concentration for anaesthetic effects may be higher in FHM than RBT. This view is supported by the study of Hunn (1970) that showed that the brain MS-222 concentration associated with stage II in RBT was approximately 100 mg kg^{-1} but was 200 mg kg^{-1} in catfish (*Ictalurus melas*).

Monochlorobenzene

In the circumstances of IP injection with the same doses of monochlorobenzene, the survivorship of FHM was 2- to 3-fold longer than RBT (Table 4.6). Differences in branchial uptake cannot contribute to differences in the tolerance of injected monochlorobenzene. However, detoxification and tissue resistance may be the source of the greater resistance of FHM. Detoxification of monochlorobenzene in the liver is believed to occur by phase I and phase II enzymes including glutathione-S-transferase, eventually forming mercapturic acid (Dalich et al., 1985). In this study, I showed that control glutathione-S-transferase activity was higher in FHM than RBT. This suggests that differences in detoxification may have an important role in tolerance differences between

the two species. In addition, differences in detoxification between the two species are probably more important than differences in excretion of monochlorobenzene. This is because the high lipid solubility of monochlorbenzene (log K_{ow} 3.3; MacKay et al., 1995) suggests that detoxification (i.e., biotransformation) would have to occur before monochlorobenzene could be excreted by aqueous channels such as paracellular pathways in the gill. Furthermore, gill excretion of monochlorobenzene that was not biotransformed (i.e., excreted via diffusion through the cell membrane and not excreted by aqueous paracellular pathways) should be higher in RBT than FHM because of the larger lamellar surface area in RBT available for lipid diffusion.

Finally, differences in tissue resistance to monochlorobenzene between the two species, like those suggested for MS-222, may also be important. For example, the study of Sijm et al. (1993) showed that FHM had approximately 2-fold higher lethal body burdens of 1, 2 dichlorobenzene than guppies (*Poecilia reticulata*). However, in the Sijm et al. (1993) study there were no significant differences in the acute toxicity of 1,2 dichlorobenzene between the two studies (i.e., 96h LC50 of approximately 40 mg L^{-1} for both species). It is possible that differences in the lethal body burden would be more pronounced for species with a difference in acute toxicity such as FHM and RBT.

Detoxification and Resistance Mechanisms

The biotransformation of chemicals is particularly important for lipophilic chemicals. These types of reactions tend to make chemicals more water soluble and therefore, more easily excreted by the kidney, bile, or the gill. The reactions are divided into phase I and phase II reactions. Phase I reactions are performed by enzymes such as the monooxygenases (i.e., P450 system) and include oxidation, reduction and hydrolysis. Phase II reactions involve the conjugation of the altered chemical with endogenous substrates such as glutathione, sulfate, or glucuronide and are performed by enzymes such as glutathione-s-transferase (Heath, 1997). Fathead minnows had approximately 2-fold

greater glutathione-S-transferase activity than RBT under control conditions. It is possible that this greater activity might contribute to the higher tolerance of FHM to organic toxicants. In fact, the study of Ankely and Agosin (1987) showed that channel catfish had approximately 3-fold higher liver glutathione-S-transferase rates than bluegills (*Lepomis macrochirus*) and they postulated that this difference might explain the general greater tolerance of catfish than bluegills to toxicants. A caveat to this hypothesis is that these enzyme activity differences would only matter if the toxicant in question was biotransformed by glutathione-S-transferase, and induction rates and glutathione availability also differed between two species. However, it does suggest that detoxification differences play a role in the general greater tolerance of FHM than RBT to organic non-electrolyte toxicants.

Lastly, the higher liver lipid concentrations in FHM may help to increase tissue resistance to organic toxicants because lipid soluble compounds could be sequestered in these fats (Table 4.7). This view is supported by the model developed by Lassiter and Hallam (1990) that predicted that fish with higher lipid content would survive longer upon toxicant exposure than lean fish. However, more recently the study by Van Wezel et al. (1995) was unable to show a relationship between survival time and fat content in FHM, although they did show a positive relationship between lethal body burden and fat content for several chlorobenzenes.

Summary

Fathead minnows showed physiological traits that could consistently explain greater tolerance to organic non-electrolytes than RBT in each area investigated in this study. For example, FHM had lower non-electrolyte uptake, greater xenobiotic clearance rates, greater detoxification enzyme activity and higher tissue resistance than RBT. Thus, this study emphasizes the need to consider multiple origins of greater tolerance between species.

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Table 4.1 : Description of the stages of anaesthesia taken from Ryan (1992).

Stage	General Response
0	Normal
I	Sedation
II	Loss of Equilibrium
III	Loss of Locomotion
IV	Loss of Ventilatory Movements

Table 4.2 : Uptake rates of radiolabelled ethanol (1 mM external concentration; N = 6 per species) and urea (2.5 mM external concentration; N = 6 per species) for FHM (ethanol, 2.4 ± 0.3 g; urea 1.2 ± 0.1 g) and RBT (ethanol, 5.4 ± 0.5 g; urea 1.1 ± 0.1 g).

Species	Non Electroyte Uptake (nmol g^{-1} h^{-1})	
	Urea	Ethanol
rainbow trout	93.4 ± 2.9	159.9 ± 21.5
fathead minnow	77.5 ± 6.3	97.3 ± 5.6†

Values are means ± SEM. Significant differences between species are indicated by a dagger ($p<0.05$).

Table 4.3: The uptake coefficients of urea, and ethanol for RBT and FHM (see methods for calculations; N = 6 per species per treatment).

Species	Non Electroyte	
	Urea	Ethanol
rainbow trout	0.037 ± 0.001*	0.160 ± 0.021
fathead minnow	0.031 ± 0.003*	0.097 ± 0.006†

Values are means ± SEM. Significant differences within species are indicated by an asterisk, and significant differences between species are indicated by a dagger ($p<0.05$).

Table 4.4: Gill dimensions in FHM and RBT.

	Species	
	Rainbow trout	Fathead minnow
weight (g)	2.02 ± 0.11	3.37 ± 0.98
Gill basket weight (mg g^{-1})	70.8 ± 7.2	72.0 ± 5.3
Lamellar Surface Area (cm^2 g^{-1})	2.46 ± 0.24	1.13 ± 0.14†
Filaments per gram	152 ± 12	71 ± 12†
Total filament length (mm g^{-1})	246 ± 21	139 ± 20†
Ave. filament length (mm)	1.51 ± 0.03	1.95 ± 0.08†
Lamellae per millimeter	22 ± 1	27 ± 2
Ave. Lamellar height (µm)	77 ± 1	78 ± 4
Ave. Lamellar width (µm)	17.7 ± 0.4	12.3 ± 0.3
Mucus cells per filament (m.c. mm^{-1})	50 ±7	78 ± 7†
volume of mucus cells (mm^3)	238 ± 58	525 ± 38†
sialic acid content (µM NANA g^{-1} wet gill)	4.29 ± 2.02	15.26 ± 0.81†

Values are means ± SEM (N = 5 for all measurements except sialic acid content where N = 3). Significant differences between species are indicated by a dagger ($p<0.05$).

Table 4.5: The clearance rates of intraperitoneally injected radiolabelled paracellular markers, PEG (m.w. 4000), and mannitol (m.w. 182) in FHM (1.86 ± 0.14 g) and RBT (3.52 ± 0.22 g; N = 5 per species per marker). Both species were injected with either 26.8 nmol PEG, or 0.0759 nmol mannitol. Loss rates were measured over a 4 h period in Na^+-free water in both species.

Species	Loss rates ($\mu l\ g^{-1}\ h^{-1}$)	
	PEG	Mannitol
rainbow trout	36.1 ± 5.1	31.9 ± 13.1
fathead minnow	$59.8 \pm 2.4^{\dagger}$	$147.3 \pm 28.6^{\dagger}$

Values are means ± SEM. Significant differences between species (same marker) are indicated by a dagger ($p<0.05$).

Table 4.6: Estimated time to 50% mortality of RBT (1.88 ± 0.15 g) and FHM (3.26 ± 0.21 g) intraperitoneally injected with 89 or 178 nmol g^{-1} monochlorobenzene (N = 7 per species per dose). Experiments were done at 15 ± 1°C and there was 100% mortality.

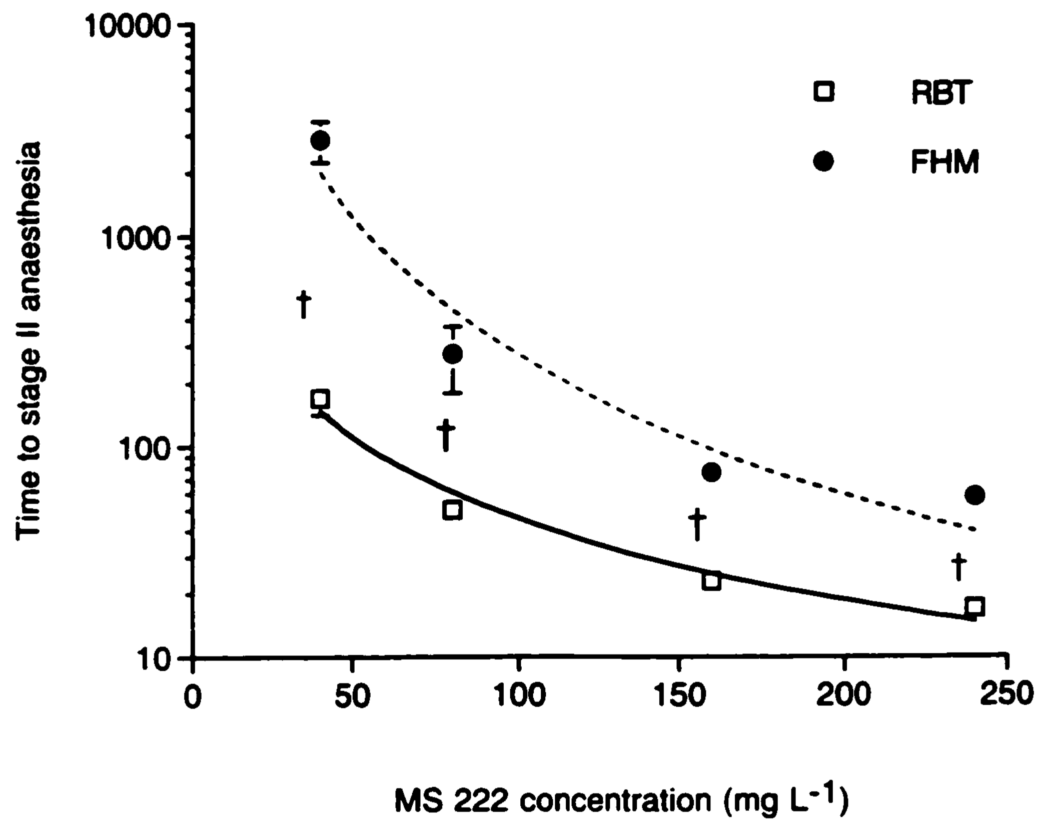
Species	Time to death (hours)	
	98 nmol g^{-1}	196 nmol g^{-1}
Rainbow trout	2.36 ± 0.68	1.53 ± 0.43
Fathead minnow	5.07 ± 0.71†	4.07 ± 0.14†

Values are means ± SEM. Significant differences between species are indicated by a dagger ($p < 0.05$).

Table 4.7 Lipid content in whole body, liver, and muscle tissues of RBT and FHM (N = 10 for whole body and livers, N = 5 for muscle tissues per species).

Species	Lipid Levels (mg lipid g^{-1} tissue)		
	liver	muscle	whole body
Rainbow trout	21.94 ± 2.95†	11.72 ± 1.18	24.18 ± 2.48
Fathead minnow	37.71 ± 2.95	10.50 ± 0.92	24.48 ± 2.48

Values are means ± SEM . Significant differences in lipid content of tissues between species is indicated by a dagger ($p < 0.05$).


10000
1000
100
10
Time to stage II anaesthesia
RBT
FHM
†
0
50
100
150
200
250
MS 222 concentration (mg L-1)

Chapter 5

Assessing the use of sprint tests to measure swimming performance in fathead minnow (*Pimephales promelas*): A comparison with rainbow trout (*Oncorhynchus mykiss*)

Introduction

The assessment of chronic effects of toxicants usually relies on such endpoints as survival, reproduction, and growth. However, measurement of the latter two endpoints can take anywhere from days to months. As a result, a number of studies (reviewed by Beitinger and McCauley, 1990; and Hammer, 1995) have used swimming as a much faster (e.g., minutes - hours) measure of the effects of toxicants. Moreover, swimming performance has been shown to be at least as sensitive an indicator of effects of sub-lethal toxicant exposure as growth (i.e., Waiwood and Beamish, 1978).

The most common method used to assess swim performance is the swim speed or critical velocity (Ucrit). To determine Ucrit, swimming velocity is increased in increments of 0.5 to 1 body length s^{-1} (BL s^{-1}) at intervals set between 5 and 60 min. until the fish fatigues. The Ucrit is the penultimate velocity plus the time weighted average at the final velocity (Brett, 1964; Hammer 1995). The Ucrit measurement can take between 1 and 6 hours to complete (depending on the interval duration and velocity increment selected). However, there is an alternative swim performance test that is much more rapid to perform, the sprint test. In the protocol for the sprint test developed by McDonald et al. (1998) on stream dwelling salmonid species, groups of fish are quickly accelerated to a fixed velocity and the time to fatigue is measured. This protocol typically takes less than 20 min. to complete and the rank order of individual performance in the test is reproducible. Moreover, studies using earlier variations of the sprint test have shown that sprint performance is sensitive to physiological stressors such as air exposure, and electroshock stress (Mitton and McDonald, 1994) as well as temperature (Bernatchez and Dodson, 1985). Thus, sprint tests may be equally as useful as Ucrit for the evaluation of sub-lethal effects of toxicants.

However, the sprint protocol of McDonald et al. (1998) was developed and tested only on salmonid species. In salmonid species (i.e., rainbow trout (RBT)) continuous swimming or high speed bursts can arguably be considered natural events. Therefore, it would be useful to establish the applicability of this test on non-current dwelling species. More specifically, it would be useful to establish the applicability of the sprint test for fish species whose life history and habitat preferences suggest they would not normally swim for an extended duration or swim at high velocities. At the same time the comparison requires a fish species that will swim when a current is imposed. Fathead minnows (FHM) met these requirements. They prefer slow-moving water (Scott and Crossman, 1973), and several studies have shown that they will swim when a current is imposed (MacLeod and Smith, 1966; Kolok and Oris, 1995; Kolok et. al, 1998). In this study the sprint performance of FHM was examined and compared to the better-characterized swim performance of RBT. The purpose of this study was to examine factors that might affect the interpretation of sprint performance in the two species. The factors examined included scaling, velocity, fuel use, repeat sprint trials, reproducibility of individual performance, and the acute and seasonal effects of temperature change.

This study also assessed the effect of ammonia on sprint performance. Here, ammonia was used as a reference toxicant for the purpose of evaluating the effects of toxicants on sprint performance. This choice was based upon Beaumont et al. (1995), which showed a correlation between decreased Ucrit and increased plasma ammonia concentration in brown trout (*Salmo trutta*).

Thus, this study addressed the following specific questions: What are the behavioural and physiological responses of FHM to sprint tests? What velocity should be used? How does performance correlate with body size? What is FHM anaerobic energy expenditure relative to that of RBT? Do FHM recover as quickly from sprint tests as RBT?

What are the effects of temperature on sprint performance? Is sprint performance affected by exposure to ammonia in FHM and RBT?

Materials and Methods

Experimental Animals

Rainbow trout (0.5 - 3.0 g) were obtained from Rainbow Springs Hatchery in Thamesford, ON. Fathead minnows (0.5 - 4.5 g) were obtained either from Rainbow Springs Hatchery or from Aquatic Biosystems in Boulder, CO. All fish were held in 50 L circular tanks supplied either with flow-through, aerated, dechlorinated Hamilton tap water at ambient temperature (seasonal variation of 6 - 20 °C; RBT and FHM from Rainbow Springs) or with partial replacement of aerated, dechlorinated Hamilton tap water maintained at 20 ± 2 °C (FHM from Aquatic Biosystems) for at least three weeks prior to use. Both species were fed approximately 1% body weight once daily with either trout chow (RBT) or a mix of Nutrafin flakes and frozen brine shrimp (FHM).

Experimental Apparatus

Fish were swum in an open, recirculating swim flume of 100 L total volume with a rectangular working section of 11 x 20 x 74 cm (W x L x H; McDonald et al., 1998). Water flow was generated by a propeller driven by a 373-W DC motor controlled by a solid state controller with internal feedback. Velocity was adjustable from zero to approximately 60 cm s^{-1} and was calibrated with a Marsh-McBirney Model 2000 electromagnetic flow meter.

Swim test protocols

1. Sprint Tests

The protocol for sprint tests, adapted from McDonald et al. (1998), was as follows: fish were transferred to the swim flume in groups of 5 - 15, and then given a 5 min. period to acclimate and orient to a low velocity (5 cm s^{-1}). Velocity was then increased over 2 min. to the final test velocity (20 to 50 cm s^{-1} depending on the specific trial) and timing of the sprint was begun. As fish tired they fell to the back screen, at which point they were encouraged to resume swimming by light prodding with a thin metal rod.

If this was ineffective the time was recorded, fish were removed from the swim flume, and body lengths and weight were recorded. After 30 min. at the final velocity, the test was terminated, and non-exhausted fish were removed and weighed. In some trials fish were euthanized upon exhaustion with a lethal dose of anesthesia (0.1 g L^{-1} tricaine methanesulphate, MS222, buffered to pH 7.0 with $NaHCO_3$). The carcasses were freeze-clamped between two aluminum blocks pre-cooled with liquid nitrogen and then body mass and length were recorded. White muscle samples were excised from the frozen fish, ground to a fine powder and stored at -80 °C for later analysis of lactate, ATP, phosphocreatine (PCr), and glycogen. For analysis of sprint performance the times to fatigue from each trial at a given speed were pooled. Time to fatigue was not normally distributed. Therefore, time to fatigue was log transformed and the geometric mean ± standard error of mean (SEM) was reported.

2. Ucrit Tests

The protocol for the Ucrit tests was adapted from Brett (1964). Fish were allowed a 10 min. acclimation period in the tunnel at 10 cm s^{-1}, before increasing the tunnel velocity by 5 cm s^{-1} (which corresponded to approximately 1 BL s^{-1} in both species) at intervals of either 10 min. or 30 min. until all fish were exhausted. Ucrit was calculated according to the following equation from Brett (1964):

$$\text{Ucrit} = V_p + ((t_f / t_i) + V_i) \qquad (1)$$

where "V_i" is the velocity increment (cm s^{-1}), "V_p" is the penultimate velocity at which the fish swam before fatigue, "t_f" is the elapsed time from the velocity increase to fatigue, and "t_i" is the time between velocity increments. In the 30 min. interval trials, fish were terminally anaesthetized and muscle tissue was taken for further analysis using the protocol described above.

Experimental Series

1. The effect of test velocity on sprint performance

Rainbow trout (N = 180) were sprinted at one of nine different velocities (22.5 to 45 cm s^{-1}) and FHM (N = 146) were sprinted at one of seven different velocities (20 to 50 cm s^{-1}). These tests were done in the winter when the ambient water temperature was 6 ± 1 °C (approximately 9 °C below optimum for RBT: Scott & Crossman, 1973; approximately 19 °C below optimum for FHM: Denny, 1987). Because of the difference in optima, the holding temperature of FHM was increased at a rate that did not exceed 2 °C per day to 14 °C, and then held at 14 ± 1°C for at two weeks. After this adjustment period, the FHM sprint trials were done at the new holding temperature (i.e., each species was approximately 10 °C below optimal values).

2. Seasonal Variations in Sprint Performance

The effect of seasonal variations in temperature on sprint performance was examined in both species. Fathead minnows (N = 164) were sprinted at 35 cm s^{-1} at 11, 14, 18, 19, or 22 °C and RBT (N = 101) were sprinted at 30 cm s^{-1} at 6, 9, 12, or 18 °C.

3. Ucrit

Differences in Ucrit and the effect of interval duration on Ucrit within and between species was examined. In the first test a 10 min. interval (N = 10 per species; 11 ± 1 °C) was used. In the second test, a 30 min. interval (N = 30 and N = 20 for FHM and RBT respectively, 14 ± 1 °C) was used. Ucrit was calculated for each species using equation 1.

4. Reproducibility of Individual Sprint Performance

The reproducibility of individual FHM rank order in repeated sprints was examined. Individual FHM were identified by weight. Fathead minnows (N = 30; 2.85 ± 0.11 g, 6.12 ± 0.08 cm) were sprinted at 35 cm s^{-1} five times with a 30. min. recovery period between trials.

5. Recovery from Sprints

The recovery time between repeated sprint trials was examined in FHM and RBT. All fish were sprinted at 35 cm s^{-1} at T = 0 and then sprinted again at either 24, 48 or 72 h (N = 15 or 20 per interval). Rainbow trout were held and tested at 9 °C and FHM at 18 °C. The reproducibility of an individual's rank order between the initial and subsequent sprint tests was assessed in a subset of FHM and RBT. Individual FHM and RBT were identified by body size. Only a small number of fish could be clearly recognized by body size so the subsets consisted of N = 8 RBT and N = 6 FHM.

6. Acute Effect of Temperature

The effect of a 9 °C increase in temperature on sprint performance in FHM and RBT was examined. Control temperatures were 9 °C for RBT and 19 °C for FHM, and test speed was 35 cm s^{-1} for both species. FHM and RBT were first tested at their respective control temperatures (19 °C and 9 °C, FHM and RBT respectively; FHM N = 60; RBT N = 45). Holding temperature was increased 9 ± 1 °C over a 24 h period to 18 °C in RBT and 28 °C in FHM (N = 20 per species). At 24 h, naive fish (i.e., not sprinted at T= 0) from each species were sprinted and time to fatigue was compared to time to fatigue at their respective control temperatures.

7. Anaerobic Energy expenditure

Anaerobic energy expenditure of FHM and RBT was compared after a sprint test (N = 14 for RBT; N = 15 FHM, both done at 9 °C) , Ucrit test (N = 20 for RBT; N = 30 FHM, done at 14 °C) and a forced swim (N = 10 for each species; done at 9 °C). The forced swim was carried out as follows: fish were forced to swim by chasing them in a half filled circular holding tank (38 cm diameter) through continuous manual stimulation for eight minutes after which they were terminally sampled. This method has been shown to produce maximum exertion in salmonid species (McDonald et al., 1998). For the sprint

test, and forced swim test N = 10 control fish (9 °C) were sampled from each species and for the Ucrit tests N = 9 (14 °C) control fish were sampled from each species. Resting concentrations of lactate, ATP and PCr were determined on these control fish. Anaerobic energy expenditure was calculated using equation 2.

8. Effect of Ammonia on Sprint Performance

The effect of sub-lethal ammonia exposure at pH 7.8 on sprint performance in both species was examined. Exposures were done statically in aerated glass aquaria maintained at either 16 or 22 °C for RBT and FHM, respectively. Fish were sprint tested after 4 h of ammonia exposure as were fish held and tested under identical conditions but without ammonia (i.e., $< 30\ \mu M$). Rainbow trout (N = 20 per treatment; mean weight 1.74 ± 0.08 g, mean length 5.8 ± 0.08 cm) were exposed to 1 mM and FHM (N = 20 per treatment; 0.62 ± 0.12 g, 3.8 ± 0.1 cm) were exposed to 1 and 1.5 mM total ammonia. These concentrations are equivalent to 63% of the 96-h LC50 for RBT, and to 33% and 52% of the 96-h LC50 for FHM (Thurston et al., 1983, Thurston and Russo, 1983). Ammonia was not present in the swim tunnel during the sprint test.

Analytical Methods

Tissue Analysis for Anaerobic Energy Expenditure

Frozen ground white muscle samples were measured into aliquots of approximately 100 mg. Sample aliquots were then analyzed for lactate, PCr, and ATP. In control fish, glycogen was also analyzed. For analysis of lactate, PCr, and ATP, sample aliquots were homogenized with 1 ml of 8% perchloric acid (PCA) and decanted into microcentrifuge tubes. Samples were then centrifuged on a microcentrifuge (235 A, Fisher) at 13 600 G for 1 min. Enzymatic analysis of the supernatant was carried out based upon the procedures in Bergmeyer (1983). For glycogen analysis, sample aliquots were digested according to the methods of Hassid and Abraham (1957). Glucose was enzymatically analyzed according to the procedures in Bergmeyer (1983).

The anaerobic energy expenditure (AEE) in ATP equivalents was calculated, from an adaptation of the equation described by Pearson et al. (1990), with the following formula:

$$AEE = ((\Delta \text{ lactate}) \times 1.5) + \Delta \text{ ATP} + \Delta \text{ PCr} \quad (2)$$

where "Δ" represents the difference between resting and exhausted animals, the factor 1.5 is used to represent the amount of ATP produced per molecule of lactate, and 1 PCr = 1 ATP. Additional energy during anaerobic metabolism is derived from the hydrolysis of ADP to AMP. However, this additional energy was ignored because it makes only a small contribution to the total AEE (less than 10 % of AEE; Pearson et al., 1990).

Statistics

Means ± one standard error of the mean (SEM) are reported throughout. Comparisons among species and treatments were made by analysis of variance ($p < 0.05$). Tukey's multiple range test was used to resolve differences between species and treatments. The reproducibility of an individual's rank order between sprint trials was assessed using Spearman's rank correlation coefficient (r_s).

Results

Body Weight and Length

Juvenile RBT and adult FHM used in this study were within the same approximate size range (0.5 to 5 g). Nonetheless, there was a slight difference in scaling of weight (W) to length (L) between the two species ($W = L^{3.03}$ for RBT and $W = L^{3.45}$ for FHM, N = 399 and 369, respectively). However, most of the swimming comparisons were done on fish in the length range of 3.5 to 6 cm where the length-weight relationship was virtually identical (Fig. 5.1).

Behaviour

Although FHM were similar to RBT in weight and length, they responded quite differently to the imposition of current in the swim flume. When the initial low velocity current was imposed (approximately 1 BL s^{-1}), RBT quickly oriented in an upstream direction. In contrast, FHM only began to orient in an upstream direction once the current was increased beyond 2 BL s^{-1}. Moreover, at the lower current velocities (i.e., 1 - 3 BL s^{-1}) FHM exhibited more burst and coast behaviour and were more easily startled when the velocity was increased in the Ucrit trials or in the acceleration phase of a sprint test. During the mid-portion of the Ucrit test (4 - 5 BL s^{-1}), FHM appeared to be less agitated than they were at lower speeds. However, during this portion of the Ucrit test FHM exhibited more current avoidance behaviour (i.e., moving vertically in the swim flume) than RBT. At higher speeds (i.e., 5 - 7 BL s^{-1} in Ucrit trials and sprint tests) FHM exhibited more burst and coast, and current avoidance behaviour than RBT. These behaviours became more pronounced in both species immediately prior to fatigue.

Performance

Ucrit

Rainbow trout had significantly higher Ucrit than FHM in both the 10 min. interval and 30 min. interval Ucrit tests. For the 10 min. interval test, the Ucrit was 5 cm s^{-1} higher in RBT than FHM. However, because FHM were 1.2-fold longer than RBT, their 10 min. interval Ucrit were 2 BL s^{-1} lower than RBT (6.0 vs. 7.9 BL s^{-1}; Table 5.1). In 30 min. interval Ucrit test, RBT had a higher absolute speed but a relative speed similar to FHM (48 *vs.* 33 cm s^{-1}; 6.9 *vs.* 6.8 BL s^{-1}; Table 5.1).

Sprint performance

Despite differences in the 10 min. Ucrit, there were no significant differences between the two species in sprint performance (i.e., fatigue time) when the fish were sprinted at fixed velocities above 27 cm s^{-1} (Fig. 5.2). Furthermore, both species showed much more rapid onset of fatigue when rapidly accelerated in a sprint test to the final Ucrit test velocity as compared to the fatigue time at the final velocity in Ucrit tests. For example, RBT sprinted at 40 cm s^{-1} had an average fatigue time of 0.82 ± 0.11 min. after a 2 min. acceleration period (Fig. 5.2) compared to 5.11 ± 1.05 min. at the final velocity in the 10 min. interval Ucrit, after an average acceleration period of 73 ± 3 min. Similarly, FHM sprinted at 35 cm s^{-1} had an average fatigue time of 1.68 ± 0.16 min. after a 2 min acceleration period compared to 4.87 ± 0.67 min. at the final velocity in the 10 min. interval Ucrit trial, after an average acceleration period of 69 ± 5 min.

Both species took less time to fatigue when the velocity was increased (Fig. 5.2). There was a trend of longer fatigue times in RBT than FHM at velocities below 27 cm s^{-1}; however, in both species, most fish did not fatigue at a sprinting velocity of 20 cm s^{-1}.

Scaling of Swim Performance

For RBT, approximately 50% of the variation in Ucrit and 30% the variation in sprint performance could be explained by variation in body length using the model: fatigue

time = a L^b where "L" represents the length of the fish in cm (Table 5.2 and 5.3). In contrast, FHM swim performance was independent of body size for both Ucrit and sprint tests (Table 5.2 and 5.3).

Repeated Sprints

The rank order of individual fatigue time in repeated sprint tests was reproducible in both species when assessed by the Spearman rank coefficient (r_s). The rank order of FHM fatigue time was significantly reproducible both in sprint tests repeated 5 times in one day on the same fish (Table 5.4) and between an initial sprint and one done 24 h later ($N = 6$; $r_s = 0.952$, $p < 0.02$). The rank order of individual RBT fatigue times was also significant between an initial sprint and one done 24 h later ($N = 8$; $r_s = 0.915$, $p < 0.005$). However, there was a significant difference in performance recovery time between FHM and RBT when the sprint interval was 24 h long. Time to fatigue in FHM declined by 70% between an initial sprint and one done 24 h later, but there were no significant differences between initial and subsequent sprints in RBT (Fig. 5.3). In contrast, there were no significant differences in fatigue time among the 5 sprint tests done on FHM in the same day (Table 5.4).

Effects of Temperature on Sprint Performance

An acute increase in temperature led to an improvement in sprint performance in both species (Table 5.5). In RBT, there was a 2.3-fold increase in fatigue time when temperature was increased by 9 °C over a 24 h period (9 to 18 °C). In FHM there was a 3.4-fold increase in fatigue time from 19 to 28 °C (Table 5.5). However, there were no seasonal temperature effects on sprint performance in either species. A temperature range of 11 to 22 °C had no impact on fatigue time in FHM (Fig. 5.4). Similarly, there was no significant effect of seasonal temperature (range- 6 to 19 °C) on fatigue time in RBT (Fig. 5.4).

Muscle Glycolytic Fuel Utilization

There were differences in the concentration of fuels in resting FHM and RBT (Table 5.6). Fathead minnow had significantly higher concentrations of glycogen (approximately 1.4 times greater), and mean resting phosphocreatine (approximately 1.4 times greater) than RBT. In contrast, RBT had higher mean resting ATP (approximately 1.4 times greater) than FHM.

Anaerobic Energy Expenditure

There were no significant differences in anaerobic energy expenditure between FHM and RBT both after a forced swim and after a sprint test (Table 5.7). However, anaerobic energy expenditure of RBT in the sprint test was significantly lower than anaerobic energy expenditure in the forced swim. In contrast, there was no significant difference between anaerobic energy expenditure in the sprint test and the forced swim in FHM. Furthermore, FHM had significantly higher anaerobic energy expenditure in the Ucrit test than RBT. In the Ucrit test anaerobic energy expenditure was 57% of the forced swim anaerobic energy expenditure in FHM, whereas RBT had negligible anaerobic energy expenditure in the Ucrit test.

Effects of Ammonia on Sprint Performance

Four hours of exposure to high ammonia concentrations negatively affected sprint performance in both species (Table 5.8). There was also no difference in the sensitivity of sprint performance to ammonia in FHM and RBT. Rainbow trout showed a 68% decline in fatigue time after 4 h exposure to 1 mM ammonia, compared to their paired controls. Fathead minnow showed a 73% and 52% decline in fatigue time after 4 h exposure to 1 mM and 1.5 mM ammonia respectively, when compared to their paired controls.

Discussion

This study is the first to directly compare FHM and RBT sprint performance. Despite the fact that FHM do not frequently swim at high velocity there were several similarities in fixed velocity sprint performance between FHM and RBT. These were as follows: 1) the rank order of individual sprint performance was reproducible in both species (Table 5.4); 2) time to fatigue (fatigue time) increased when sprint velocity was decreased in both species (Fig. 5.2); 3) a longer acceleration period also increased fatigue time in FHM and RBT (Table 5.1 and Fig 5.2); 4) there were effects of acute temperature changes but no effect of seasonal temperature changes on sprint performance in FHM and RBT(Table 5.5 and Fig. 5.4); and 5) acute ammonia exposure caused significant decreases in sprint performance in both species (Table 5.8).

These results suggest that fixed velocity sprint tests can be used in both FHM and RBT. The reproducibility of rank order of individual performance suggests that fatigue is based upon physiological and behavioral characteristics of an individual fish in both species. If individual performance was not reproducible then sprint performance could be interpreted as being based upon a random response of an individual (Bennett, 1987). Moreover, the similar responses of FHM and RBT sprint performance to increased velocity and acceleration period suggest that there are some similar physiological and behavioural factors underlying fatigue in the two species.

The effects of acute temperature change and acute ammonia exposure on sprint performance in both species suggest that fixed velocity sprint tests are good candidates for evaluating the effects of toxicants in both FHM and RBT. Furthermore, the results of this study and the study of Beaumont et al. (1995) suggest that sprint testing may be at least as sensitive as Ucrit for evaluating the effects of toxicants. Beaumont et al. (1995) found that an increase in plasma ammonia levels from approximately 100 to 700 μmol L^{-1} caused a decrease in Ucrit in adult brown trout. This is likely comparable to the plasma ammonia

concentrations in both species in this study. The study of Wilson et al. (1994) showed that 4 h exposure to 1 mM ammonia increased plasma ammonia concentrations in adult RBT to 765 μmol L^{-1}. Although fish were not swum in ammonia in this study, at the completion of the sprint test they had typically been in low external ammonia for less than 10 min. This time period is probably not long enough for fish to excrete all of the elevated plasma ammonia (Croke, unpublished results). Finally, in this study ammonia exposure caused sprint performance to decline more than the decrease in Ucrit reported by Beaumont et. al. (1995; sprint performance was approximately 65% lower than controls in both species in this study *vs.* 26% lower Ucrit in brown trout).

There were also four important differences in the response of FHM and RBT to sprint testing. These were as follows: 1) the behaviour of FHM in the swim flume was more erratic than RBT; 2) fathead minnows had greater anaerobic exertion in Ucrit tests than RBT (Table 5.7); 3) fathead minnows took longer to recover between sprints done on subsequent days than RBT (Fig. 5.3); and 4) sprint performance correlated with body size in RBT but not in FHM (Table 5.3).

The differences in sprint performance between FHM and RBT do not mean that FHM cannot be used in fixed velocity sprint tests. However, the differences showed that the effect of fixed velocity sprint tests on FHM and RBT was not the same. Fathead minnows relied more on anaerobic energy to fuel swimming than RBT. For example, the Ucrit test in RBT was largely aerobic, whereas it had a significant anaerobic component in FHM (Table 5.7). This difference in anaerobic energy expenditure agrees with the more erratic swimming behaviour of FHM than RBT because turning behaviours are more energetically costly than maintaining position in current (Brett, 1995). In addition, the difference in anaerobic exertion between FHM and RBT may be a characteristic of cyprinid *vs.* salmonid comparisons. Jones' (1981) review compared the literature on goldfish (*Carassius auratus*; Smit, 1971) and salmon (Brett, 1964) Ucrit performance. He

highlighted that goldfish used anaerobic energy sources at swimming speeds that represented 50% of Ucrit whereas salmonid species did not use anaerobic energy sources until swimming speeds exceeded 70 to 80% of Ucrit (Jones, 1981). Like the differences in anaerobic energy expenditure reported here between FHM and RBT, this would result in a larger anaerobic energy expenditure in goldfish than in salmon at Ucrit.

Whether or not FHM had greater anaerobic exertion in the sprint test is not as clear. The two species had similar anaerobic energy expenditure in the sprint tests and forced swims. McDonald et al. (1998) showed that anaerobic energy expenditure in a chased swim test represented the maximal anaerobic energy expenditure (i.e., the realized capacity) in salmonid species. If this is also true of FHM, the realized capacity of anaerobic energy expenditure is not significantly different between FHM and RBT. However, RBT had significantly greater anaerobic energy expenditure in the forced swim than the sprint test, but FHM did not. This suggests that FHM had greater anaerobic exertion in sprint tests (i.e., as a portion of their realized capacity) than RBT.

Another difference between FHM and RBT was that FHM took longer to recover their performance after a fixed velocity sprint test (Fig. 5.3). The simplest explanation for this is that FHM took longer to recover metabolites than RBT. However, sprint performance in FHM declined significantly only after an extended recovery period (i.e., > 24 h). With short term recovery (i.e., 30 min.) there was no significant decrease in FHM performance (Table 5.4). This suggests that immediate metabolite recovery is not the source of longer recovery periods in FHM. An alternative possible explanation is that part of the post-sprint response is impeding performance in FHM but not in RBT. Exhaustive exercise has been shown to cause a stress response (i.e., catecholamine and cortisol release) in salmonid species (Pagnotta et. al, 1994; Eros and Milligan, 1996). Catecholamine release occurs immediately, but cortisol release takes 1-2 h to develop after exhaustive exercise. Moreover, cortisol release has been shown to impede metabolite

recovery after exercise in salmonids (Pagnotta et. al, 1994; Eros and Milligan, 1996). Perhaps cortisol (i.e., greater release or more pronounced and prolonged effect) is the cause of longer recovery periods in FHM. However, the 5 repeated trials took approximately 3 h and 40 min to complete. Perhaps repeated sprint trials delay the release of cortisol, or the cortisol effect takes longer to develop in FHM.

Indirect evidence suggests that FHM were more stressed than RBT after sprint tests. In this study and other experiments, FHM that were repeatedly sprint tested over a series of days were more likely to develop pathogenic infections such as tail rot or fungus than RBT (unpublished results). Elevated cortisol concentrations have been correlated with immunosuppression in fish (Pickering and Pottinger, 1989; Barton and Iwama, 1991). In addition, swimming may be more stressful for other cyprinids than for salmonid species. Davison and Goldspink (1978) showed that continuous swimming at low speeds (28 days, 1.5, 3.0 BL s^{-1}) had a negative effect on growth in goldfish. In contrast, growth was promoted in brown trout swum continuously at low speeds (28 days, 1.5, 3.0 BL s^{-1}; Davison and Goldspink, 1977). Moreover, Postlethwaite and McDonald (1995) showed that prolonged swimming at low speeds reduced cortisol levels in RBT. In addition, Milligan et al. (2000) showed that RBT allowed to swim continuously at low speeds after exhaustive exercise had approximately 5-fold lower cortisol levels than RBT held in still water. Furthermore, RBT repeatedly exercised with sprints (i.e., 2-3 sprints over several days) improved their sprint performance (McFarlane and McDonald, submitted). This suggests that swimming is less stressful for RBT than FHM and cyprinids.

The lack of scaling in FHM between body size and sprint performance or Ucrit confirms and expands the findings of Kolok et al. (1998). He similarly showed no relationship between body size and Ucrit in adult FHM. One possible reason for a lack of scaling in adult FHM *vs.* the presence of scaling in juvenile RBT (Tables 5.2 and 5.3), is the relative age difference between the two groups of fish. Garenc et al. (1999) found that

burst swimming speed increased as a function of size in juvenile stickleback, (*Gasterosteus aculeatus*) but no such relationship existed in adult stickleback. They attributed the difference in body size scaling between adults and juveniles to reduced muscle metabolic capacities (i.e., phosphofructokinase, creatine phosphokinase, and lactate dehydrogenase) in adult stickleback.

Summary and Recommendations

This study showed that sprint performance has the potential to be a useful tool for assessing the sub-lethal effects of toxicant exposure in both FHM and RBT. Moreover, sprint performance has the benefit of being more rapid than the more commonly used Ucrit test. However, before assessing sprint performance in a species (particularly in one that does not normally live in current), several factors should be taken into consideration. 1) It is necessary to establish the relationship between body size and performance. In this study there was no correlation between body size and performance in FHM but there was one in RBT. This is important when comparing performance between groups within a species as some species will require size correction whereas others will not. 2) It is necessary to determine what velocity should be used for the tests. An average fatigue time of 100 s will ensure the rapidity of the test (individuals will typically fatigue among 10 and 600 s), without fatigue occurring so quickly that it might obscure differences among treatment groups. In this study, a velocity of 35 cm s^{-1} was ideal for FHM. 3) It is necessary to decide what acceleration period will be used. The duration of the acceleration appears have a profound effect on fatigue time, and therefore a difference in the acceleration period could affect comparisons. 4) Acute changes in temperature should be avoided although it appears that seasonal changes in temperature will not affect performance. 5) It must be established whether sprint performance is reproducible. 6) It is recommended that the experimenter determine whether or not individuals can be used as their own controls or paired controls would be better. This study showed that the use of paired controls is necessary in FHM

because performance declined on subsequent days (Fig. 5.3). Moreover, the training effect of repeated sprints in RBT shown by McFarlane and McDonald (submitted) suggests that paired controls should also be used for RBT. A separate point is that not all toxicants will affect sprint performance. However, elevated concentrations of ammonia, the toxicant used in this study, have been shown to depolarize white muscle in brown trout (Beaumont et al., 2000). This may cause a loss of electrical excitement in these muscle fibres, thereby decreasing swimming performance (Beaumont et al., 2000). Furthermore, acute temperature change, the other environmental factor shown to influence sprint performance in this study is believed to affect swimming by influences on muscle contractility through catalytic and/or diffusive limitations (Gerlach et al., 1990; Guderley, 1990; Johnston et al., 1990). For example, acclimation to cold water causes an increase in enzymatic myosin ATPase, whereas acclimation to warm water causes a decrease (Gerlach et al., 1990). Thus, sprint performance impairment or improvement appears to be sensitive to toxicants or environmental changes that act directly at the muscle.

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Table 5.1: The maximum sustainable swimming speed of FHM and RBT after a 10 min. and 30 min. Ucrit test. The 10 min. test was done at 11°C (N = 10 for both species), while the 30 min. Ucrit test was done at 14°C (N = 30 for FHM, N = 20 for RBT).

Test	Species	
	Rainbow trout	Fathead minnow
30 min. Ucrit test (cm s^{-1})	40.0 ± 0.8†	32.6 ± 0.9
(BL s^{-1})	6.9 ± 0.1	6.8 ± 0.2
mass (g)	3.9 ± 0.3	1.4 ± 0.1
length (cm)	7.0 ± 0.2	4.8 ± 0.1
10 min. Ucrit test (cm s^{-1})	40.5 ± 2.0 †	35.5 ± 2.2
(BL s^{-1})	7.9 ± 0.3†	6.1 ± 0.4
mass (g)	1.6 ± 0.1	1.9 ± 0.1
length (cm)	5.1 ± 0.2	5.9 ± 0.2

Values are means ± SEM. Significant differences between species are indicated by a dagger.

Table 5.2: The relationship between body length and mass with Ucrit in FHM and RBT. Body size and Ucrit were fitted to the model Ucrit = a(body size)^b. The 10 min. test was done at 11°C (N = 10 for both species), while the 30 min. Ucrit test was done at 14°C (N = 30 for FHM, N = 20 for RBT).

Species	Test	Test Parameters				
		N	length range (cm)	r^2	weight range (g)	r^2
Fathead minnow	10 min. test	10	5.1 - 6.4	0.000	1.3 -2.4	0.004
	30 min. test	30	4.4 - 6.3	0.275	1.1 - 3.3	0.328
Rainbow trout	10 min. test	10	4.6 - 6.1	0.610*	1.2 - 2.6	0.560*
	30 min. test	20	5.6 - 8.5	0.466*	1.9 - 7.3	0.434*

An asterisk indicates a significant relationship between the two parameters ($p < 0.05$).

Table 5.3: The relationship between body length and time to fatigue in FHM and RBT at various velocities. Body length and time to fatigue were fitted to the model Time to fatigue = a(body size)^b.

Species	Velocity ($cm\ s^{-1}$)	N	length range (cm)	r^2
Fathead minnow	25	73	4 - 6.2	0.013
	30	16	4.5 - 5.8	0.017
	35	150	3.0 - 7.2	0.112
	40	28	3.4 - 6.5	0.147
Rainbow trout	25	13	3.6 - 5.2	0.288
	30	89	3.2 - 5.3	0.310*
	35	61	4.2 - 5.6	0.315*
	40	45	3.8 - 5.9	0.295

An asterisk indicates a significant relationship between the two parameters ($p < 0.05$).

Table 5.4: The individual reproducibility of sprint performance in FHM (N = 30; 2.85 ± 0.11g; 6.12 ± 0.08 cm). Fish were sprinted at 35 cm s^{-1} and there was a 30 min. rest between trials.

Trial	Time to fatigue (min)	Spearman Rank Coefficient
1	2.60 ± 0.39	
2	2.03 ± 0.29	0.52 < p 0.005
3	1.88 ± 0.32	0.73 < p 0.001
4	1.93 ± 0.25	0.90 < p 0.001
5	1.75 ± 0.19	0.73 < p 0.001

Values are means ± SEM.

Table 5.5 : The effect of a 9 ± 1 °C increase in temperature on time to fatigue at 35 cm s^{-1} in FHM (0.59 ± 0.02 g, 4.05 ± 0.05 cm) and RBT (1.30 ± 0.03 g, 5.0 ± 0.04 cm). The holding temperatures were 18 °C for FHM and 9 °C fro RBT, and the increased temperatures were 28 °C and 18 °C for FHM and RBT respectively.

Species	Time to Fatigue (min)	
	holding temperature	increased temperature
Fathead minnow	1.62 ± 0.25 (N = 60)	5.45 ± 1.78* (N = 20)
Rainbow trout	2.05± 0.22 (N = 45)	4.70 ± 0.96* (N = 20)

Values are means ± SEM. An asterisk indicates signifcant differences within species ($p < 0.05$).

Table 5.6: Anaerobic energy stores in the muscles of resting FHM (N = 29) and RBT (N = 28).

	Species	
	Fathead minnow	Rainbow Trout
mass (g)	1.5 ± 0.2	2.3 ± 0.1
glycogen (μmol g^{-1})	43.4 ± 4.6†	31.8 ± 2.9
ATP (μmol g^{-1})	2.7 ± 0.2†	3.8 ± 0.2
PCr (μmol g^{-1})	9.6 ± 0.4†	6.7 ± 0.4

Values are presented as means ± SEM. A dagger indicates significant differences between species ($p < 0.05$).

Table 5.7: The anaerobic energy expenditure (AEE) in FHM and RBT after a forced swim (N = 10 for both species), a sprint test at 25 cm s^{-1} (N = 14 for RBT and N = 15 for FHM), and a 30 min. Ucrit test (N = 20 for RBT, and N = 30 for FHM). The AEE were calculated using equation 2 in the methods with the data from resting fish collected prior to each test.

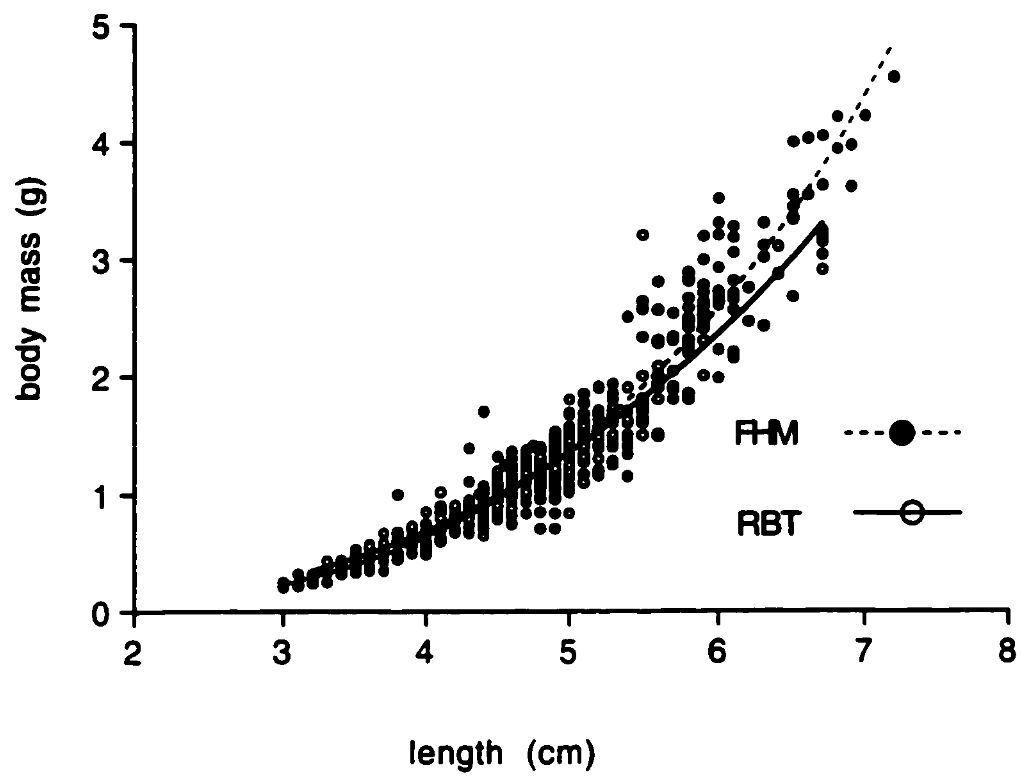
Species	AEE (μmol g^{-1})		
	forced swim	sprint	30 min. Ucrit
Fathead minnow	11.1 ± 1.4	10.1 ± 1.1	6.3 ± 0.6†*
Rainbow trout	12.8 ± 1.0*	7.9 ± 0.7*	- 1.6 ± 0.6†*

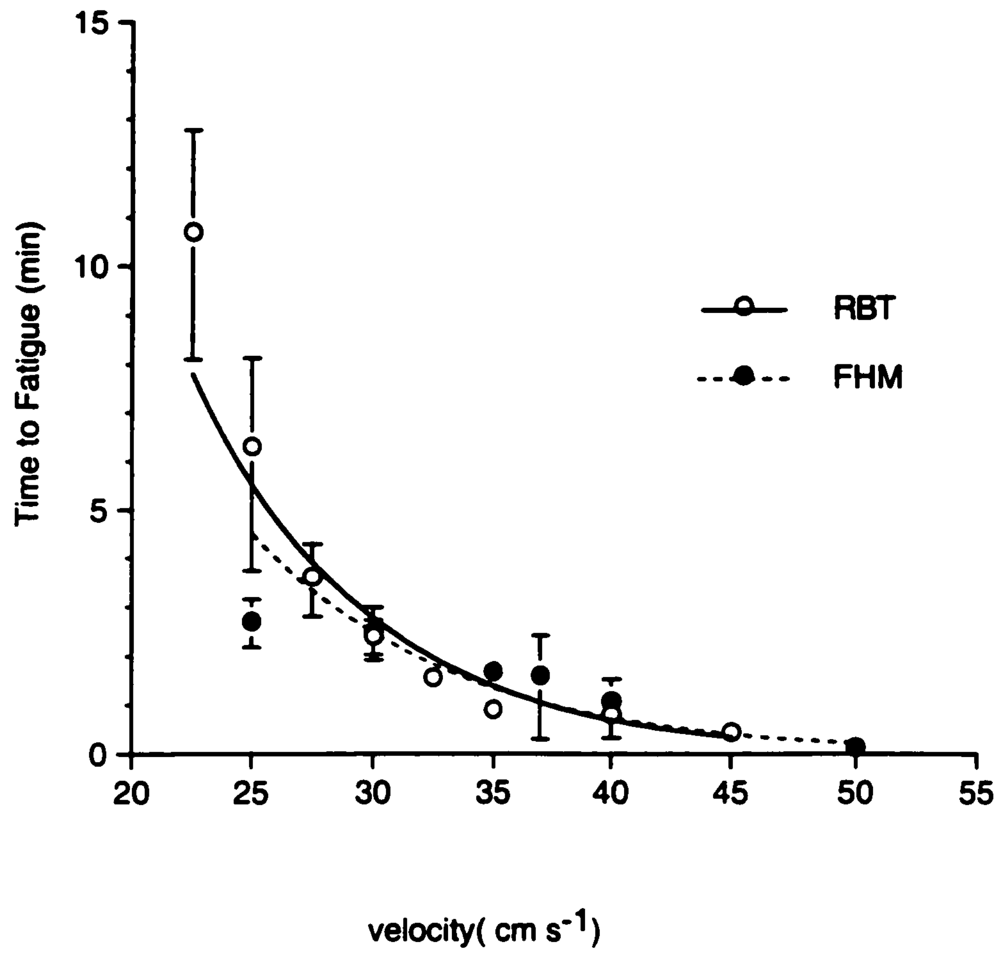
Values are means ± SEM. An asterisk indicates significant differences within species and a dagger indicates significant differences between species.

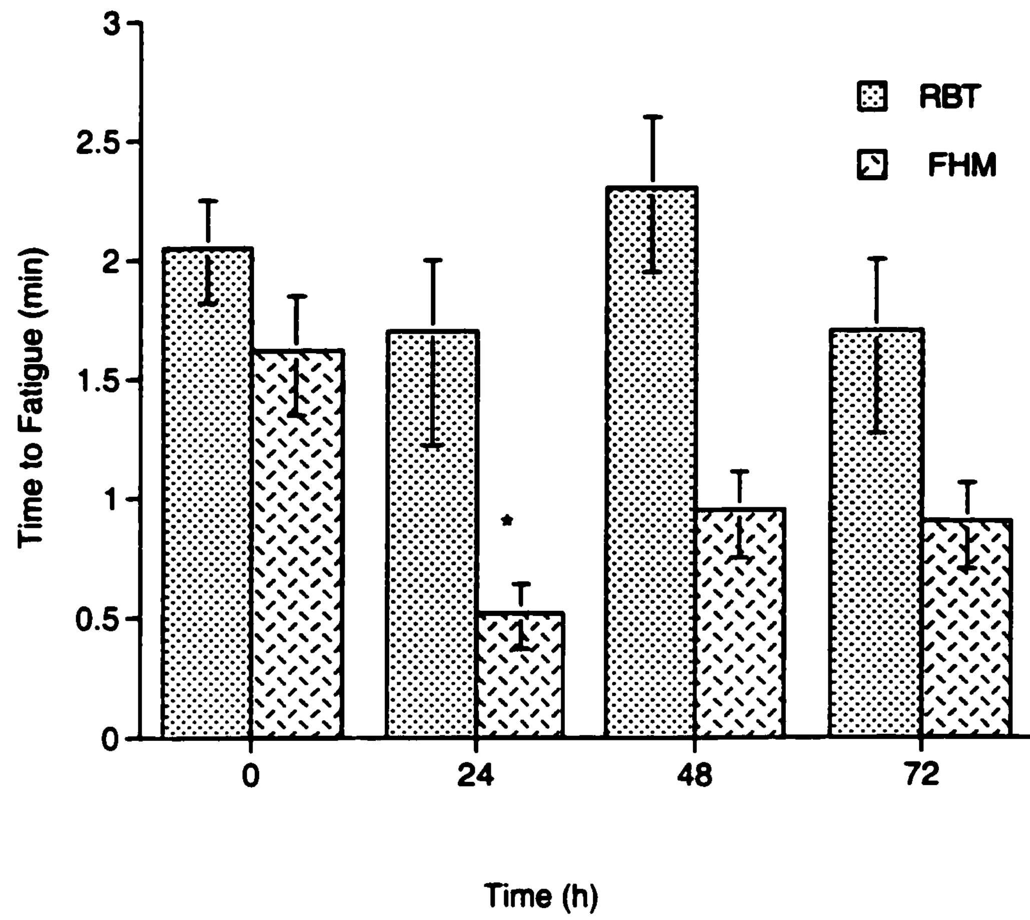
Table 5.8: The effect of 4 h 1mM and 1.5 mM ammonia exposure on sprint performance of FHM (N = 20 per treatment; 0.62 ± 0.12 g, 3.8 ± 0.1 cm) and RBT (N = 20 per treatment; 1.74 ± 0.08 g, 5.8± 0.08 cm). Both species were sprinted at 35 cm s^{-1}.

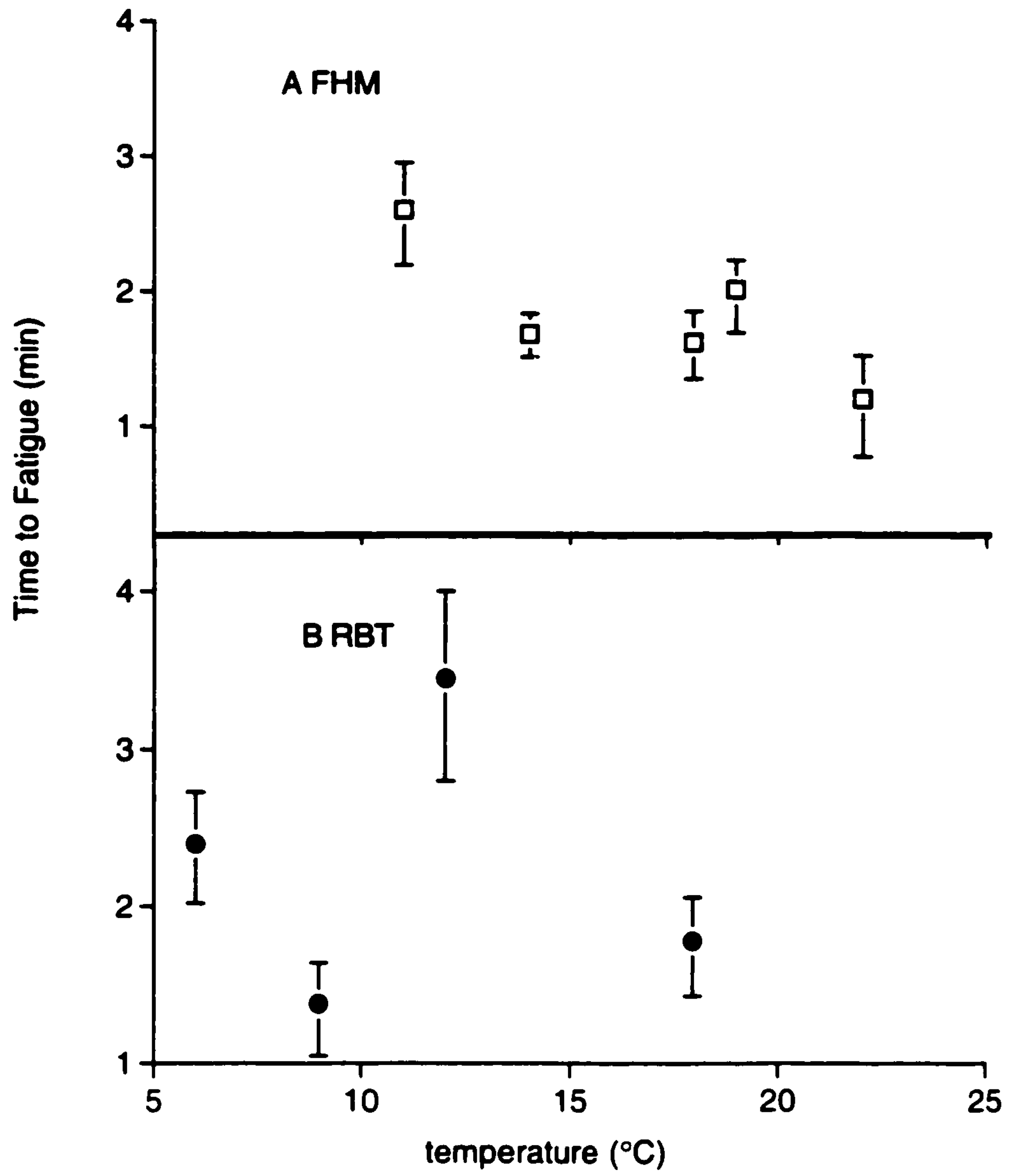
Species	Time to fatigue (min)		
	controls	1 mM ammonia	1.5 mM ammonia
Fathead minnow	1.35 ± 0.45	0.36 ± 0.12*	0.65 ± 0.19*
Rainbow trout	3.05 ± 0.71	0.98 ± 0.21*	

Values are means ± SEM. An asterisk indicates significant differences from controls within species.


body mass (g)
0
1
2
3
4
5
2
3
4
5
6
7
8
length (cm)
FHM
RBT


15
10
5
0
Time to Fatigue (min)
20
25
30
35
40
45
50
55
velocity(cm s-1)
RBT
FHM


3
2.5
2
1.5
1
0.5
0
Time to Fatigue (min)
RBT
FHM
*
0
24
48
72
Time (h)



A FHM
B RBT
Time to Fatigue (min)
temperature (°C)
4
3
2
1
4
3
2
1
5
10
15
20
25

Chapter 6

General Discussion

General Discussion

In this thesis I compared two unrelated freshwater fish species with different sensitivities to toxicants. This was done in an effort to determine any differences between the physiological systems of the two species that could provide insights into their tolerance to toxicants and the way that the effect of toxicants is assessed. The whole organism approach and wide variety of challenges examined made this thesis novel. I assumed that the mechanism of action of a toxicant was similar in the two species and that differences in sensitivity were based upon differences in the threshold concentration required for effect. Differences in threshold, I assumed, could occur either because of a higher cellular resistance in one species *vs.* the other, a greater capacity to detoxify the toxicant in one species *vs.* the other, or that the physiological system being affected was more vulnerable to the action of the toxicant in one species *vs.* the other. As expected I found some clear differences between the physiological responses of FHM and RBT. However, one of the key findings of this thesis was that there are often multiple origins of greater tolerance in one species *vs.* another so no one explanation may be sufficient.

The three main differences in physiology found between the two species were differences in gill function and morphology, cellular resistance, and detoxification. In addition, there were also important differences in the way the two species responded to the sprint test, a method of measuring swim performance. The contribution of the results of each chapter to each of these areas are summarized below.

There were clear differences between the two species in gill function and morphology. First, the following results support the view that paracellular pathways in the gills of FHM are leakier than those of RBT. Fathead minnows experienced greater changes in Na^+ balance (i.e., greater loss or gain) than RBT when external Ca^{2+} was reduced acutely (Fig. 2.6), when both species were stressed by epinephrine and/or saline intraperitoneal injection (Table 2.3, Fig. 2.8), and when both species were exposed to

increased salinity (Fig. 2.7). One factor common to each of these challenges is that each could affect the tight junctions between adjacent gill cells. For example, Ca^{2+} removal could affect stabilizing cross bonds within tight junctions (Hunn, 1985), stress (i.e., catecholamine increase) would increase the transmural and intralamellar pressure of the gills which could distort and stretch the tight junctions (McDonald and Rogano, 1986; McDonald and Milligan, 1996), and osmotic shock could stretch the tight junctions by causing cell shrinkage (Gonzalez and McDonald, 1992). In addition, FHM had greater clearance rates of intraperitoneally (IP) injected mannitol and PEG (Table 4.5). In chapter 4, I discussed how these clearance rate differences could also be linked to leakier paracellular pathways in FHM than RBT. Therefore, the greater response of FHM to each of these challenges supports the view that tight junctions in FHM are weaker and losses of electrolytes or other substances from paracellular pathways have the potential to be greater in FHM than RBT.

The second difference between gill function of FHM and RBT was that FHM did not regulate Na^+ uptake to the same extent as RBT. After confinement stress, and substantial losses of whole body Na^+ in the two species (approximately 25%), RBT significantly increased Na^+ influx, whereas there were no significant changes in Na^+ influx in FHM (Fig. 2.9). In addition, there was an effect of temperature on Na^+ influx in FHM but not RBT, even after fish had been chronically exposed to the temperature (Fig 2.2).

The third difference between gill function of FHM and RBT was that FHM had lower non-electrolyte uptake rates than RBT. For example, FHM had lower uptake rates of ethanol (Table 4.2). In addition, FHM appeared to have lower uptake of ammonia (Fig. 3.6 and 3.7) and MS-222 (Fig. 5.1). Lastly, there were some differences in the morphology of the two gills. Fathead minnows had a smaller lamellar surface area, and greater gill mucus content than RBT (Table 4.4). All of these differences in gill function could contribute to the differential sensitivity of the two species to toxicants. In the first

study (chapter 2) I linked the differences in gill ion regulation between the two species to the greater sensitivity of FHM to low pH. In addition, a lower uptake of non-electrolytes could also help to explain the greater tolerance of FHM to organic toxicants (chapter 4).

There were also differences in detoxification and cellular resistance between the two species. Under control conditions FHM had higher glutamine synthetase activity rates in the brain (Table 3.6), and higher glutathione-s-transferase activity in crude liver homogenates (see Chapter 4). Glutamine synthetase catalyzes the formation of glutamine from glutamate, ATP, and ammonia thereby reducing ammonia concentrations in affected tissues (Jeney et al., 1992). Glutathione-s-transferase is one of the biotransformation enzymes. It is involved in the conjugation of chemicals with endogenous glutathione which helps the animal excrete the chemical and often renders it less toxic (Heath, 1997). The higher rates of both of these enzymes under control conditions suggest that FHM are better prepared to detoxify toxicants upon exposure than RBT. Moreover, it seems likely that this pattern is repeated for other detoxifying enzymes such as the monooxygenases, other transferases, or other ammonia detoxification enzymes (i.e., glutamate dehydrogenase). Induction of many enzymes occurs over a period of days (Heath, 1997). For example, 48 h of exposure to elevated ammonia had no significant effect on brain glutamine synthetase activity in either species (Table 3.6). This suggests that higher rates under control conditions could play an important protective role, particularly in acute toxicity. It is worth noting that other factors such as substrate availability will affect the activity of these enzymes in the fish, and differences may be greater or smaller than those reported here. However, a second factor, greater cellular resistance, may also be important for the greater tolerance of one fish species *vs.* another to toxicants.

Fathead minnows were more tolerant of IP injected ammonia (Table 3.4) and IP injected monochlorobenzene (Table 4.6) than RBT. Because of the short time frame before death after IP injection of these toxicants (i.e., minutes to hours) I am confident that these

differences are at least partially attributable to differences in cellular resistance to toxicants. In addition, whole body ammonia concentrations associated with death were much higher in FHM than RBT (Fig. 3.1). Differences between species in the tolerated tissue concentrations of compounds such as ammonia (Walsh, 1997), anaesthetics (Hunn, 1970), and 1, 2 dichlorobenzene (Sijm et al., 1993) have been reported for a variety of species comparisons. This suggests differences in cellular resistance to potentially toxic compounds are common among species. These differences would also contribute to the greater tolerance of FHM to organic toxicants than RBT. In addition, these differences may also play a role in determining the tolerance of FHM and RBT to metals, particularly once a metal is inside the fish, and not just acting at the gill surface.

It was also determined that sprint testing could be used as a performance measure to assess the effects of toxicant exposure in both species. In both species, the rank order of individual performance was reproducible, and there was an effect of acute ammonia exposure, and acute temperature change on performance. However, sprint performance appeared to be a more stressful experience for FHM than RBT as performance declined over intervals of 24 h in FHM but not RBT (Fig. 5.3), and FHM developed infections such as tail rot. Furthermore, swim performance scaled with size in RBT but not FHM (Tables 5.2 and 5.3).

It is interesting to speculate on why FHM and RBT differ in the ways listed above. Perhaps, the differences between the ionoregulatory strategies of FHM and RBT are related to the fact that adult RBT are anadromous, whereas FHM are a strictly freshwater species. One of the striking findings was that the greater tolerance of FHM to internally active toxicants (i.e., ammonia and organic toxicants) was consistent with differences between the two species in uptake, excretion, detoxification, and cellular resistance. It is possible that FHM evolved a greater tolerance to internally acting toxicants such as ammonia and organic toxicants because the slow, still moving water that they

prefer would be more susceptible to increased ammonia levels and anoxia. Regardless, the fact that greater tolerance of a species can have multiple origins should be considered when examining differences in tolerance between species.

Finally, it is possible that the differences in physiological responses found between FHM and RBT are representative of differences between cyprinids and salmonids in general. Many of the differences found between FHM and RBT in this thesis agreed with earlier studies on cyprinid and salmonid species respectively. This suggests that the patterns reported for FHM and RBT could be extrapolated to differences in tolerance between other species, and to the assessment of the effect of toxicants on other species. The findings of this thesis have implications in the development of biomarkers to predict differences in species sensitivity to toxicants, in understanding mechanisms or action, and in explaining and further examining tolerance differences among species.

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