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The Role of Oxidative Stress in the Mechanisms of Ammonia-Induced Brain Swelling and Tolerance in the Goldfish (Carassius auratus)

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By

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ABSTRACT

Toxic build-ups of ammonia can cause potentially fatal brain swelling in mammals, but such swelling is reversible in the anoxia- and ammonia-tolerant goldfish (*Carassius auratus*). The mechanisms of ammonia-induced brain swelling and tolerance remain elusive, but several studies have suggested a role for reactive oxygen species (ROS), which may damage proteins and lipids in the plasma membrane of astrocytes in the brain. As a result, osmotic gradients across cell membranes may be altered leading to water uptake by astrocytes and swelling. While a role for ROS has been proposed in mammals, no studies have addressed this question in teleosts, in which blood ammonia concentrations can fluctuate markedly following feeding, exercise, and exposure to environmental ammonia. This study aimed to determine if exposure to high external ammonia (HEA; 5 mmol L^{-1}) induced oxidative stress in the brain and liver of goldfish. HEA exposure led to 10-fold increases in internal ammonia and oxidative stress in the liver and brain. Oxidative damage was most pronounced in the brain, in which there were 114% increases in thiobarbituric-acid reactive substances (TBARS) and 3–fold increases in protein carbonyl content after 72 h HEA in warm-acclimated (14°C) goldfish. Notably, cold-water acclimation (4°C) completely attenuated the oxidative stress response in the goldfish brain and liver. This was accompanied by a marked diminution of the brain swelling response in cold-acclimated goldfish, whereas brain water volume increased by 20% in normothermic (14°C) individuals after 72 h HEA. The present study also demonstrated an increase in the activity of key antioxidant enzymes in the brain (CAT, GPx, GR) and liver (SOD, CAT, GR) during HEA, suggesting that goldfish are able to upregulate their antioxidant capacity in response to ammonia. In conclusion, oxidative

stress appears to play a central role in the brain swelling process during acute hyperammonemia. Moreover, goldfish brains appear to have a high capacity to withstand oxidative stress in response to variations in internal ammonia. This likely explains why goldfish are more resilient to this homeostatic disturbance than mammalian brains.

CONTRIBUTIONS BY PRINCIPLE & CO-INVESTIGATORS

Dr. M.P. Wilkie and D.F.J. Lisser developed the concepts and also designed the experiments. D.F.J. Lisser performed all high external ammonia exposures of goldfish (*Carassius auratus*), water ammonia determinations, and tissue collection. D.F.J. Lisser also optimized and performed all oxidative damage and antioxidant enzyme assays. Dr. G.R. Scott financially contributed to the antioxidant enzyme analyses and advised on enzyme kinetics through collaboration with Dept. Biology, McMaster University, Hamilton, ON. Z. Lister quantified brain water volume at different temperatures in partial fulfillment of an undergraduate honours thesis with technical assistance from D.F.J. Lisser and P.Q. Pham-Ho. Brain ammonia and glutamine measurements were quantified by D.F.J. Lisser with technical assistance by P.Q. Pham-Ho. D.F.J. Lisser conducted all statistical analyses and thesis writing. Dr. M.P. Wilkie advised on all experimental, analytical, and writing components of the thesis.

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List of Abbreviations

$\Delta\Psi_m$	inner mitochondrial membrane potential
ALF	acute liver failure
AP-1	activator protein-1
ARE	antioxidant response element
ASC	ascorbate
ATP	adenosine triphosphate
BAY11-7082	(E)-3-(4-methylphenylsulfonyl)-2-propenenitrile
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
Ca^{2+}	calcium
$CaCO_3$	calcium carbonate
CAM	cell adhesion molecule
CAT	catalase
CBF	cerebral blood flow
Cl^-	chloride
CNS	central nervous system
CO_2	carbon dioxide
CsA	cyclosporine A
CYP	cytochrome P450 system
DHA	docosahexaenoic acid

DNA	deoxyribonucleic acid
DNPH	dinitrophenylhydrazine
EAAT	excitatory amino acid transporter
EDTA	ethylenediaminetetraacetic acid
Fe^{2+}	ferrous iron
GDH	glutamate dehydrogenase
GLU	glutamate
GLN	glutamine
GLNase	glutaminase
GLnT	neuronal Na^+/glutamine co-transporter
GPx	glutathione peroxidase
GR	glutathione reductase
GS	glutamine synthetase
GSH	reduced glutathione
GSSG	oxidized glutathione disulfide
GST	glutathione-*S*-transferase
H_2O_2	hydrogen peroxide
HCl	hydrochloride, hydrochloric acid
HE	hepatic encephalopathy
HEA	high external ammonia
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
J_{Amm}	ammonia excretion rate
K^+	potassium

KOH	potassium hydroxide
KPi	potassium phosphate
LPO	lipid hydroperoxide
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
MK801	dizocilpine
MPT	mitochondrial permeability transition
MSO	methionine sulfoxamine
N_2	nitrogen
Na^+	sodium
NADPH	nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	sodium bicarbonate
NF-κβ	nuclear factor kappaB
NH_3	ammonia
NH_4^+	ammonium
NH_4Cl	ammonium chloride
NH_4HCO_3	ammonium bicarbonate
NKCC	$Na^+/K^+/2Cl^-$ co-transporter
NMDA	N-methyl-*D*-aspartate
NMDAr	N-methyl-*D*-aspartate receptor
Nrf2	nuclear factor (erythroid-derived 2)-like 2
NO•	nitric oxide radical
cNOS	constitutive nitric oxide synthase

eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
NOX	NADPH oxidase
NQO1	NADPH:quinone oxidoreductase
NSCC	non-selective cation channel
$O_2\bullet^-$	superoxide anion radical
OH•	hydroxyl radical
$ONOO^-$	peroxynitrite
OUC	ornithine urea cycle
PAG	phosphate-activated glutaminase
PCA	perchloric acid
pKa	acid dissociation constant
PLA_2	phospholipase A_2
PMSF	phenylmethanesulfonyl fluoride
PTP	permeability transition pore
PUFA	polyunsaturated fatty acid
Rh	Rhesus glycoprotein
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
mRNA	messenger RNA
ROS	reactive oxygen species
RONS	reactive oxygen and nitrogen species
SDS	sodium dodecyl sulfate

SEM	standard error of the mean
SN1	astrocytic Na^+/glutamine co-transporter
SOD	superoxide dismutase
T_{Amm}	total ammonia
TBA	thiobarbituric acid
TBARS	thiobarbituric acid-reactive substances
TBI	traumatic brain injury
TCA	trichloroacetic acid
TH	therapeutic hypothermia
TK	Tukey-Kramer post-hoc test
WM	wet tissue mass
WST-1	water-soluble tetrazolium salt

Chapter 1

General Introduction

INTRODUCTION

Ammonia Toxicity

Increased internal ammonia concentrations, a condition referred to as hyperammonemia, can lead to ammonia toxicity characterized by seizures, hyperexcitability, cognitive deficits, coma, and life-threatening brain swelling (Felipo and Butterworth 2002). Brain swelling is a characteristic feature of several hyperammonemic syndromes including acute liver failure (ALF), fulminant hepatic failure, Reye's syndrome, and congenital urea cycle pathologies (Felipo and Butterworth 2002). The brain swelling observed in patients suffering from various liver insufficiencies is referred to clinically as hepatic encephalopathy (HE; Blei and Cordoba 2001). Such brain swelling is accompanied by increases in intracranial pressure, brain herniation, and is the most frequent cause of death in patients exhibiting HE (Felipo and Butterworth 2002; Norenberg et al. 2007). Currently, effective treatments for the brain swelling observed in HE are limited to emergency liver transplants (Hoofnagle et al. 1995).

The vertebrate liver is responsible for detoxifying ammonia, which inevitably arises from the breakdown of proteins and subsequent deamination of amino acids (Mommsen and Walsh 1992; Felipo and Butterworth 2002; Weihrauch et al. 2009). In mammals, ammonia is converted to urea via the ornithine-urea cycle enzymes contained in the liver, and is subsequently excreted via the urine (Felipo and Butterworth 2002). In most freshwater fishes, nitrogenous waste is excreted predominantly as ammonia directly across the gill epithelium via Rhesus (Rh) glycoproteins (Weihrauch et al. 2009; Wright and Wood 2009). Defects in the liver's ability to detoxify ammonia, owing to congenital anomalies or liver failure, can cause ammonia to accumulate in mammalian tissues. In

aquatic ecosystems, agricultural run-off containing ammonia-rich fertilizers, industrial effluent, and overcrowding can lead to high levels of environmental ammonia (Randall and Tsui 2002). Moreover, stress, prolonged periods of starvation, and routine activities such as feeding and vigorous swimming can also lead to hyperammonemia in fish tissues (Mommsen and Walsh 1992; Wang et al. 1994a; Wood 2001; Wicks and Randall 2002; Ortega et al. 2005).

In the central nervous system (CNS), ammonia permeates the blood-brain barrier by simple diffusion rather than via a saturable transport system, and this process is highly pH-dependent (Lockwood et al. 1980; Felipo and Butterworth 2002). In aqueous solution, unionized ammonia (NH_3) exists in equilibrium with the ionized ammonium form (NH_4^+). The relative ratio of these two species is a function of pH, as described by the Henderson-Hasselbalch equation:

$$\log_{10}[NH_3/NH_4^+] = pH - pKa \qquad (1)$$

Consequently, at normal physiological pH (pH 7.8 at 15°C in fish blood; Wilkie and Wood 1991), the majority of ammonia is present as NH_4^+. Unionized NH_3 permeates cell membranes more freely than the ionized form, NH_4^+. However, researchers suggest that under physiological conditions, 25% of ammonia may enter the brain as NH_4^+ (Felipo and Butterworth 2002). Since NH_4^+ has ionic properties similar to K^+, it can compete with K^+ ions and be transported across the blood-brain barrier through K^+ channels, Na^+/K^+-ATPases, and $Na^+/K^+/2\ Cl^-$ co-transporters (Ip and Chew 2010).

The molecular basis by which ammonia induces brain swelling is poorly understood, but it is generally accepted that astrocytes are the primary target. The central nervous system (CNS) of vertebrates is composed of two main classes of cells: neurons and glial cells. Astrocytes are specialized glial cells that provide structural and metabolic support for neurons. These cells are most susceptible to the neurotoxic effects of high levels of ammonia. Primary cultures of mammalian astrocytes undergo significant cell swelling when treated with ammonia (Norenberg 1988). Furthermore, astrocyte size has been shown to increase significantly in mammals following experimentally-induced HE *in vivo* (Swain et al. 1991).

It is unclear why astrocytes are prone to swelling in acute hyperammonemia, but the exclusive localization of glutamine synthetase (GS) in astrocytes may explain their particular vulnerability. Under normal conditions, astrocytes recycle excess glutamate neurotransmitter from the synaptic cleft via GS (Figure 1.1; Cooper and Plum 1987; Mommsen and Walsh 1992; Felipo and Butterworth 2002, Murray et al. 2003). Excess glutamate is taken up by nearby astrocytes via Na^+/glutamine co-transporters EAAT-1 and EAAT-2, terminating the excitatory signal (Walsh et al. 2007). Astrocytic GS catalyzes the addition of ammonia to glutamate, forming glutamine at the expense of ATP (Walsh et al. 2007). The glutamine is then transported back to the pre-synaptic neuron where it is processed into glutamate and packaged into synaptic vesicles for release.

This glutamate-glutamine cycle has traditionally been viewed as the principle means of ammonia detoxification in the brain (Cooper and Plum 1987; Ip et al. 2004; Felipo and Butterworth 2002). Some researchers, on the other hand, have challenged this

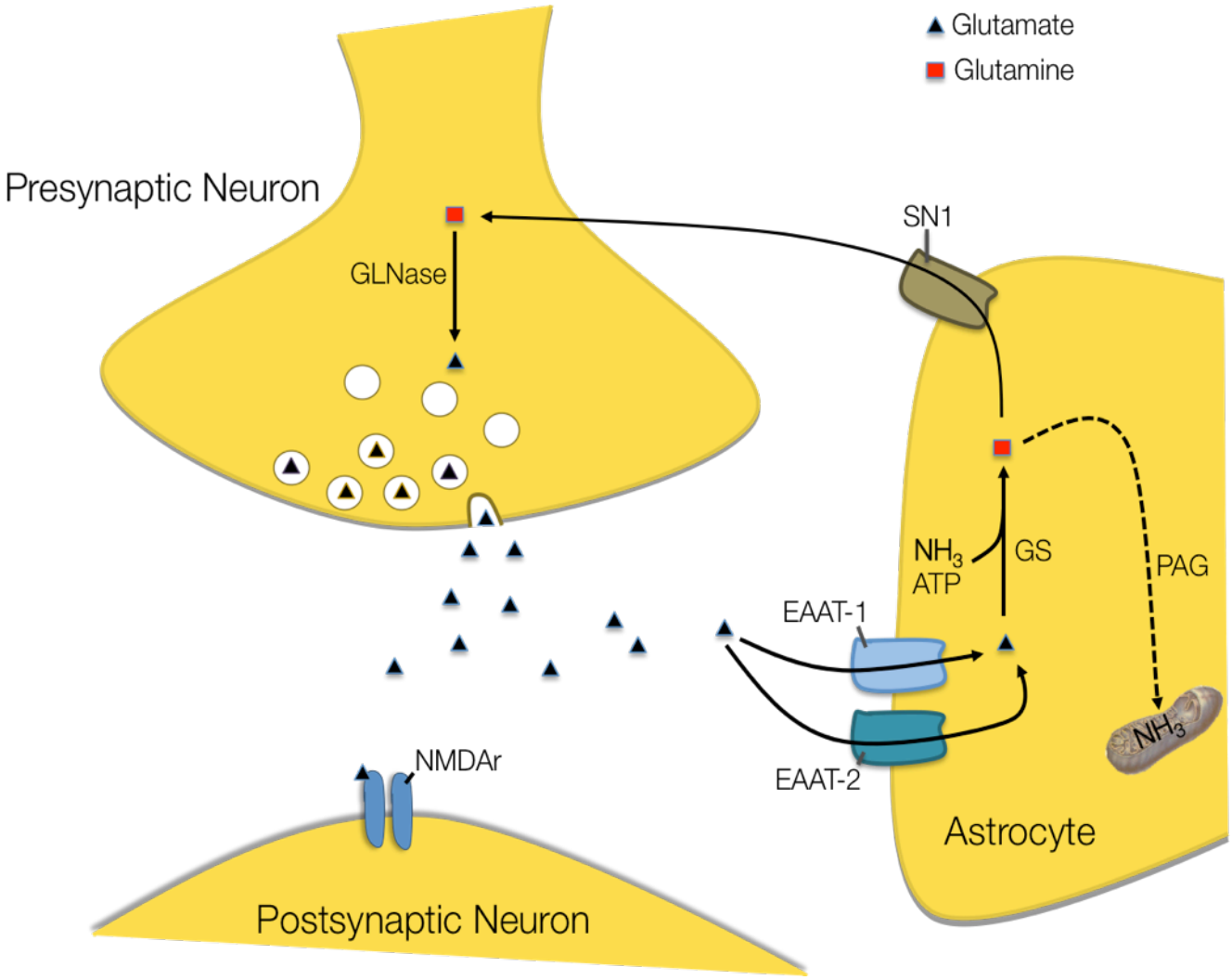
beneficial view suggesting that glutamine accumulation along with inhibition of the astrocytic glutamine transporter (SN1), results in increased intracellular osmotic pressure leading to astrocyte swelling (Brusilow 2002; Walsh et al. 2007). However, there is a lack of correlation between the extent of cell swelling and cellular levels of glutamine (Jayakumar et al. 2006b). While glutamine may not be acting as an osmolyte, it has been proposed that glutamine-mediated oxidative and nitrosative stress may be responsible for the swelling characteristics of astrocytes exposed to hyperammonemia (for a review see Albrecht and Norenberg 2006).

Oxidative and Nitrosative Stress

The mechanisms of ammonia-induced brain swelling remain unclear, but may involve the generation of reactive oxygen species (ROS), such as superoxide radicals ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hydroxyl radicals ($OH^{\bullet}$). These highly reactive molecules are generated as byproducts of oxidative metabolism. When overproduced, ROS can interact with other molecules in the cell and cause damage to lipids and proteins in the plasma membrane of astrocytes in the brain. As a result, ionic and osmotic gradients across cell membranes may be altered leading to water uptake by astrocytes and brain swelling. Oxidative/nitrosative stress is induced when cellular antioxidant defense systems can no longer counteract the elevated ROS levels and/or repair oxidative cellular damage (Dorval and Hontela 2003; Prieto et al. 2009; Zhao et al. 2009).

The potential role of ROS in ammonia-induced neurotoxicity originated from work by O'Connor and Costell (1990) who observed evidence of lipid peroxidation in mice exposed to acute hyperammonemia. Lipid and protein oxidative damage have also

Figure 1.1: Glutamate-Glutamine Cycling By Astrocytes. Glutamate is released into the synaptic cleft by excitatory presynaptic neurons where it activates *N*-methyl-*D*-aspartate receptors (NMDAr). Excess glutamate is taken up by nearby astrocytes via Na^+/glutamine co-transporters EAAT-1 and EAAT-2, terminating the excitatory signal. Within astrocytes, glutamine synthetase (GS) catalyzes the addition of ammonia (NH_3) to glutamate to form glutamine in an ATP-dependent reaction. The glutamine product is exported into the extracellular space via a Na^+/glutamine co-transporter (SN1) and taken up by the presynaptic neuron. Within the presynaptic neuron, glutamine is converted via a glutaminase (GLNase)-catalyzed reaction back to glutamate and packaged into synaptic vesicles for further release. Some researchers have suggested that newly synthesized glutamine in astrocytes may be transported in excess into mitochondria, where it is subsequently hydrolyzed back into ammonia via mitochondrial phosphate-activated glutaminases (PAG). Pathologically high levels of this glutamine-derived ammonia within the relatively small mitochondrial compartment may uncouple oxidative phosphorylation, causing the overproduction of ROS (not shown). Adapted and modified from Walsh et al. 2007.


Glutamate
Glutamine
Presynaptic Neuron
GLNase
SN1
NH3
ATP
GS
PAG
EAAT-1
EAAT-2
NH3
NMDAr
Astrocyte
Postsynaptic Neuron

been demonstrated in cultured astrocytes exposed to high levels of ammonia (Murphy et al. 1992; Reinehr et al. 2007). Kosenko et al. (1997) observed the production of superoxide directly in rat brain and liver via the superoxide-dependent oxidation of epinephrine to adrenochrome following acute ammonia exposure *in vivo*. Likewise, free radical production has also been demonstrated directly via the oxidation of 2',7'-dichlorofluorescin diacetate dye in primary cultures of mammalian astrocytes treated with ammonia *in vitro* (Murthy et al. 2001).

The oxidative and nitrosative stress resulting from acute ammonia toxicity has been shown to cause cell swelling *in vivo* (Chan et al. 1982) and in cultured mammalian astrocytes (Chan et al. 1989). Subsequent treatment with various antioxidants (superoxide dismutase, catalase, vitamin E, *N*-tert-butyl-α-phenylnirone) mitigated the ammonia-induced brain swelling response in cultured rat astrocytes (Jayakumar et al. 2006a). Thus, there is growing evidence that ammonia exposure can lead to oxidative/nitrosative stress in mammals and that this stress is capable of inducing brain swelling.

ROS may be produced in several complex, interactive ways upon exposure to high levels of ammonia, including glutamate excitotoxicity and the induction of the mitochondrial permeability transition (MPT; see Figure 1.2). Glutamate excitotoxicity is caused by the overactivation of N-methyl-*D*-aspartate receptors (NMDArs), located on the surface of neurons and astrocytes in the brain. NMDArs primarily act as calcium (Ca^{2+}) channels when activated by NMDA or glutamate (Wenthold et al. 2003). Overactivation of the NMDAr facilitates the entry of sodium (Na^{+}) and large amounts of Ca^{2+} into the post-synaptic neuron. Increased intracellular Ca^{2+} concentrations can stimulate ROS production through effects on superoxide ($O_2\bullet^-$) producing enzymes in the

mitochondrial respiratory chain (see Figure 1.3; for reviews see Kowaltowski et al. 2001; Rama Rao et al. 2003, 2005). Ca^{2+} can also promote ROS generation by activating cytosolic Ca^{2+}-dependent enzymes, such as constitutive nitric oxide synthase (cNOS), phospholipase A_2 (PLA_2), and NADPH oxidase (NOX), liberating nitric oxide (NO•) and $O_2•^-$ (Reinehr et al. 2007; Norenberg et al. 2009).

The induction of the mitochondrial permeability transition (MPT) may also contribute to ROS production and astrocyte dysfunction in acute hyperammonemia. The MPT refers to a pathological increase in the permeability of the inner mitochondrial membrane caused by the opening of a non-selective pore (permeability transition pore, PTP) in the inner mitochondrial membrane (Crompton 1999). This process is stimulated by the accumulation of excess Ca^{2+} and oxidative stress in the mitochondrial matrix (Figure 1.3; Kowaltowski et al. 2001; Norenberg et al. 2009). The opening of the PTP results in the deterioration of the inner mitochondrial membrane potential ($\Delta\Psi_m$), impaired oxidative phosphorylation, and ROS generation (Kowaltowski et al. 2001; Rama Rao et al. 2003, 2005; Norenberg et al. 2009).

The MPT may also contribute to the swelling characteristics of astrocytes to ammonia by promoting secondary oxidative stress. Firstly, the direct production of ROS by the MPT has been demonstrated (Votyakova and Reynolds 2001; Zorov et al. 2006). Secondly, the opening of the PTP may cause the release of mitochondrial Ca^{2+} into the cytosol, stimulating even further ROS production through effects on Ca^{2+}-dependent enzymes (positive feedback), thereby promoting unrelenting oxidative and nitrosative stress (Norenberg et al. 2009). Ammonia has previously been shown to induce the MPT in cultured mammalian astrocytes (Bai et al. 2001). Moreover, ammonia-induced

astrocyte swelling was mitigated in mammalian cells treated with the MPT inhibitor, cyclosporin A (CsA; Rama Rao et al. 2003).

Collectively, numerous studies in mammals indicate that ROS generation represents a key step for the initiation of various downstream events leading to ammonia-induced brain swelling, including the induction of the MPT causing further ROS production, and when severe, cell death via necrosis/apoptosis (Figure 1.1). While a role for ROS has been proposed in mammals (for reviews see Albrecht and Norenberg 2006; Norenberg et al. 2007, 2009), very few studies have addressed this question in fishes, where blood ammonia concentrations can fluctuate markedly following feeding (Wicks and Randall 2002), exposure to environmental ammonia (Eddy 2005), and/or following exercise (Wang et al. 1994a).

Therapeutic Hypothermia and Neuroprotection

The deliberate reduction of core body temperature, or therapeutic hypothermia (TH), has demonstrated to be a strong neuroprotective measure against traumatic brain injury (TBI), cardiopulmonary resuscitation, stroke, and various other neurological disorders in mammals (Busto et al. 1987; Busto et al. 1989; Meden et al. 1994; Kammersgaard et al. 2002; Polderman 2004; Gonzalez-Ibarra et al. 2011). Although poorly understood, the mechanisms of action are varied and shown to block the cascade of cerebral ischemic injury on multiple levels. The earliest rationale for the neuroprotective effects of TH focused on the slowing of cellular metabolism resulting from a drop in body temperature, diminishing the impact of substrate-limiting neural insults such as edema, hypoxia, and hypoglycemia.

Figure 1.2. Proposed signaling events in astrocytes exposed to high levels of ammonia, implicating the major role of reactive oxygen and nitrogen species (RONS) in initiating many of the downstream events of ammonia neurotoxicity. NH_4^+, ammonium ion; Ca^{2+}, intracellular calcium; cNOS, constitutive nitric oxide synthase; NOX, NADPH oxidase; PLA2, Phospholipase A_2; GS, Glutamine Synthetase; PAG, Phosphate-Activated Glutaminase; GLU, Glutamate; MPT, mitochondrial permeability transition, NMDAr, *N*-methyl-*D*-aspartate receptor. Adapted and modified from Norenberg et al. 2007.

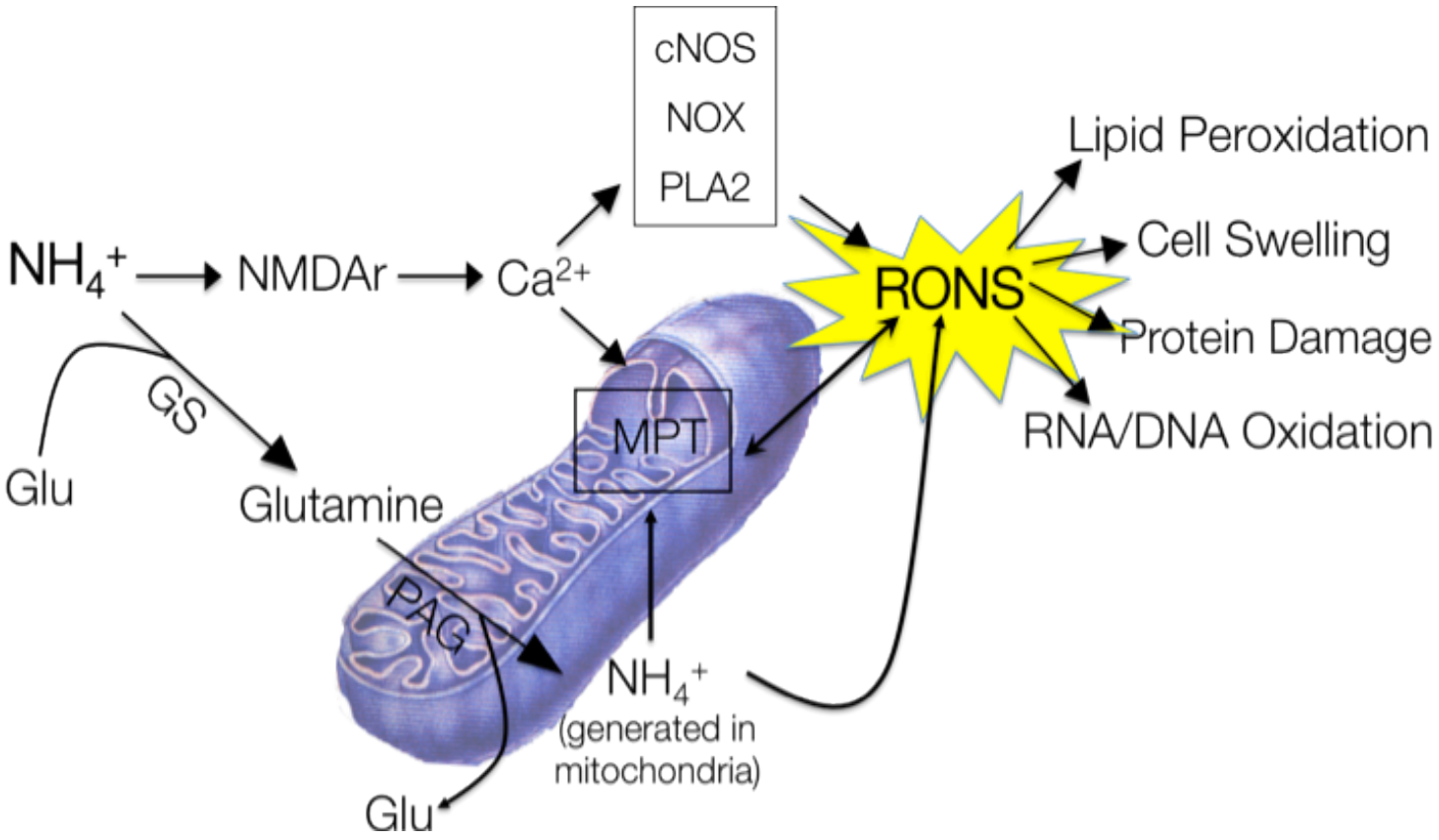
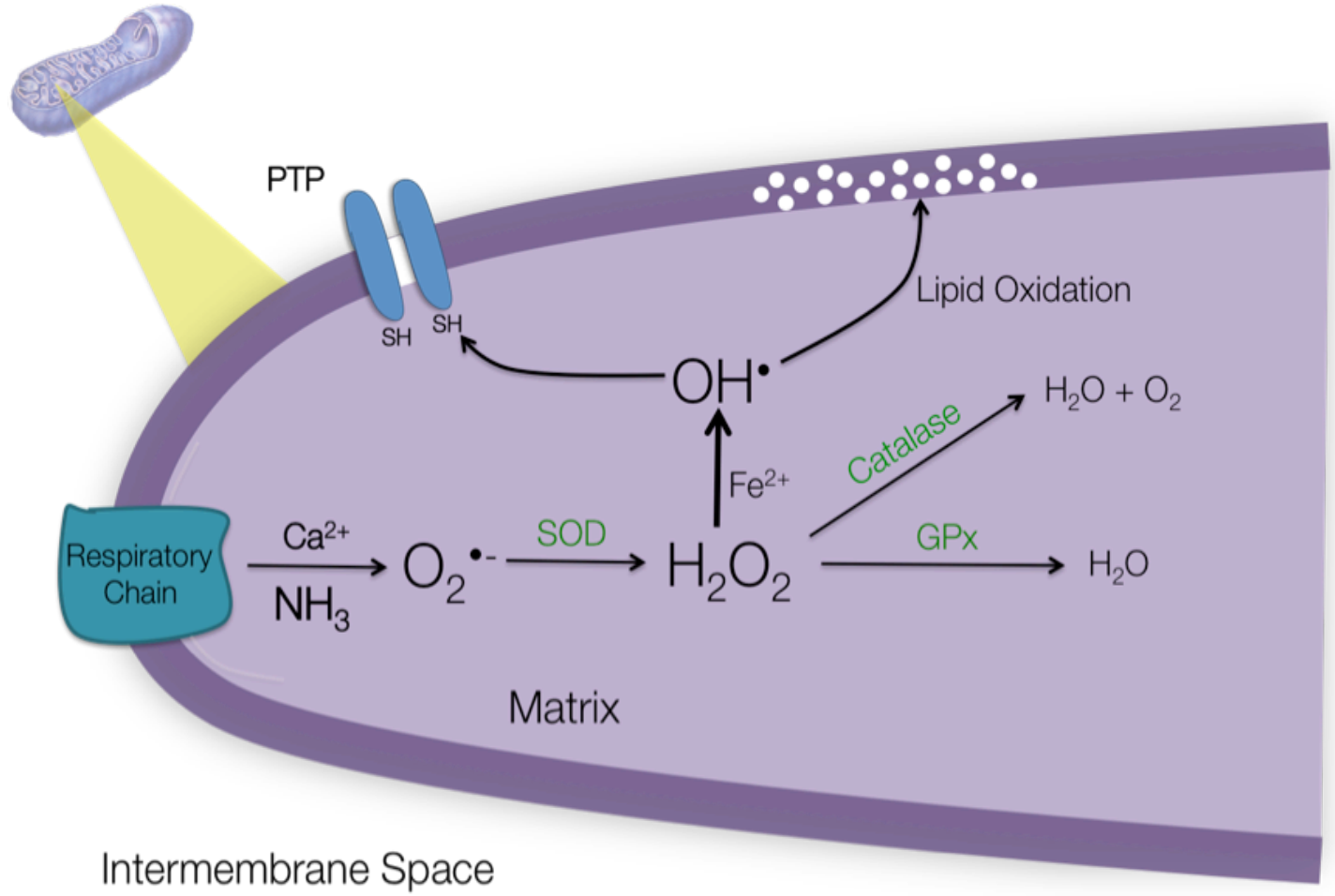

cNOS
NOX
PLA2
NH_4^+
NMDAr
Ca^{2+}
Lipid Peroxidation
Cell Swelling
RONS
Protein Damage
RNA/DNA Oxidation
GS
Glu
Glutamine
MPT
PAG
NH_4^+
(generated in
mitochondria)
Glu

Figure 1.3. The Mitochondrial Permeability Transition (MPT) Hypothesis. Small amounts of superoxide radicals are constantly produced by the mitochondrial respiratory chain. These radicals are normally neutralized by Mn-superoxide dismutase (SOD), which generates hydrogen peroxide (H_2O_2). This peroxide is reduced to water by the antioxidant enzymes, glutathione peroxidase (GPx) and catalase (CAT) in the mitochondrial matrix. However, superoxide production may increase in the presence of calcium (Ca^{2+}) and ammonia (NH_3), causing peroxide to accumulate in amounts that cannot be effectively removed by local antioxidants. If iron (Fe^{2+}) is present, the highly reactive hydroxyl radical is generated, which oxidizes thiol groups of the permeability transition pore (PTP), causing the pore to open. The MPT is thereby induced, uncoupling oxidative phosphorylation, causing secondary oxidative stress (positive feedback) and cell swelling. Reactive Oxygen Species ($O_2\bullet^-$, superoxide; H_2O_2, hydrogen peroxide; $OH\bullet$, hydroxyl radical). Adapted and modified from Kowaltowski et al. 2001.


PTP
SH
SH
Lipid Oxidation
OH•
$H_2O + O_2$
Catalase
Fe^{2+}
Respiratory Chain
Ca^{2+}
NH_3
$O_2^{\bullet-}$
SOD
H_2O_2
GPx
H_2O
Matrix
Intermembrane Space

For every one degree Celsius drop in temperature, cerebral metabolism slows by 5-6%, thereby decreasing cerebral blood flow (CBF), oxygen consumption, glucose utilization, and preserving glycogen reserves in the brain (Rosomoff and Holaday 1954; Hagerdal et al. 1975; Gonzalez-Ibarra et al. 2011). The production of ROS is also temperature-dependent, and decreased rates of ROS production have been observed in rats following hypoxia/ischemia *in vivo* (Globus et al. 1995a, b; Katz et al. 2004). More recently, TH has also been shown to attenuate oxidative stress after severe traumatic brain injury (TBI) in infants and children (Bayir et al. 2009).

TH likely also affects pathways that extend beyond a decrease in cellular metabolism, such as membrane integrity and excitotoxicity. Even modest decreases in temperature can help stabilize cell membranes, preventing the influx of unwanted ions and/or the release of excitatory neurotransmitters, such as L-glutamate (Okuda et al. 1986; Illievich et al. 1994; Ooboshi et al. 2000; Phillips et al. 2013).

Along with its known inhibitory effect on excitotoxicity, loss of neuronal membrane integrity, and oxidative stress, TH has also been shown to attenuate brain swelling in mammals. Hsu et al. (2006) demonstrated that brain cooling decreased intracranial pressure and oxidative stress in rat brain following experimentally-induced heat stroke. Moreover, hypothermia has also been shown to stabilize uncontrollable brain edema in individuals following TBI (Shiozaki et al. 1993; Gonzalez-Ibarra et al. 2011).

Given the marked similarities between the cascades of hypoxic/ischemic injury and hyperammonemia in the mammalian brain, it is not surprising that hypothermia has also been shown to diminish brain swelling and oxidative stress in mammalian models of

ammonia toxicity (Rose et al. 2000; Zwingmann et al. 2004; Jiang et al. 2009) and in ALF patients (Stravitz et al. 2008). Currently, temperature is assumed to have only minor effects on ammonia toxicity in fish (Ip et al. 2001). However, no studies have examined the neuroprotective effect of cold temperatures in teleosts, where increases in internal ammonia can be a daily challenge.

Mechanisms of Ammonia-Tolerance (*Carassius* spp.)

Many animals have evolved a number of different strategies to handle endogenous ammonia (for review see Randall and Tsui 2002; Ip and Chew 2010). Most vertebrates are capable of enzymatically-converting ammonia to less noxious compounds such as urea, glutamine, and other free amino acids (although, the role of glutamine synthesis in ammonia detoxification is currently under investigation; Albrecht and Norenberg 2006). Mudskippers (*Periophthalmodon schlosseri, Boleophthalmus boddaerti)* prevent the accumulation of endogenous ammonia by reducing the rates of protein and amino acid catabolism (Lim et al. 2001). Furthermore, many teleosts are capable of augmenting rates of ammonia excretion by upregulating Na^+/NH_4^+ exchange metabolon complexes via Rh glycoproteins located along the gill epithelium (Wright and Wood 2009). Additionally, some tropical air-breathing fish species volatize significant amounts of NH_3 gas during aerial exposure (Rozemeijer and Plaut 1993; Tsui et al. 2002; Frick and Wright 2002). While these adaptations act to limit further increases in internal ammonia following exposure to HEA, they do not address the downstream consequences of ammonia toxicity to cells. The extremely high neural tolerance to ammonia in the anoxia-tolerant goldfish (*Carassius auratus*) has been recently demonstrated (Wilkie et

al. 2011), but the cellular mechanisms underlying this adaptation have not yet been fully elucidated.

Oxygen starvation (anoxia) can have similar effects to ammonia, leading to brain damage within a matter of minutes. Moreover, the cascade of events that characterize ammonia-induced neurotoxicity closely resembles those associated with anoxic injury. A key mechanism of hypoxic injury is the overactivation of NMDArs and subsequent induction of the MPT, culminating in unrelenting oxidative/nitrosative stress. Although studies surrounding ammonia and anoxia tolerance in fish are few, there appears to be a relationship between the ability to tolerate these two neurotoxic insults (for review see Walsh et al. 2007). The goldfish (*Carassius auratus*) and crucian carp (*Carassius carassius*) are the most anoxia-tolerant fish species known (Bickler and Buck 2007; Wilkie et al. 2011). The adaptations that allow the goldfish to tolerate anoxia have been investigated extensively (for reviews see Lutz and Nilsson 2004; Bickler and Buck 2007). Their ability to (1) conserve ATP via metabolic depression, (2) exploit large reserves of glycogen in the brain, (3) convert lactate arising from anaerobic metabolism into ethanol and CO_2, and, (4) reduce NMDAr currents during anoxia, all act to prolong anoxic survival time.

On the other hand, anoxia-tolerant organisms must also express adaptations that deal with the consequences of oxygen re-introduction. Reoxygenation following anoxic episodes is associated with significant ROS production and subsequent oxidative damage to cellular components (Lushchak et al. 2001; Bickler and Buck 2007). Similar to other vertebrates, aquatic organisms have evolved numerous low-molecular mass antioxidants such as reduced glutathione (GSH) and ascorbate (ASC), and high-molecular mass

antioxidants such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione reductase (GR; Lushchak et al. 2001; Guerriero et al. 2002; Basha and Rani 2003; Lushchak 2011; Sinha et al. 2014). These metabolites and enzymes scavenge ROS, preventing oxidative damage to cellular components, such as lipids, proteins, and nucleic acids. The expression of cellular antioxidant defenses may differ considerably amongst teleosts depending on their relative resistance to stressors inducing oxidative damage (Stephensen 2002; Eyckmans et al. 2011; Sinha et al. 2014). In response to anoxia and reoxygenation, the anoxia-tolerant goldfish upregulates the activity of several antioxidant enzymes to effectively manage post-anoxic ROS generation, thereby limiting the extent of oxidative damage occurring in the brain and other tissues (Lushchak et al. 2001). Other researchers have also demonstrated similar increases in antioxidant capacity in tissues of goldfish exposed to a wide array of stressors capable of inducing ROS production (Lushchak et al. 2005; Shi et al. 2005; Matviishyn et al. 2014).

The anoxia-tolerant goldfish and crucian carp (*Carassius carassius*) are also extremely tolerant to high levels of external ammonia (Wilkie et al. 2011). Yet, exposure of goldfish to millimolar concentrations of ammonia results in significant brain swelling (Wilkie et al. 2015). While this process can cause life-threatening brain swelling in mammals and other teleosts, recent work indicates that such swelling is physiological and reversible in these fishes (Wilkie et al. 2015). Although the mechanisms remain elusive, goldfish provide an excellent model to study ammonia-induced neurotoxicity and tolerance. Unraveling the adaptive response of the goldfish brain to ammonia could

provide new insights into neuroprotective strategies for the brain swelling in HE and related neurological disorders.

Objectives and Hypotheses

The overarching goal of this thesis was to characterize the role of oxidative stress in the mechanisms of ammonia-induced neurotoxicity in goldfish. My central hypothesis was that the underlying mechanism of ammonia-induced brain swelling in teleosts involves the generation of reactive oxygen species (ROS). Due to their known tolerance to post-anoxic oxidative stress, I also predicted that the goldfish brain would exhibit a pronounced antioxidant response, which would limit ammonia-induced oxidative damage and brain swelling. Lastly, I tested the hypothesis that ammonia-induced neurotoxicity, including brain swelling and oxidative stress, would be attenuated at colder temperatures. With this background, the specific objectives of the study were to:

(1) Quantify the level of oxidative cellular damage occurring in goldfish liver and brain during acute (72 h) hyperammonemia.

(2) Characterize the antioxidant defense response to acute hyperammonemia in the goldfish.

(3) Determine the effect of temperature acclimation on oxidative stress responses and brain swelling in goldfish during acute exposure to high environmental ammonia (HEA).

Chapter 2

Oxidative Stress, Antioxidant Defenses, and Brain Swelling in Goldfish (*Carassius auratus*) During Exposure to High Environmental Ammonia

INTRODUCTION

The deamination of excess amino acids following feeding, vigorous swimming, and prolonged periods of starvation can cause ammonia to accumulate to toxic levels in the blood and tissues of fish (Mommsen and Walsh 1992; Wang et al. 1994a; Wood 2001; Wicks and Randall 2002; Ortega et al. 2005). Low millimolar (mmol L^{-1}) concentrations of ammonia can also build-up in confined, aquaculture pens and in aquatic ecosystems due to anthropogenic inputs that include agricultural run-off, industrial effluent, and sewage waste (Randall and Tsui 2002). The effect of high environmental ammonia (HEA) in fish can be devastating, causing ammonia excretion (J_{Amm}) via the gills to be inhibited leading to both the accumulation of endogenous ammonia and uptake of exogenous ammonia.

Increased internal ammonia concentrations, or hyperammonemia, can lead to decreased growth rates, alterations in energy metabolism, disruption of ionic balance, alterations in hormone regulation, and increased vulnerability to disease (Arillo et al. 1981; Soderberg and Meade 1992; Wilkie 1997; Dosdat et al. 2003; McKenzie et al. 2003; Lemarie et al. 2004; Foss et al. 2004; Pinto et al. 2007; Sinha et al. 2012a, b). Although fish are more tolerant to ammonia than mammals, their relative resistance to ammonia's toxic effects varies considerably among different fish species (Ip et al. 2001; Randall and Tsui 2002; Eddy 2005). In mammals, hyperammonemia resulting from acute liver failure (ALF) or congenital defects in the ornithine urea cycle (OUC), can cause significant neurotoxicity characterized by hyperexcitability, glutamate excitotoxicity, coma, convulsions, and life-threatening brain swelling (Felipo and Butterworth 2002). Such brain swelling is often accompanied by increased intracranial pressure, brain herniation, and is the most frequent cause of death in hyperammonemic mammals (Felipo and Butterworth 2002; Norenberg et al. 2007). Teleosts exhibit similar symptoms to toxic build-

ups of internal ammonia, including hyperventilation and hyperexcitability, convulsions, coma, and death (Eddy 2005). Wilkie et al. (2015) recently demonstrated that goldfish (*Carassius auratus*) and crucian carp (*Carassius carassius*) brains undergo significant, albeit reversible swelling in response to hyperammonemia brought about by acute exposure to HEA. However, the cellular mechanisms underlying such brain swelling and tolerance still remain poorly understood.

While the underlying mechanisms of brain swelling (encephalopathy) remain elusive, some researchers have suggested that reactive oxygen species (ROS), such as superoxide radicals ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hydroxyl radicals ($OH^{\bullet}$), could mediate the downstream events leading to ammonia neurotoxicity (for reviews see Walsh et al. 2007; Norenberg et al. 2007, 2009). Oxidative/nitrosative stress, characterized by the accumulation of oxidized lipids and proteins in the cell, is induced when cellular defense systems can no longer counteract the elevated ROS levels and/or repair oxidative cellular damage (Dorval and Hontela 2003; Prieto et al. 2009; Zhao et al. 2009). ROS can cause damage to lipids and proteins in the plasma membrane of neurons and glial cells of the brain, altering ionic and osmotic gradients, leading to water uptake and brain swelling (for reviews see Norenberg et al. 2007, 2009).

Similar to other vertebrates, teleosts have evolved a wide array of antioxidant defenses to protect themselves from the deleterious effects of ROS (Lushchak et al. 2001; Guerriero et al. 2002; Basha and Rani 2003; Lushchak 2011; Sinha et al. 2014). In vertebrates, these comprise low molecular weight antioxidants such as reduced glutathione (GSH) and ascorbate (ASC), and enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione-S-transferases (GST). These enzymatic and non-

enzymatic constituents scavenge ROS elements and protect cells against oxidative damage. The induction of antioxidant defenses can vary considerably between fish species depending on their resilience to stressors inducing oxidative damage (Stephensen 2002; Eyckmans et al. 2011; Sinha et al. 2014). In response to anoxia and reoxygenation, goldfish upregulate antioxidant defenses to effectively manage the post-anoxic increase in ROS production, limiting the extent of oxidative damage occurring in the brain and other tissues (Lushchak et al. 2001). Similar increases in antioxidant capacity have also been observed in goldfish exposed to a wide array of stressors capable of inducing ROS production (Lushchak et al. 2005; Shi et al. 2005; Matviishyn et al. 2014), including ammonia (Sinha et al. 2014).

Numerous studies exist on ammonia toxicity in different fishes, but ammonia-induced oxidative stress and antioxidant defenses in fish are not fully understood. A number of studies have characterized how hepatic tissue responds to ammonia-induced oxidative stress, but much less is known about the response of the fish brain to ammonia. The aim of the present study was to elucidate how the brain of the ammonia- and anoxia-tolerant goldfish responds to ammonia-induced neurotoxicity. Due to their known tolerance to post-anoxic oxidative stress, I predicted that the goldfish brain would exhibit a pronounced antioxidant response, which would limit ammonia-induced oxidative damage. Moreover, I predicted that ammonia-induced neurotoxicity, including brain swelling and oxidative stress, would be attenuated at colder temperatures.

MATERIALS AND METHODS

Experimental Animals and Holding Conditions

Juvenile goldfish (*Carassius auratus; n* = 150) of both sexes, weighing approximately 25 ± 5g, were purchased from a commercial supplier (Aquality Tropical Fish Wholesale Inc., Mississauga, ON) and transferred to the fish aquatics facility at Wilfrid Laurier University. Fish were held in 110 L aquaria continuously receiving filtered, aerated well water (pH ~8.0 ± 0.2, temperature ~14.0 ± 1°C, dissolved oxygen content >85 % saturation, ammonia <0.01 mmol L^{-1}, water hardness ~200 mg $CaCO_3$ L^{-1}) at a rate of 0.5 – 1.0 L min^{-1}. A subset of fish (*n = 65*) was held and acclimatized in an aquarium connected to a Delta Star®4 air-cooled water chiller (Aqua Logic Inc., San Diego, California, USA), in which the water temperature was cooled gradually to a set point of 4.0 ± 1°C. Goldfish were acclimated to these holding conditions for at least three weeks before experimentation. During this time, fish were fed three times weekly with commercially available pellets (3.0 mm; Martin Profishent™ Classic Floating Fish Feed, Martin Mills Inc. Elmira, ON), but were starved two days prior to experimentation to minimize the confounding effects that ammonia accumulation and defecation could have on water quality during exposure to high external ammonia (HEA). All experiments were approved by the Wilfrid Laurier University Animal Care Committee, and followed the guidelines of the Canadian Council on Animal Care.

Experimental Protocol

The day before experiments, goldfish (*n* = 85) were transferred one at a time, to individual, rectangular fish-holding chambers that were approximately 3 L in volume, and receiving aerated well water at a rate of 0.5 – 1.0 L min^{-1}. The chambers were darkened to minimize stress to the fish and were contained within a 150 L re-circulating system comprising a

head tank, which drained via a flow-splitter into each individual, fish-holding chamber. Fish were either held under control conditions (no ammonia) or exposed to HEA, which was initiated by temporarily cutting-off water flow to each chamber and adding a sufficient volume of 5 mol L^{-1} NH_4Cl stock solution to the water to achieve a nominal total ammonia (T_{Amm}) concentration of 5 mmol L^{-1}. At the same time, 5 mol L^{-1} NH_4Cl stock was also added to the head tank to yield a target total ammonia concentration of 5 mmol L^{-1} in the recirculating system. Once the T_{Amm} concentration in the re-circulating system equilibrated (~2h), water flow was restored to the individual containers, and fish were held under these conditions for 24 h or 72 h. Water pH was continuously monitored in the head tank with a Radiometer PHM84 pH meter, connected to a Radiometer GK2401C pH electrode (Radiometer, Copenhagen, Denmark), and spot-checked in each fish-holding container using a portable pH meter (Oakton® Instruments, pH 11 Model, Vernon Hills, IL). Water pH was controlled around pH 8.07 ± 0.01 by the drop-wise addition of 0.1 N HCl into the head tank using a solenoid valve connected to a TTT80 Autotitrator (Radiometer, Copenhagen, Denmark) connected to the PHM84 meter.

Cold-acclimated (4°C) goldfish were transferred one at a time, to individual, darkened, fish-holding chambers that were approximately 1 L in volume. Fish were acclimatized overnight and either held under control conditions (no ammonia) or exposed to HEA. HEA was initiated by cutting-off water flow to the holding aquarium and adding a sufficient volume of 5 mol L^{-1} NH_4Cl stock solution to the water to achieve a nominal total ammonia (T_{Amm}) concentration of 5 mmol L^{-1}. Water pH was continuously monitored in the aquarium and spot-checked in each fish-holding container using a portable pH meter (Oakton® Instruments, pH 11 Model, Vernon Hills, IL). Water pH was controlled around pH 8.12 ± 0.09 by the drop-wise addition of 0.1 N HCl into the aquaria.

Water samples were collected at different time intervals (2 h, 8 h, 24 h, 36 h, 48 h, 60 h, 72 h) throughout HEA and water T_{Amm} concentrations measured to ensure that target concentrations were maintained throughout the exposure period. At each sampling period (C, 24 h, 72 h), fish were euthanized with 1.0 g L^{-1} tricaine methanesulfonate buffered with 2.0 g L^{-1} $NaHCO_3$. Blood was collected immediately by caudal puncture using a 1.0 mL syringe fitted with a 21G needle pre-rinsed with sodium heparin (~50.0 Units mL^{-1}) to prevent coagulation. Whole brain and liver were then carefully removed from each animal, snap-frozen in liquid N_2, and stored at -80°C until analyzed for the determination of ammonia accumulation, indices of oxidative stress, and antioxidant enzymes.

Analytical techniques

Tissue and Water Ammonia Concentrations

Whole brain and liver tissues were prepared for ammonia and glutamine analysis by homogenization, and subsequent deproteination, in 7% ice-cold perchloric acid (PCA) using a Precellys®24 bead tissue homogenizer (Bertin Technologies**,** France; Wang et al. 1994b). The resulting slurry was incubated on ice for 10 min and centrifuged for 10 min at 10,000 x *g* at 4°C. The supernatant was withdrawn and neutralized with 2N KOH for the immediate quantification of tissue ammonia and glutamine content. T_{Amm} concentrations in untreated plasma and neutralized extracts of brain and liver were quantified enzymatically using a commercially available assay kit (Sigma-Aldrich Cat. No. AA0100), in which [T_{Amm}] quantification was based on the glutamate dehydrogenase (GDH)-catalyzed oxidation of NADPH, measured at 340 nm on a Spectramax190 plate spectrophotometer (Molecular Device, Sunnyvale, CA). Water T_{Amm} concentrations were measured colorimetrically at 650 nm via the salicylate-hypochlorite method (Verdouw et al. 1978).

Tissue Glutamine Concentrations

Tissue extracts of whole brain and liver were deproteinated and neutralized for glutamine analysis as described above. Briefly, tissue glutamine content was quantified colorometrically at 540 nm using glutamine synthetase, followed by the addition of ferric chloride (Mecke 1985).

Indices of Oxidative Damage

The concentration of malondialdehyde (MDA), a naturally occurring end product of lipid peroxidation and peroxidative tissue injury (Janero 1990), was measured using the thiobarbituric acid-reactive substances (TBARS) assay (Ohkawa et al. 1979). For this assay, whole brain or liver tissue was sonicated in ice-cold radio-immunoprecipitation (RIPA) buffer (10% w/v) and centrifuged at 1,600 x *g* for 10 min at 4°C. The supernatant was withdrawn and used for the determination of tissue MDA content. Homogenates were mixed with sodium dodecyl sulfate (SDS), thiobarbituric acid (TBA), and butylated hydroxytoluene (BHT) in an acetate buffer (pH 3.5). BHT, an antioxidant, was added to avoid oxidation during subsequent heating, as recommended by Halliwell and Chirico (1993). Following incubation at 95°C for 60 min., the pink-red chromogen produced by the TBA-MDA adduct was mixed vigorously with an equal volume of *n*-butanol and centrifuged at 10,000 x *g* for 5 min at 4°C. The butanol phase was removed and used to quantify the level of TBARS by measuring its absorbance at 532 nm using a SpectramaxPlus340 plate spectrophotometer (Molecular Device, Sunnyvale, CA). The level of MDA was calculated and expressed in nanomoles of TBARS per gram tissue wet weight.

Protein carbonyl content, the most commonly used biomarker of protein oxidative damage in animal tissues (Stadtman and Oliver 1991), was quantified using a commercially available assay kit (Cayman Chemical Item No. 10005020, Michigan, USA). Briefly, frozen brain and liver samples were homogenized (1:5 w/v) in ice-cold buffer (50 mM KPi, 1.1 mM

EDTA, 0.1 mM PMSF, pH 6.7) and then centrifuged at 10,000 x *g* for 15 min at 4°C. Homogenates were mixed with 2,4-dinitrophenylhydrazine (DNPH) and trichloroacetic acid (TCA), incubated in the dark at room temperature for 60 min, and then centrifuged at 10,000 x *g* for 10 min at 4°C. The resulting supernatant was discarded and the precipitated protein pellets were washed three times with ethanol/ethyl acetate (1:1 v/v) mixture to remove any unreacted DNPH. The protein pellets were finally dissolved in guanidine HCl and centrifuged as above to remove insoluble debris. Protein carbonyl content in the resulting supernatants was quantified spectrophotometrically at 360 nm using a molar extinction coefficient of 22000 M^{-1} cm^{-1}, and values expressed as nanomoles of protein carbonyls per mg protein. Protein concentration in the final, resuspended pellet was quantified based on its relative absorption at 280 nm, using BSA in guanidine HCl (Cayman Chemical Item No. 10005020, Michigan, USA) as a standard.

Measurement of Antioxidant Enzymes

The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) were determined from homogenates of goldfish brain and liver (1:20 w/v) prepared in ice-cold buffer (20 mM HEPES, 1 mM EDTA, 0.1% Triton X-100, pH 7.4) using a Tenbroek all glass tissue homogenizer (2 mL; VWR CanLab, Mississauga, ON) and centrifuged for 2 min at 10,000 x *g* in a refrigerated Eppendorf 5430R centrifuge (Eppendorf, Mississauga, ON). Aliquots of the resulting homogenates were sonicated over ice for 15 seconds at 40 V using a Q125 tissue sonicator (QSonica, Newtown, CT). All enzyme activity measurements were initially optimized to obtain linear time- and concentration-dependence using a SpectramaxPlus340 plate spectrophotometer (Molecular Device, Sunnyvale, CA).

SOD percent (%) inhibition rate was determined using a commercially available SOD

assay kit (Sigma-Aldrich, Cat No. 19160). In brief, the activity of total SOD (Cu-, Mn-SOD) was based on its inhibitory action on the reduction of highly water-soluble tetrazolium salt (WST-1) by xanthine oxidase, which produces a water-soluble formazan dye that can be detected colorimetrically at 440 nm. SOD activity was determined from a standard curve using known units (U) of purified SOD enzyme (Sigma Aldrich Cat No. S2515) against the % inhibition rate of tretrazolium salt under identical conditions.

CAT and GPx activities were determined in homogenates of brain and liver tissue according to the methods of Weydert and Cullen (2010). The activity of CAT was determined by monitoring the initial rate of H_2O_2 decomposition at 240 nm. The final concentrations of the reaction components were: 20 mM KPi buffer (pH 7.4), 20 mM H_2O_2, and 10 µL of appropriately diluted sample. Total GPx activity was determined by monitoring the reduction in NADPH absorbance at 340 nm in the presence of 0.25 mM tert-butyl hydroperoxide in a reaction medium containing (final concentrations): 50 mM KPi buffer (pH 7.0), 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 mM GSH, and 1 unit mL^{-1} GR. The activity of GR was determined by monitoring the oxidation of NADPH in the presence of 10 mM oxidized glutathione (GSSG) at 340 nm in a medium containing (final concentrations): 50 mM KPi buffer (pH 7.0), 1 mM EDTA, and 0.25 mM NADPH. The activity of GPx and GR were calculated using a molar extinction coefficient of 6220 M^{-1} cm^{-1}, and one unit (U) of CAT, GPX, and GR defined as the amount of enzyme consuming 1 µmole of substrate per minute at 25°C. All antioxidant enzyme activities were expressed as international units (or millunits) per milligram protein. Protein content in homogenates of brain and liver tissue was determined via the Bradford method, using bovine serum albumin (BSA) as a standard (Bradford 1976).

Calculations and Statistics

The speciation of water-borne ammonia was determined through re-arrangement of the Henderson-Hasselbalch equation (Wright and Wood 1985; Wilkie et al. 2015) using the appropriate apparent dissociation constant (pKa), according to the equation below:

$$[NH_4^+] = \left\{ \frac{T_{Amm}}{[1 + anti\log(pH - pKa)]} \right\} \quad (1)$$

$$[NH_3] = T_{Amm} - [NH_4^+] \quad (2)$$

Brain water content was quantified by the wet-weight/dry-weight method, as previously described by Wilkie et al. (2015). Briefly, whole brains were carefully excised and the mass determined in pre-weighed 1.5 mL tubes before and after 72 h incubation in a 65 ± 5°C oven. Tissue H_2O content is typically reported as % tissue H_2O, according to the following formula:

$$Brain\ Water\ Content = \left[\frac{(Mass_{wet} - Mass_{dry})}{Mass_{wet}} \right] \times 100\% \quad (3)$$

Where mass is expressed in g, water volume in mL, and brain water content in mL H_2O g^{-1} wet tissue mass. The volume of water per gram dry tissue mass (mL H_2O g^{-1} dry mass) was then calculated by dividing the brain water content (mL H_2O g^{-1} wet tissue mass; Eqn. 3) by the mass of dry tissue (g dry mass) over the wet tissue mass (g wet mass) according to the following formula:

$$Brain\ Water\ Volume = \left[\frac{Brain\ Water\ Content}{(Mass_{dry} \div Mass_{wet})} \right] \quad (4)$$

Using this approach, it was possible to directly calculate how much the volume of H_2O changed in the brain during HEA, as a function of brain swelling.

All values are presented as the mean ± 1 standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey-Kramer (TK) post-

hoc test. Student's unpaired two-tailed t-test was used for single comparisons between tissues. In all determinations, the null hypothesis was rejected if $p<0.05$.

RESULTS

Ammonia Accumulation

Warm-acclimated goldfish (14°C) were exposed to a measured T_{Amm} concentration of 5.3 ± 0.1 mmol L^{-1}, which was not significantly different from the target HEA concentration of 5 mmol L^{-1} with concentrations fluctuating between 5.10 ± 0.043 mmol L^{-1} and 5.79 ± 0.16 mmol L^{-1} during the exposure period (Table 2.1). Cold-acclimated (4°C) goldfish were exposed to a similar measured T_{Amm} concentration of 5.3 ± 0.2 mmol L^{-1}, with concentrations fluctuating between 5.16 ± 0.01 mmol L^{-1} and 5.41 ± 0.02 mmol L^{-1} during the exposure period (Table 2.2). Warm-acclimated (14°C) goldfish survived HEA despite 10-fold increases in plasma [T_{Amm}] from approximately 150 μmol L^{-1} under control conditions to approximately 1440 μmol L^{-1} after 24 h, and approximately 1325 μmol L^{-1} after 72 h (Figure 2.1A). Cold-acclimated (4°C) goldfish experienced similar increases in plasma [T_{Amm}] during HEA, which increased to approximately 1740 μmol L^{-1} after 72 h from control values of approximately 100 μmol L^{-1} (Figure 2.1B). Mortality was not observed in control fish or in any experimental group throughout the experimental period.

Following exposure to HEA, ammonia accumulation in the goldfish brain was pronounced (Figure 2.2). In warm-acclimated goldfish, brain T_{Amm} concentrations of ~1 μmol g^{-1} wet mass (WM) under control conditions increased nearly 6-fold to ~5.7 μmol g^{-1} WM after 72 h exposure to HEA (Figure 2.2B). In cold-acclimated individuals, brain [T_{Amm}] increased nearly 7-fold from control values of ~0.7 μmol g^{-1} WM to 5 μmol g^{-1} WM and 5.2 μmol g^{-1} WM after 24 h and 72 h HEA, respectively (Figure 2.2B).

Glutamine Accumulation

Glutamine concentrations were elevated 5–6 fold after 24 h and 72 h HEA from control

values of approximately 4 μmol g^{-1} wet tissue in warm-acclimated goldfish brain (Figure 2.3A). On the other hand, brain glutamine concentrations in cold-acclimated goldfish increased from control values of approximately 5 μmol g^{-1} wet tissue to approximately 17 μmol g^{-1} wet tissue after 24 h, and increased further to nearly 24 μmol g^{-1} wet tissue after 72 h HEA exposure (Figure 2.3B). No significant differences in glutamine concentration were noted between cold- and warm-acclimated goldfish after 72 h HEA.

Brain Water Volume

Cold-temperature acclimation mitigated the brain swelling response in goldfish exposed to HEA. No significant differences in basal brain water volume were noted between cold- and warm-acclimated control goldfish. Brain water volume increased significantly by 20% in warm-acclimated (14°C) goldfish following 72 h exposure to HEA from control values of approximately 5.14 mL H_2O g^{-1} dry mass to approximately 6.38 mL H_2O g^{-1} dry mass (Figure 2.4A). However, water content in cold-acclimated goldfish brains remained largely unaffected from control values of ~5 mL g^{-1} dry mass through 72 h HEA (Figure 2.4B).

Indices of Oxidative Stress

In control goldfish, baseline concentrations of TBARS in the liver were 6-fold higher than in the brain (Figure 2.5A). Following 24 h and 72 h exposure to HEA, the level of TBARS did not change significantly from control values in the liver of warm-acclimated (14°C) goldfish. In the brain, however, there was a pronounced 114 % increase in TBARS following 72 h of HEA (Figure 2.5A). However, the level of TBARS remained unchanged from control values in the brain and liver of cold-acclimated goldfish throughout HEA (Figure 2.5B).

In contrast to lipid peroxidation, the liver and brain were much more susceptible to protein oxidation following increases in internal ammonia at 14°C. In control fish, protein

carbonyl content was similar in the liver and the brain at both acclimation temperatures. Following 24 h HEA, however, protein carbonyl content in the liver increased by nearly 3-fold to 3.5 nmol mg protein^{-1} before returning to control values after 72 h HEA in warm-acclimated goldfish (Figure 2.6A). On the other hand, protein carbonyl content increased in a time-dependent manner in the warm-acclimated goldfish brain, increasing 2-fold from approximately 1 nmol mg protein^{-1} in control fish to approximately 2 nmol mg protein^{-1} after 24 h, and increasing further to 2.5 nmol mg protein^{-1} following 72 h exposure (Figure 2.6A). Treatment at 4°C mitigated the increase in protein carbonyl content, which remained unaffected from control values in the liver and brain of cold-acclimated goldfish (Figure 2.6B).

Table 2.1: Measured concentrations of total ammonia (T_{Amm}) that warm-acclimated (14°C) goldfish were exposed throughout 72 h exposure to high external ammonia (Nominal $[T_{Amm}] = 5$ mmol L^{-1}).

Time (h)	Measured $[T_{Amm}]$ (mM)	$[NH_3]$ (µM)	pH
2	5.10 ± 0.09 (19)	164.27 ± 6.86 (19)	8.17 ± 0.01 (19)
8	5.33 ± 0.06 (19)	150.14 ± 4.65 (19)	8.10 ± 0.01 (19)
24	5.10 ± 0.04 (19)	141.28 ± 4.07 (19)	8.10 ± 0.01 (19)
36	5.79 ± 0.16 (10)	143.52 ± 5.93 (10)	8.05 ± 0.01 (10)
48	5.18 ± 0.15 (10)	125.89 ± 6.72 (10)	8.04 ± 0.01 (10)
72	5.17 ± 0.20 (10)	106.28 ± 11.06 (10)	7.95 ± 0.03 (10)

Measurements are expressed as the mean ± 1 SEM (N), where N is the number of independent measurements of water $[T_{Amm}]$ and pH that were made throughout the exposure period. The $[NH_3]$ was calculated from the measured water $[T_{Amm}]$ and water pH by rearrangement of the Henderson-Hasselbalch equation with an apparent dissociation constant (pK') of 9.65 determined previously by using relationships outlined in Cameron and Heisler (1983). **Mean measured water $[T_{Amm}]$** = 5.3 ± 0.1 mmol L^{-1}; **Mean measured water pH** = 8.07 ± 0.01.

Table 2.2: Measured concentrations of total ammonia (T_{Amm}) that cold-acclimated (4°C) goldfish were exposed throughout 72 h exposure to high external ammonia (Nominal $[T_{Amm}] = 5$ mmol L^{-1}).

Time (h)	Measured $[T_{Amm}]$ (mM)	$[NH_3]$ (µM)	pH
2	5.16 ± 0.01 (8)	148.06 ± 0.19 (8)	8.12 ± 0.00 (8)
8	5.37 ± 0.05 (8)	140.73 ± 1.22 (8)	8.08 ± 0.00 (8)
24	5.25 ± 0.03 (8)	122.25 ± 0.89 (8)	8.03 ± 0.004 (8)
36	5.36 ± 0.04 (8)	137.30 ± 1.03 (8)	8.07 ± 0.00 (8)
48	5.38 ± 0.03 (8)	157.71 ± 0.89 (8)	8.13 ± 0.00 (8)
72	5.41 ± 0.02 (8)	183.26 ± 1.76 (8)	8.19 ± 0.003 (8)

Measurements are expressed as the mean ± 1 SEM (N), where N is the number of independent measurements of water $[T_{Amm}]$ and pH that were made throughout the exposure period. The $[NH_3]$ was calculated from the measured water $[T_{Amm}]$ and water pH by rearrangement of the Henderson-Hasselbalch equation with an apparent dissociation constant (pK') of 9.65 determined previously by using relationships outlined in Cameron and Heisler (1983). **Mean measured water $[T_{Amm}]$** = 5.3 ± 0.02 mmol L^{-1}; **Mean measured water pH** = 8.12 ± 0.09.

Figure 2.1. The effect of acute ammonia exposure (measured ammonia = 5.3 mmol L^{-1}) on plasma total ammonia levels [T_{Amm}] in the goldfish at (A) 14°C, and (B) 4°C; Data expressed as the mean ± SEM (*n* = 8). Asterisks (*) denote statistically significant differences from the corresponding control groups as assessed by ANOVA followed by Tukey-Kramer post-test, $P < 0.05$.

FIGURE 2.1.

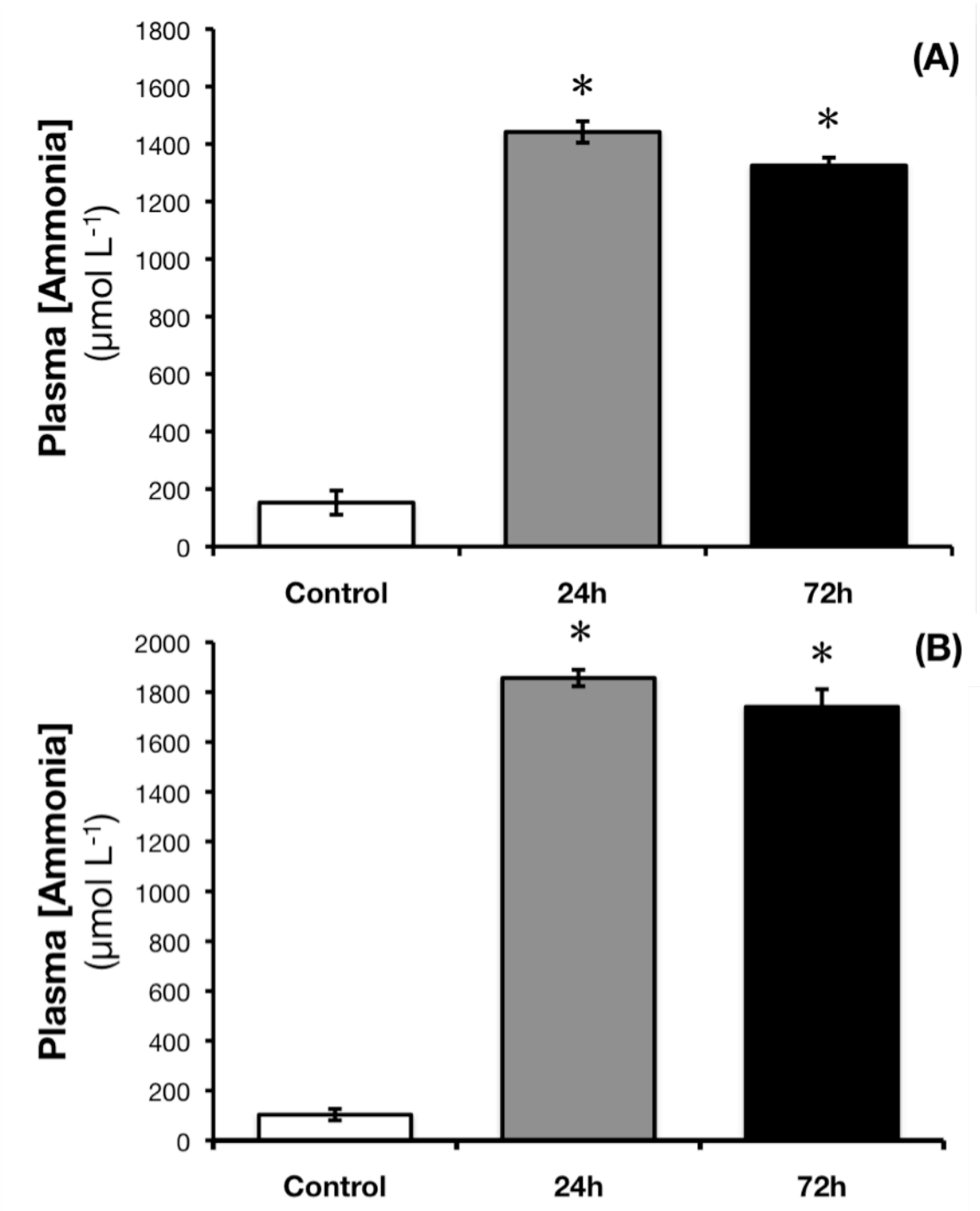

(A)
Plasma [Ammonia] (µmol L-1)
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
Control
24h
72h
(B)
Plasma [Ammonia] (µmol L-1)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
Control
24h
72h

Figure 2.2. The effect of acute ammonia exposure (measured ammonia = 5.3 mmol L^{-1}) on ammonia concentration in goldfish brain at (A) 14°C, and (B) 4°C; Data expressed as the mean ± SEM (*n* = 8). Asterisks (*) denote statistically significant differences from the corresponding control groups as assessed by ANOVA followed by Tukey-Kramer post-test, $P < 0.05$.

FIGURE 2.2.

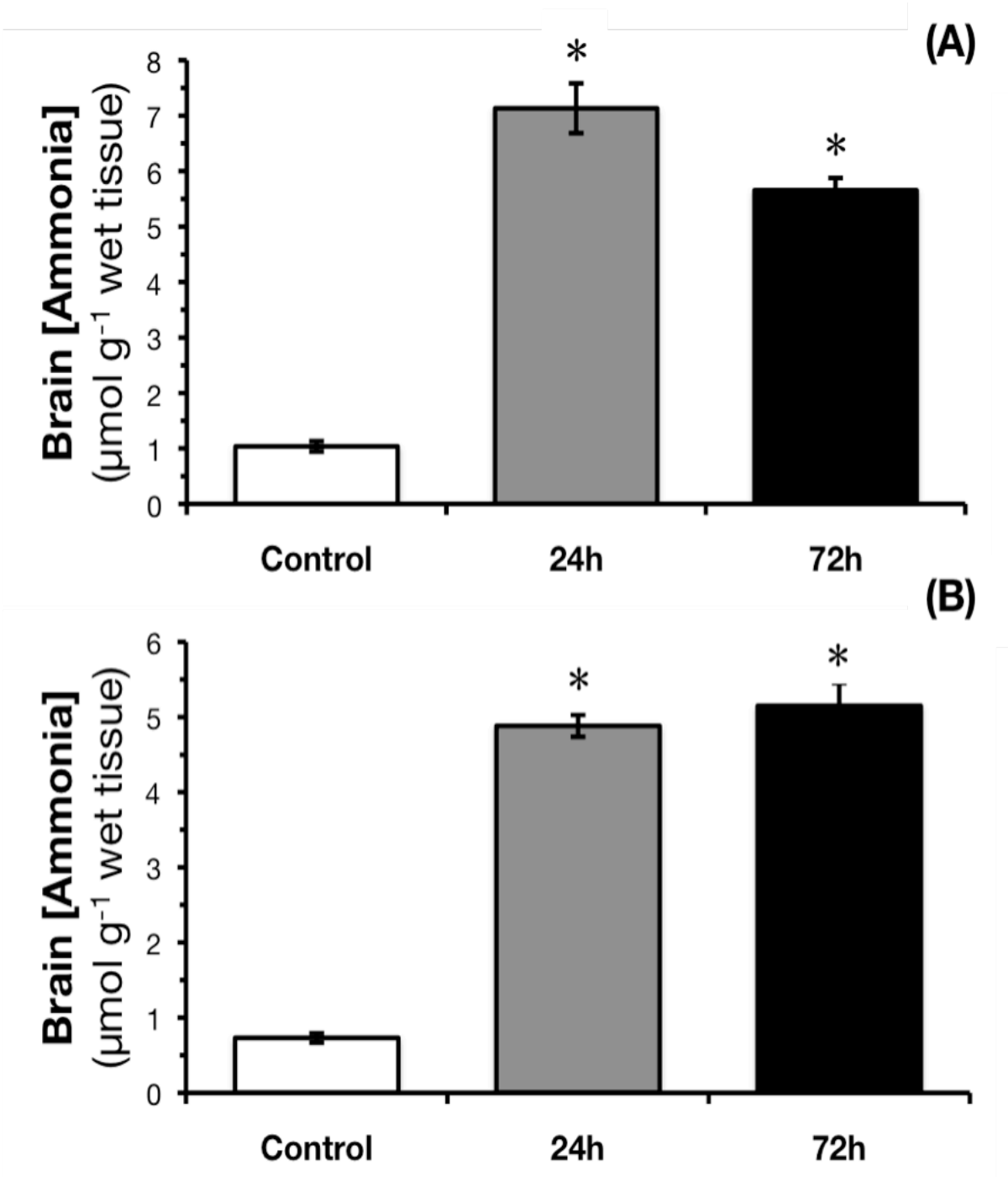

(A)
Brain [Ammonia]
(µmol g-1 wet tissue)
0
1
2
3
4
5
6
7
8
*
*
Control
24h
72h
(B)
Brain [Ammonia]
(µmol g-1 wet tissue)
0
1
2
3
4
5
6
*
*
Control
24h
72h

Figure 2.3. The effect of acute ammonia exposure (measured ammonia = 5.3 mmol L^{-1}) on glutamine concentration in goldfish brain at (A) 14°C, and (B) 4°C; Data expressed as the mean ± SEM (*n* = 8). Asterisks (*) denote statistically significant differences from the corresponding control groups and (**) 24 h treatment groups as assessed by ANOVA followed by Tukey-Kramer post-test, $P < 0.05$.

FIGURE 2.3.

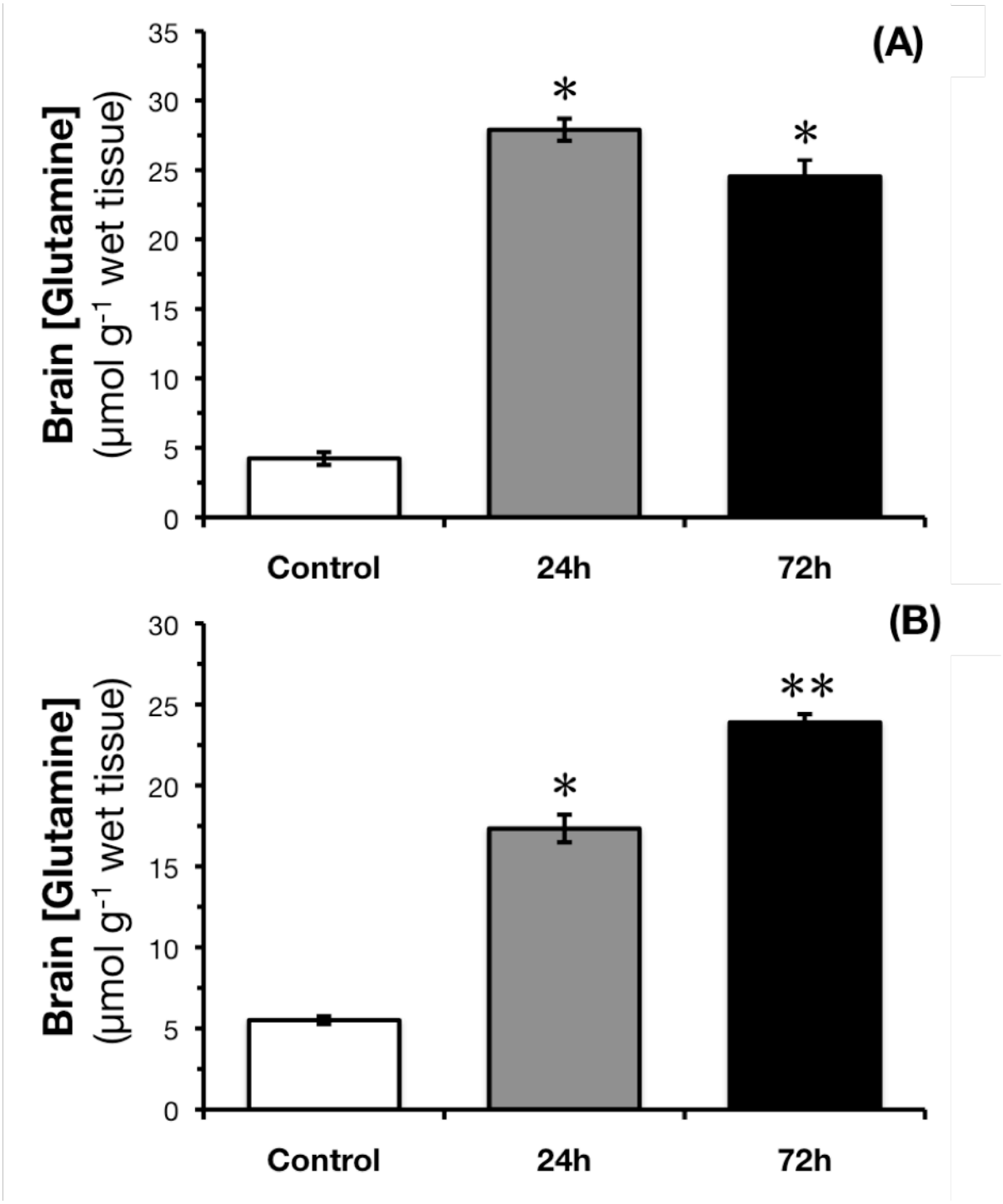

(A)
Brain [Glutamine]
(µmol g-1 wet tissue)
35
30
25
20
15
10
5
0
*
*
Control
24h
72h
(B)
Brain [Glutamine]
(µmol g-1 wet tissue)
30
25
20
15
10
5
0
*
**
Control
24h
72h

Figure 2.4. The effect of acute ammonia exposure (measured ammonia = 5.3 mmol L^{-1}) on water volume in goldfish brain at (A) 14°C, and (B) 4°C. Data are presented as the mean ± SEM (n = 6-8). Asterisks (*) denote statistically significant differences from the corresponding control groups as assessed by ANOVA followed by Tukey-Kramer post-test, $P < 0.05$. Data obtained from Z. Lister (Honours thesis, 2014).

FIGURE 2.4.

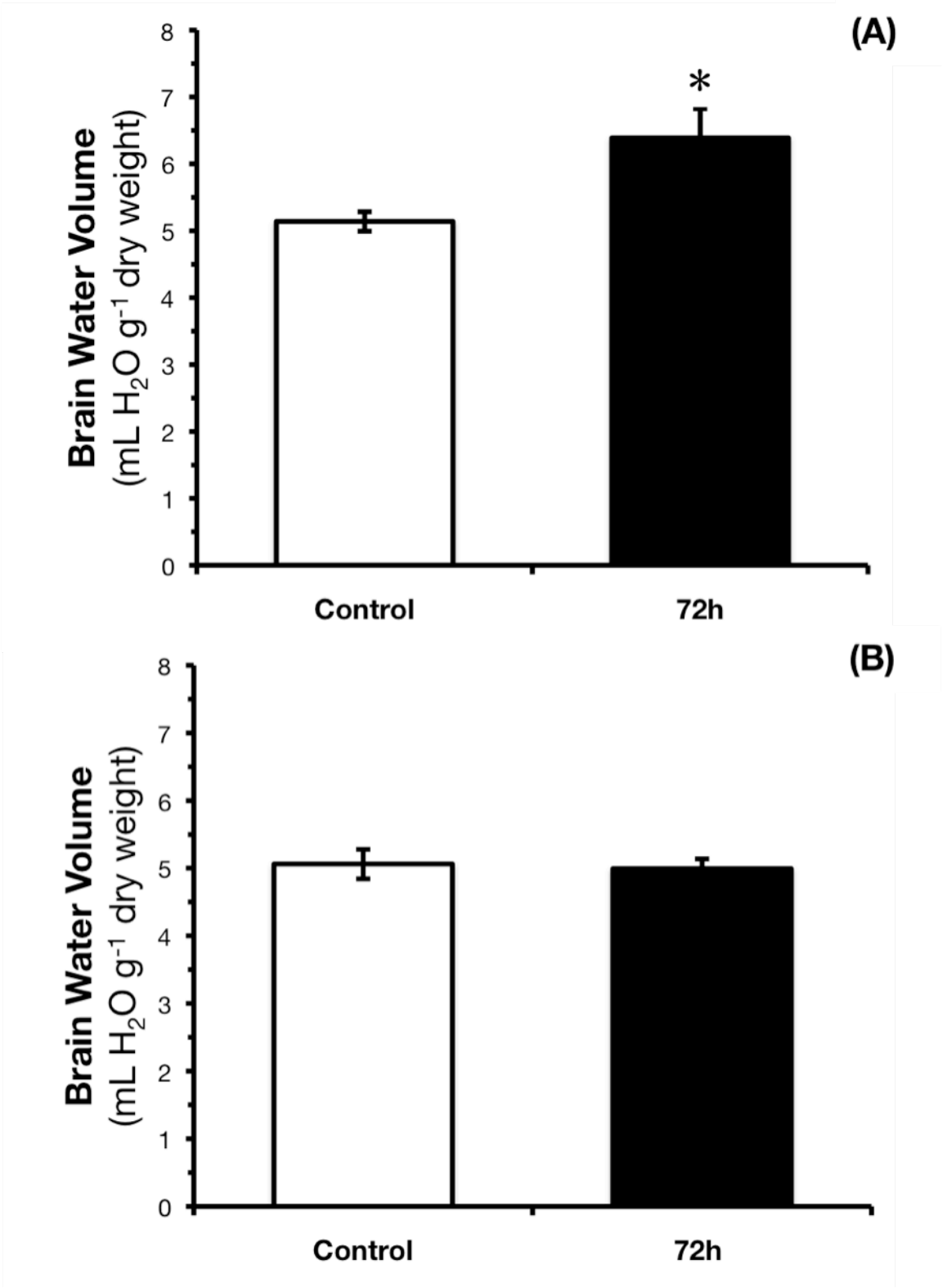

(A)
Brain Water Volume
(mL H_2O g^{-1} dry weight)
8
7
6
5
4
3
2
1
0
*
Control
72h
(B)
Brain Water Volume
(mL H_2O g^{-1} dry weight)
8
7
6
5
4
3
2
1
0
Control
72h

Figure 2.5. Level of Thiobarbituric Acid Reactive Substances (TBARS) in tissues of goldfish exposed to acute ammonia toxicity, measured ammonia = 5.3 mmol L^{-1} at (A) 14°C, and (B) 4°C; Data are presented as the mean ± 1 SEM, *n* = 6-8. Asterisks (*) denote statistically significant differences from the corresponding control groups as assessed by ANOVA followed by Tukey-Kramer post-test, $P < 0.05$. † Significantly different from corresponding control or experimental group in liver tissue, $P < 0.05$.

FIGURE 2.5.

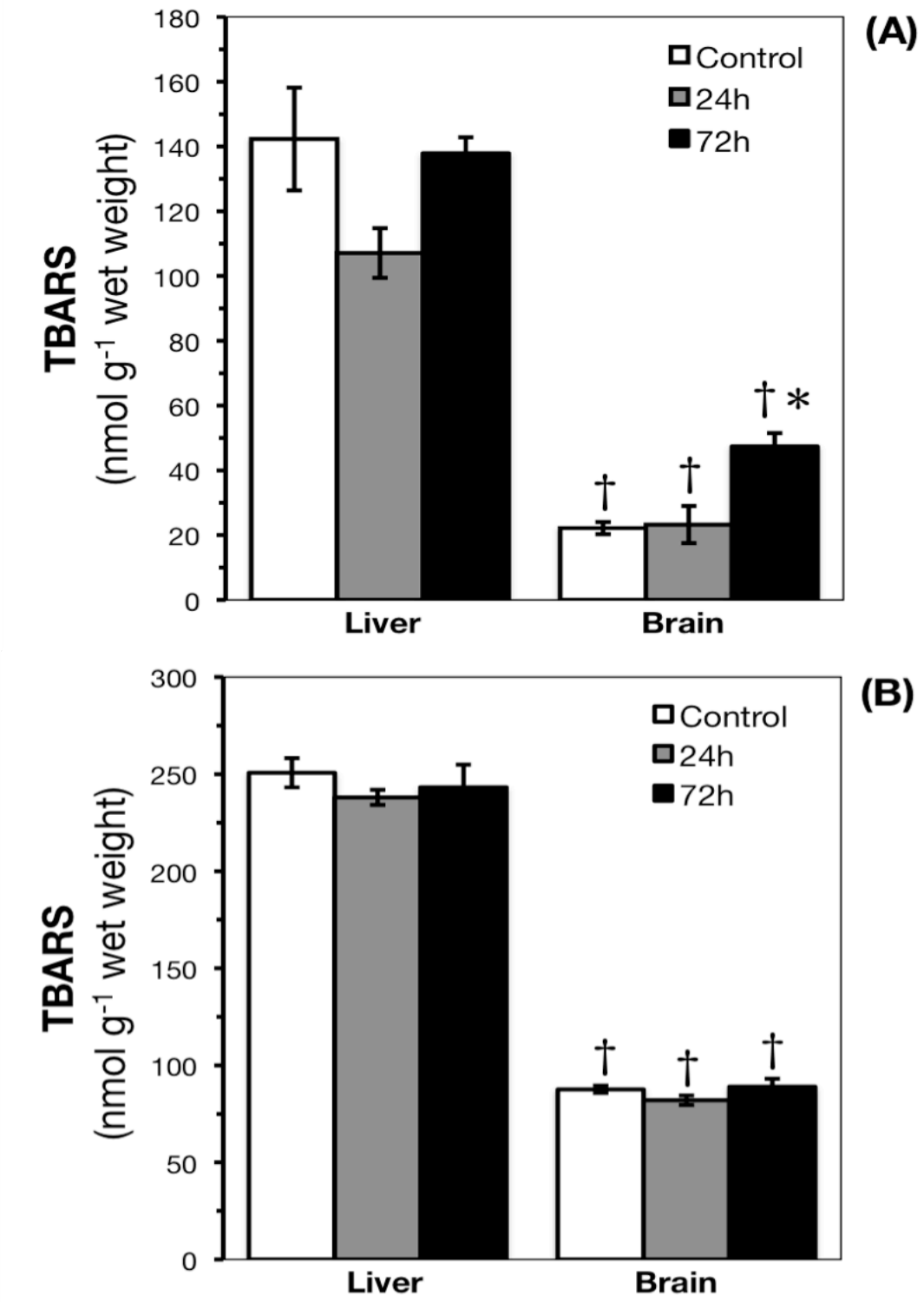
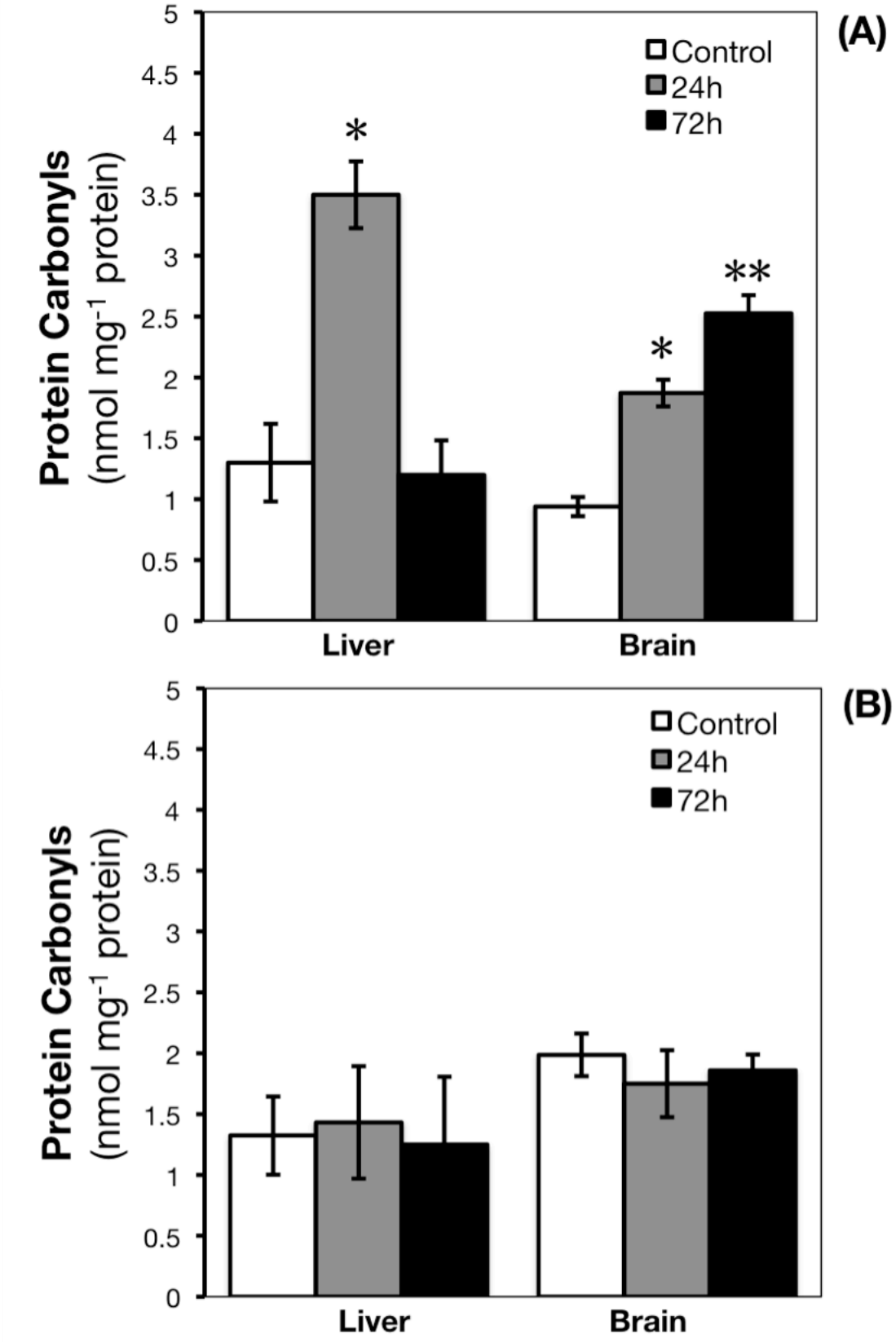

(A)
TBARS
(nmol g-1 wet weight)
180
160
140
120
100
80
60
40
20
0
Control
24h
72h
† *
†
†
Liver
Brain
(B)
TBARS
(nmol g-1 wet weight)
300
250
200
150
100
50
0
Control
24h
72h
†
†
†
Liver
Brain

Figure 2.6. Level of protein carbonyls in tissues of goldfish exposed to acute ammonia toxicity, measured ammonia = 5.3 mmol L^{-1}, at (A) 14°C, and (B) 4°C; Data are presented as the mean ± 1 SEM (*n* = 6-8). Asterisks denote statistically significant differences from the corresponding control groups (*) or 24 h treatment groups (**) as assessed by ANOVA followed by Tukey-Kramer post-test, $P < 0.05$. † Significantly different from corresponding control or experimental group in liver tissue, $P < 0.05$.

FIGURE 2.6.


(A)
Control
24h
72h
Protein Carbonyls
(nmol mg-1 protein)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
**
Liver
Brain
(B)
Control
24h
72h
Protein Carbonyls
(nmol mg-1 protein)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Liver
Brain

Antioxidant Enzyme Activities

No significant differences in basal SOD activity were noted between the liver and brain in control goldfish at both acclimation temperatures. Moreover, SOD activity did not change in either tissue following 24 h HEA (Figure 2.7A). However, SOD activity increased significantly by 83% in the liver of warm-acclimated goldfish following 72 h exposure to HEA (Figure 2.7A). In contrast, SOD activity in the brain remained unchanged from control values throughout 72 h HEA at both acclimation temperatures (Figures 2.7A, 2.8).

Catalase (CAT) activity in control goldfish was 3-fold greater in the liver than in the brain at both acclimation temperatures. HEA induced differential changes in CAT activity between liver and brain of warm-acclimated (14°C) goldfish (Figure 2.7B). In the liver, CAT activity did not significantly change until 72 h of HEA, when activity was 3-fold greater than in control fish (Figure 2.7B). In contrast, CAT activity in the brain increased 2-fold after 24 h HEA, and remained elevated through 72 h of HEA (Figure 2.7B). In the cold-acclimated (4°C) goldfish, however, SOD and CAT activities in the brain and liver remained unchanged from control values throughout HEA (Table 2.3).

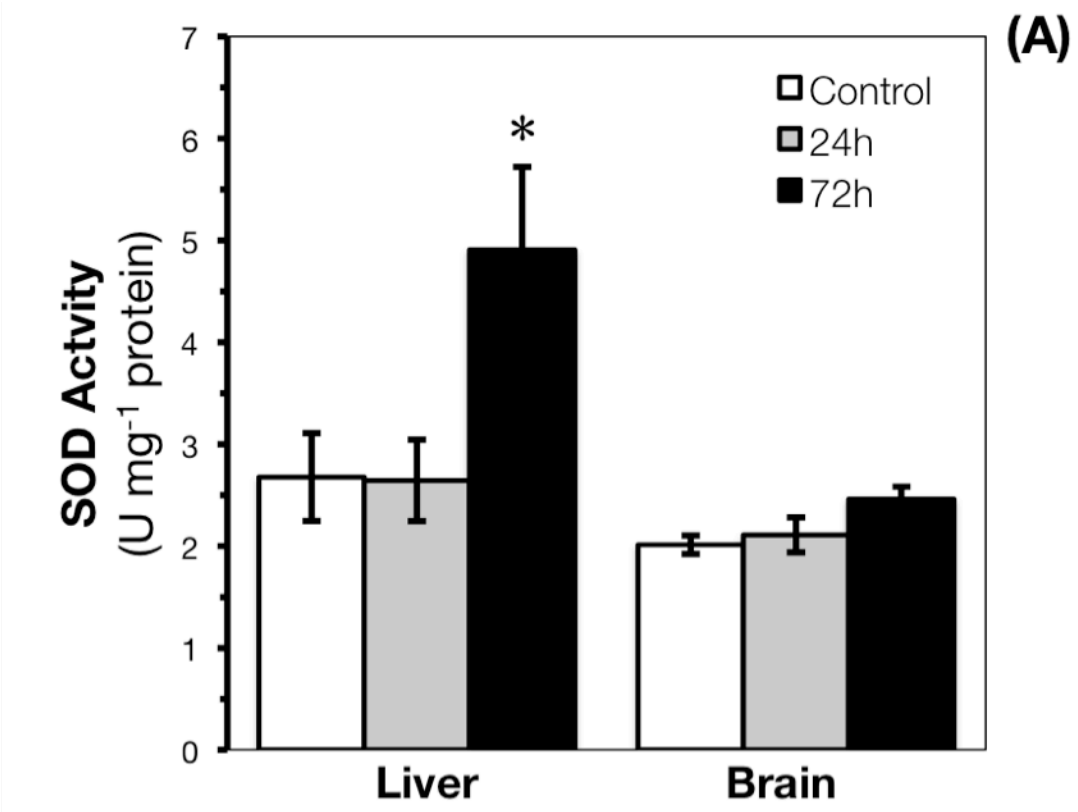
Baseline GPx activities in warm-acclimated goldfish liver were 4-fold greater compared to brain (Figure 2.8A). In the liver, HEA had no significant effect on GPx activity. The activity of GPx in the goldfish brain, however, was significantly elevated by 46% after 24 h HEA, followed by a complete recovery by 72 h (Figure 2.8A).

Baseline GR activity was also greater in the liver than in the brain by approximately 2-fold in warm-acclimated goldfish (Figure 2.8B). Ammonia exposure induced differential changes in GR activity between liver and brain in these fish. In the liver, GR activity significantly increased by 75% following 72 h HEA. In contrast, GR activity in the brain increased

significantly by 49% during 24 h and remained 38% higher than control values throughout 72 h HEA exposure (Figure 2.8B).

Figure 2.7. The effect of acute ammonia exposure (measured ammonia = 5.3 ± 0.1 mmol L^{-1}) on the specific activities of primary antioxidant enzymes in tissues of warm-acclimated (14°C) goldfish, (A) superoxide dismutase (SOD), and (B) catalase (CAT); Data are presented as the mean ± 1 SEM (*n* = 5-7). Asterisks denote statistically significant differences from the corresponding (*) control groups or 24 h treatment groups (**) as assessed by ANOVA followed by Tukey-Kramer post-test, $P < 0.05$. † Significantly different from corresponding liver groups, $P < 0.05$.

FIGURE 2.7.


(A)
Control
24h
72h
SOD Activity
(U mg-1 protein)
0
1
2
3
4
5
6
7
*
Liver
Brain

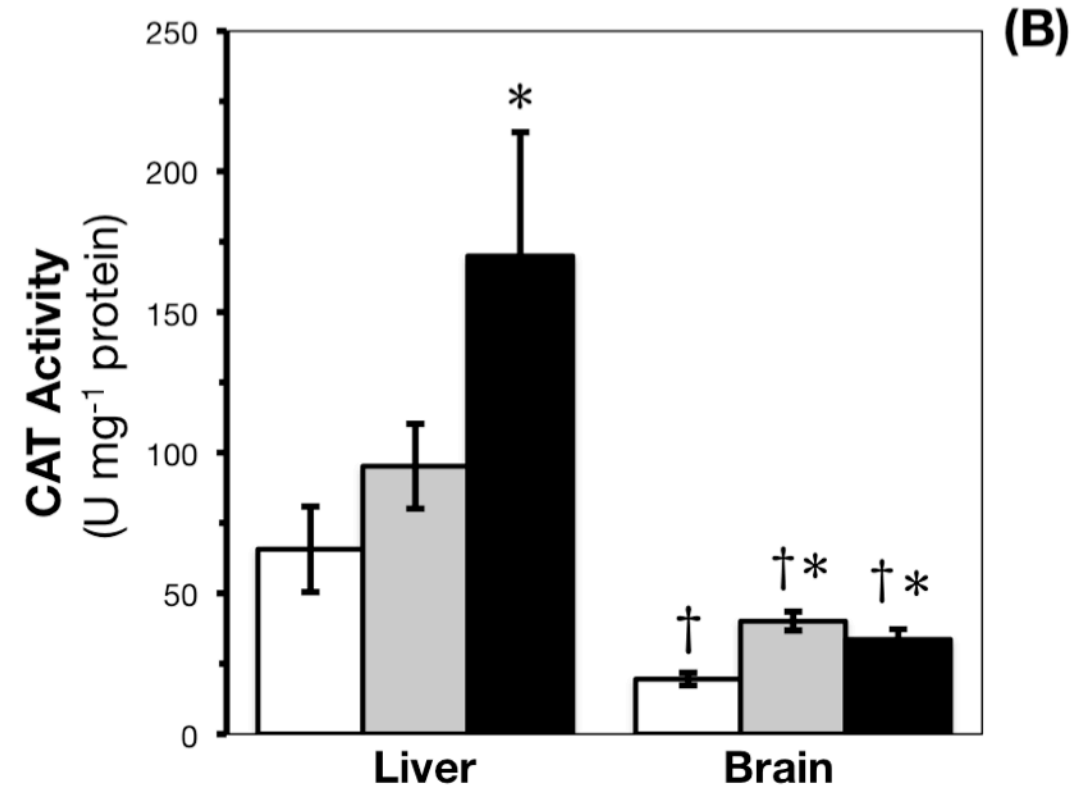

(B)
CAT Activity
(U mg-1 protein)
0
50
100
150
200
250
*
†
†*
†*
Liver
Brain

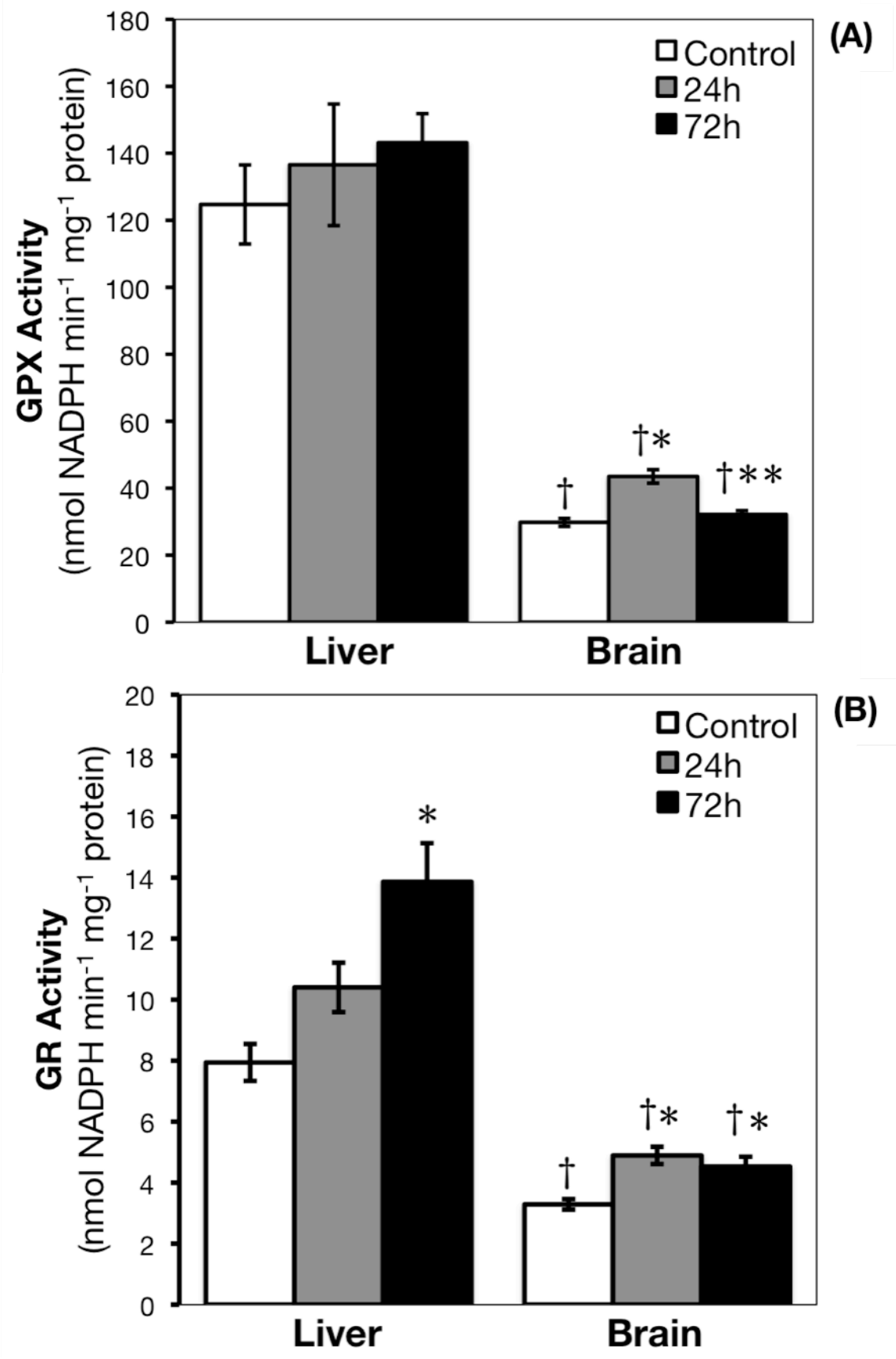
Table 2.3. The effect of acute ammonia exposure (measured ammonia = 5.3 ± 0.2 mmol L^{-1}) on the specific activities of primary antioxidant enzymes in brain of cold-acclimated (4°C) goldfish.

Brain Superoxide Dismutase (SOD)	
Control	3.4 ± 0.2 (7)
24 h HEA	3.2 ± 0.2 (7)
72 h HEA	3.3 ± 0.2 (7)
Brain Catalase (CAT)	
Control	18.9 ± 0.8 (8)
24 h HEA	19.0 ± 1.5 (7)
72 h HEA	20.3 ± 0.9 (7)

Values are presented as mean ± 1 SEM (*n*) in U mg^{-1} protein. No statistically significant differences from the corresponding control groups or 24 h treatment groups as assessed by ANOVA followed by Tukey-Kramer post-test, $P < 0.05$.

Figure 2.8. The effect of acute ammonia exposure (measured ammonia = 5.3 ± 0.1 mmol L^{-1}) on the specific activities of glutathione-dependent antioxidant enzymes in tissues of warm-acclimated goldfish, (A) glutathione peroxidase (GPx), and (B) glutathione reductase (GR); Data are presented as the mean ± SEM (*n*). Asterisks denote statistically significant differences from the corresponding (*) control groups or 24 h treatment groups (**) as assessed by ANOVA followed by Tukey-Kramer post-test, $P < 0.05$. † Significantly different from corresponding liver groups, $P < 0.05$.

FIGURE 2.8.


(A)
GPX Activity
(nmol NADPH min-1 mg-1 protein)
180
160
140
120
100
80
60
40
20
0
Control
24h
72h
†
†*
†**
Liver
Brain
(B)
GR Activity
(nmol NADPH min-1 mg-1 protein)
20
18
16
14
12
10
8
6
4
2
0
Control
24h
72h
*
†
†*
†*
Liver
Brain

DISCUSSION

Ammonia-Induced Oxidative Stress in the Goldfish

The present study demonstrates that increases in internal ammonia concentrations in the ammonia- and anoxia-tolerant goldfish (*Carassius auratus*) arising from HEA exposure lead to a temperature-dependent and tissue-specific oxidative stress response. As expected, the brain demonstrated a greater degree of oxidative stress than the liver during HEA. The results of the present study suggest that free radical-mediated damage to cellular constituents may contribute to the events that cause the significant swelling that occurs in the goldfish brain during exposure to HEA. However, unlike other studies on ammonia tolerance in fishes, the present study demonstrates a significant upregulation in the activity of some key antioxidant enzymes in the goldfish brain and the maintenance of other antioxidant enzyme activities during acute hyperammonemia. This high neural antioxidant capacity likely offsets the extent of oxidative brain damage and swelling to a level that is physiologically manageable and reversible in the goldfish (Wilkie et al. 2015).

Goldfish survived HEA despite significant increases in plasma and brain T_{Amm} concentrations. However, plasma T_{Amm} concentrations were surprisingly low and stable in goldfish exposed to nominal concentrations of 5 mmol L^{-1}, with water T_{Amm} concentrations being approximately 4-fold greater than the plasma T_{Amm} concentrations. This has been demonstrated previously (Wilkie et al. 2011, 2015), suggesting that goldfish have evolved mechanisms to limit ammonia accumulation, likely through effects on ammonia excretion (J_{Amm}) and/or uptake across the gill epithelia.

In the present study, brain T_{Amm} approached 6–7 μmol g^{-1} wet mass (WM) in goldfish exposed to a nominal T_{Amm} concentration of 5 mmol L^{-1}. These values are comparable to other

ammonia-tolerant fishes including toadfish (*Opsanus beta*), which readily withstand neural ammonia concentrations of 4–6 μmol g^{-1} WM during HEA (Wang and Walsh 2000). Immersed (4 days) climbing perch (*Anabas testudineus*) and weatherloach (*Misgurnus anguillicaudatus*) exposed to HEA had brain ammonia concentrations near to 4 μmol g^{-1} WM (Tsui et al. 2002; Tay et al. 2006). These concentrations of brain T_{Amm} greatly exceed those known to cause morbidity and death in mammals (Felipo and Butterworth 2002), suggesting that these fishes are able to prevent or minimize ammonia-induced toxicity to the central nervous system (CNS) and are not simply avoiding ammonia neurotoxicity by impermeance to ammonia.

A major consequence of hyperammonemia in mammals is the generation of reactive oxygen species (ROS), which can cause lipid peroxidation through oxidative attack on polyunsaturated fatty acids (PUFA), yielding unstable lipid hydroperoxides (LPOs) that decompose into malondialdehyde (MDA). The concentration of MDA was quantified via the TBARS assay in goldfish brain and liver to evaluate the extent of ROS-mediated attack on lipids in these tissues during HEA. The marked increase in MDA content in the brain of warm-acclimated (14°C) goldfish at 72 h HEA, therefore suggests that peroxidative lipid damage is a major consequence of HEA in teleost brains. However, lipid peroxidation was not observed in the goldfish liver. A similar study reported 40% increases in MDA content in the goldfish liver during 48 h exposure to 1 mmol L^{-1} T_{Amm} (Sinha et al. 2014). However, this increase was only transient and MDA content (quantified as TBARS) was completely restored shortly thereafter. Thus, compared to the liver, the brain appears to be more sensitive to ammonia-induced oxidative toxicity in normothermic goldfish in this study.

The relatively greater sensitivity to HEA-induced lipid peroxidative injury observed in the brain may be because the liver has a greater relative antioxidant capacity (see below). As a

result, hepatic cells have a relatively greater capacity to detoxify ROS including superoxide anions ($O_2\bullet^-$) and hydrogen peroxide (H_2O_2) from the early stages of lipid and protein oxidation, thereby limiting the extent of oxidative cellular damage occurring in the liver relative to the brain. Moreover, neural tissue contains relatively high levels of PUFAs compared to the liver, specifically docosahexaenoic acid (DHA), which is particularly prone to lipid peroxidation in the presence of ROS (Sugihara et al. 1994; Chen et al. 2008; Friedman 2011; Pifferi et al. 2012). Furthermore, high rates of O_2 consumption, the presence of ROS-producing microglia, and the relatively high ratio of membrane surface area to cytosolic volume observed in the brain may also contribute to the greater susceptibility of neural tissue to ammonia-induced oxidative damage compared to the liver (Friedman 2011).

ROS can also oxidize amine groups on several amino acids residues (i.e. lysine, proline, arginine, or histidine), transforming them into carbonyls (Dalle-Donne et al. 2003). Protein carbonyl content, a widely used indicator of protein oxidative damage, also persisted longer in the brain and failed to completely re-establish to control levels. In fact, our results indicate that protein oxidation in the brain increased in a time-dependent manner in the warm-acclimated goldfish brain during HEA exposure, suggesting that ROS-mediated damage to proteins is also substantial in this organ during acute hyperammonemia. In contrast, protein oxidation increased only transiently in the liver, returning to control levels by 72 h HEA in warm-acclimated goldfish, likely due to the activation of one or more antioxidant enzymes (see '*Upregulation of Antioxidant Defenses During Acute Hyperammonemia*' below). While studies in fish are few, similar increases in lipid and protein oxidation have been reported in Nile tilapia (*Oreochromis niloticus*) liver and white muscle (Hegazi et al. 2010; Hegazi 2011), mudskipper gill and brain (*Boleophthalmus boddarti*; Ching et al. 2009), big head carp larvae (*Hypophthalmythys nobilis*;

Sun et al. 2012), and silver carp larvae (*Hypophthalmichthys molitrix*; Sun et al. 2011) exposed to HEA, suggesting that oxidative stress is a common response to variations in increased internal ammonia in teleosts. However, since the goldfish survive HEA, the ROS damage is likely manageable.

Mechanism(s) of Ammonia-Induced Oxidative Stress and Brain Swelling

In mammalian brains, a key mechanism of ROS generation is the overactivation of N-methyl-D-aspartate receptors (NMDArs) located in the plasma membrane of neurons and astrocytes in the brain (Schliess et al. 2002; Kosenko et al. 2003; Norenberg et al. 2007, 2009). In mammals, overactivation of NMDAr facilitates the entry of sodium and large amounts of calcium into the cell, which can stimulate ROS production through effects on superoxide ($O_2\bullet^-$) producing enzymes in the mitochondrial respiratory chain (for reviews see Kowaltowski et al. 2001; Rama Rao et al. 2003, 2005) and also by activating cytosolic Ca^{2+}-dependent enzymes, such as constitutive nitric oxide synthase (cNOS), phospholipase A_2 (PLA_s), and NADPH oxidase (NOX; Reinehr et al. 2007; Norenberg et al. 2009). There is growing evidence that NMDAr-mediated events also occur in teleosts exposed to ammonia. Inhibition of NMDAr with MK801 (dizocilpine) has been shown to prevent mortality in oriental weatherloach exposed to ammonium acetate (21 mmol Kg^{-1}; Tsui et al. 2004**)**. Additionally, pre-treatment with MK801 significantly delayed unconsciousness in plainfin midshipman (*Porichtys notatus*) exposed to ammonium chloride (10 mM; Walsh et al. 2007). More recently, NMDAr inhibition with MK801 has been shown to increase survivability in rainbow trout (*Oncorynchus mykiss*) during HEA exposure (Wilkie et al. 2011). The reversible potentiation of NMDAr currents has also been observed in isolated goldfish brain slices following acute exposure to ammonia (Wilkie et al. 2011). These observations suggest that the oxidative stress observed in goldfish brain in this

study might partially be explained by NMDAr-mediated excitotoxicity, as for mammals. The exclusive localization of this neuroreceptor to neural tissue, at least in part, may also contribute to the greater sensitivity of the brain to HEA-induced oxidative damage compared to the liver in this study.

While NMDAr inhibition with MK801 increased survival in rainbow trout (*O. mykiss*) exposed to HEA, it had no effect on survival in the ammonia-tolerant goldfish (Wilkie et al. 2011) or mudskipper (*B. boddarti*; Ching et al. 2009), suggesting that additional mechanisms are involved in ammonia neurotoxicity and ROS production in these ammonia-tolerant fish. Moreover, exposure to ammonia can induce mild oxidative stress in fish gills, liver, and muscle, which do not contain NMDAr (Erdoğan et al. 2005; Ching et al. 2009; Hegazi et al. 2010; Sun et al. 2012; Sinha et al. 2014). As stated by Ching et al. (2009), increases in oxidative cellular damage by ammonia in liver suggests that NMDAr overactivation is not the only means of ROS production during acute hyperammonemia.

ROS production in neural tissue during acute hyperammonemia may also be mediated downstream of glutamine synthesis. Much of the ammonia entering the goldfish brain during HEA exposure was converted to glutamine, via the ATP-dependent glutamine synthetase (GS)-catalyzed addition of ammonia to glutamate, forming glutamine (Mommsen and Walsh 1992; Felipo and Butterworth 2002; Walsh et al. 2007). This enzymatic process has traditionally been viewed as the principle means of ammonia detoxification in the brain (Cooper and Plum 1987; Felipo and Butterworht 2002; Walsh et al. 2007). Some researchers, on the other hand, have challenged this beneficial view suggesting that intracellular glutamine accumulation along with inhibition of the astrocytic glutamine transporter (SN1), results in increased intracellular osmolarity leading to net water influx and cell swelling (Brusilow 2002; Walsh et al. 2007).

Indeed, a strong correlation between intracranial pressure and glutamine concentration was demonstrated in patients suffering from hyperammonemia (Tofteng et al. 2006).

However, the observation in the present study that brain swelling was attenuated in cold-acclimated goldfish, despite elevated brain glutamine concentrations argues against the hypothesis that glutamine accumulation is the proximate cause of brain swelling in the goldfish. In general agreement with our findings, pre-treatment of goldfish with methionine sulfoxamine (MSO) sufficient to decrease brain GS activity and intracellular glutamine concentrations, failed to attenuate ammonia-induced brain swelling *in vivo* (Wilkie et al. 2015). Moreover, several studies have reported that brain swelling was attenuated in hyperammonemic rats made mildly hypothermic (35°C), despite elevated brain glutamine concentrations (Cordoba et al. 1999; Rose et al. 2000; Jiang et al. 2009).

While glutamine may not be acting as an osmolyte necessarily, several researchers have proposed that glutamine accumulation in the brain may be associated with oxidative and nitrosative stress (Kosenko et al. 2003; Albrecht and Norenberg 2006; Jayakumar et al. 2006b; Norenberg et al. 2007, 2009). *In vitro* mammalian studies suggest that newly synthesized glutamine is transported in excess into mitochondria, in which it is subsequently hydrolyzed back into ammonia via mitochondrial phosphate-activated glutaminases (PAG; Laake et al. 1999; Albrecht et al. 2000). Pathologically high levels of this glutamine-derived ammonia within the relatively small mitochondrial compartment may lead to the production of ROS directly or indirectly through the induction of the MPT, culminating in oxidative damage to mitochondrial components (for a review see Albrecht and Norenberg 2006). Given the role that ROS appear to play in mediating the many downstream events in ammonia-induced brain swelling in mammals (for reviews see Albrecht and Norenberg 2006; Walsh et al. 2007; Norenberg et al. 2009), it

remains critical to better understand the role that glutamine plays in this process, and brain swelling, during exposure to ammonia.

Cold Temperatures Attenuate Ammonia-Induced Neurotoxicity

Further evidence that oxidative stress is implicated in the mechanisms of ammonia-induced brain swelling is provided by the findings of the present study where cold-water acclimation (4°C) sufficient to prevent brain swelling, completely attenuates the increases in protein carbonyl content and lipid peroxidation observed in normothermic indivudals during exposure to high external ammonia. Moreover, the activity of primary antioxidant enzymes remained unaffected throughout HEA in the cold-acclimated (4°C) goldfish brain and liver, indicating that the lack of oxidative damage observed in these tissues was not due to an upregulation of antioxidant defenses. This is the first direct evidence indicating that temperature can have profound impacts on the pathophysiology of ammonia in teleosts. Similar findings have been reported in hyperammonemic rats resulting from hepatic devascularization, indicating that ROS production and brain swelling during acute hyperammonemia is temperature dependent (Rose et al. 2000; Zwingmann et al. 2004; Jiang et al. 2009).

The paucity of free radical-mediated damage and brain swelling observed in ammonia-exposed mammals made mildly hypothermic (35°C) was, at least in part, attributed to a lowering of brain ammonia concentrations (Rose et al. 2000; Jiang et al. 2009). However, acclimation of goldfish to cold water (4°C) did not attenuate increases in brain ammonia concentrations following 72 h HEA exposure. This indicates that the neuroprotective effect of cold temperatures in the present study is not mediated by an effect on blood and brain ammonia accumulation. This difference is likely due to differences in the chemical-physical state of astrocytic and neuronal membranes in cold-acclimated goldfish. Earlier studies have shown that following acclimation to

cold water (5°C), goldfish synaptosomal membranes demonstrate decreased viscosity as dictated by an increase in the degree of unsaturation of membrane phospholipids - a phenomenon known as homeoviscous adaptation (Cossins 1977; Cossins and Prosser 1978). Hence, astrocytic and neuronal membranes in cold-acclimated goldfish would exhibit relatively greater permeability, allowing rates of blood-brain diffusion of ammonia (and other solutes) to be maintained despite a significant reduction in core temperature, according to Fick's law. Owing to their known susceptibility to oxidative attack (Sugihara et al. 1994), the greater abundance of membrane PUFAs would explain the greater baseline values of TBARS observed in cold-acclimated goldfish tissues relative to normothermic individuals.

On the other hand, the present study suggests that the neuroprotective effect of cold temperatures is mediated by an effect on ammonia-, or possibly, glutamine-induced ROS production. Several possibilities exist that individually or collectively may explain the beneficial effect of cold-temperature acclimation on ammonia-induced oxidative stress in the goldfish brain. Some evidence exists that suggests NMDAr-mediated excitotoxicity is temperature dependent (for a review see Gonzalez Ibarra et al. 2011). Hypothermia has been shown to prevent the release of excitatory amino acids, including L-glutamate and aspartate in rats exposed to ischemia (Ooboshi et al. 2000) and acute hyperammonemia (Rose et al. 2000). Moreover, hypothermia has also been shown to reduce brain levels of glycine, a potent co-agonist mediating NMDAr activation, thereby protecting the brain from NMDAr-mediated excitotoxicity during cerebral ischemia in mammals (Johnson and Ascher 1987; Baker et al. 1991; Kvrivishvili 2002). Given the potential role of NMDArs in ammonia-induced ROS production (for reviews see Norenberg et al. 2007, 2009), some or all of the above could explain why a reduction in body temperature limited ammonia-induced oxidative stress in cold-

acclimated goldfish in this study.

Another mechanism by which cold acclimation may attenuate HEA-induced oxidative stress is through effects on metabolic activity. As body temperature decreases, changes in the physical chemistry of the cell causes a reduction in metabolic activity (Johnston and Dunn 1987). Acclimation of goldfish to temperatures below 10°C evokes a highly regulated reduction in metabolic rate, enzyme activity, and alterations in enzyme-substrate affinity relative to warm-acclimated individuals (Kanungo and Prosser 1959; Johnson and Dunn 1987). Indeed, acetylcholinesterase activity was significantly reduced in cold-acclimated goldfish brains (Hazel 1969; Johnston and Dunn 1987). More importantly, Jiang et al. (2009) noted significant reductions in the activities of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) by hypothermia in ammonia-exposed rat brain *in vivo*, thereby effectively decreasing rates of NO production in hypothermic mammalian brains relative to normothermic individuals.

Upregulation of Antioxidant Defenses During Acute Hyperammonemia

A change in the redox state of proteins and lipids in the cell can serve as an intracellular signal of oxidative stress, which can regulate the cellular response (Nguyen et al. 2009; Lushchak 2011). This phenomenon has been suggested for goldfish exposed to variations in oxygen availability, in which a transient increase in oxidative stress was suggested to trigger an increase in antioxidant potential characterized by the up-regulation of several antioxidant enzymes in goldfish tissues (Lushchak et al. 2001; Lushchak et al. 2006). Redox-sensitive transcription factors such as AP-1, NF-κB, and Nrf2 can interact with the antioxidant response element (ARE) in cellular DNA and mediate the adaptive response by inducing the transcription of various cytoprotective genes such as, GSTA2 (glutathione-*S*-transferase A2) and NQO1

(NADPH:Quinone Oxidoreductase; Nguyen et al. 2009; Lushchak 2011). In addition to inducible gene expression, the ARE also plays a critical role in the regulation of basal expression of several genes involved in antioxidant defense in order to manage cellular redox homeostasis under non-stressed conditions. This study focused on one component of antioxidant defense – antioxidant enzymes.

Superoxide dismutase (SOD) catalyzes the conversion of highly toxic $O_2\bullet^-$ to H_2O_2. The induction of SOD during HEA exposure is suggestive of an increase in $O_2\bullet^-$, at least in hepatic tissue. Given the importance that the liver plays in the systemic metabolism of potentially harmful substances including xenobiotics and ROS (Buhler and Rasmussen 1968; Remmer 1970), it likely plays a key role in protecting the CNS from ammonia-induced ROS production. However, baseline levels of SOD were similar in liver and brain, suggesting that they have a similarly high basal capacity to scavenge $O_2\bullet^-$, as also observed by Lushchak et al. (2001) in the same species. The marked increase in SOD activity in the goldfish liver might partly explain the restoration of protein carbonyl content to control values by 72 h HEA. Similar elevations in hepatic SOD activity have been demonstrated in the liver of goldfish exposed to various metals (Vieira et al. 2002; Shi et al. 2005; Kubrak et al. 2010), color additives (Sun et al. 2006), herbicides (Fatima et al. 2007), aromatic amines (Liu et al. 2009), and 1 mmol L^{-1} NH_4HCO_3 (Sinha et al. 2014). The minimal change in SOD activity in the brain during HEA exposure suggests that nervous tissue does not rely on SOD, however. Interestingly, Luschak et al. (2001) observed no increases in SOD activity in goldfish brain and liver during post-anoxia recovery, during which time there was ample evidence of ROS generation based on increases in conjugated dienes and the induction of other antioxidant enzymes in both tissues. The observed induction of SOD in the present study supports the premise that ammonia accumulation results in a level of

oxidative stress that is similar to that associated with post-anoxia exposure in this species (Lushchak et al. 2001). However, the less marked induction of SOD in the brain compared to the liver, also suggests that this organ has a lower reserve capacity to scavenge $O_2\bullet^-$, and apparently it is not the only mechanism to manage ammonia-induced ROS production in neural tissue.

Catalase (CAT) is an important primary antioxidant that catalyzes the decomposition of H_2O_2 arising from the dismutation of $O_2\bullet^-$ radicals. As commonly observed for other lower vertebrates (Hermes-Lima and Storey 1993; Perez-Campo et al. 1993; Hermes-Lima and Storey 1996; Joanisse and Storey 1996), CAT activity in control goldfish was much higher in the liver (66 U mg protein^{-1}) than in the brain (19 U mg protein^{-1}), which likely renders the liver significantly more resistant to ammonia-induced H_2O_2 overproduction and peroxidative tissue injury. Surprisingly, despite very high constitutive expression compared to the brain, hepatic CAT activity doubled during exposure to HEA. Interestingly, CAT activities in the liver have also been shown to undergo significant activation from relatively high constitutive levels in goldfish exposed to anoxia/reoxygenation (Lushchak et al. 2001), hyperoxia (Lushchak et al. 2006), naphthalene (Shi et al. 2005), glyphosate herbicide (Lushchak et al. 2009), and low concentrations of ammonia (Sinha et al. 2014). This observation, in conjunction with marked elevations in SOD activity, likely explains the restoration of protein carbonyl content in the liver after 72 h HEA. Unlike SOD, however, CAT activity in the brain was activated at earlier exposure periods and remained elevated throughout HEA, suggesting that H_2O_2 overproduction arising from $O_2\bullet^-$ generation is a problem in the goldfish brain during acute hyperammonemia, at least at warmer temperatures. Similarly, CAT displayed a significant induction in the goldfish brain during exposure to stressors capable of inducing oxidative stress such as during post-hyperoxic recovery and exposure to 2,4-dichlorophenoxyacetic acid (Lushchak et al. 2005;

Matviishyn et al. 2014).

Glutathione peroxidase (GPx) and glutathione reductase (GR) are important secondary antioxidants involved in various reactions of the glutathione-dependent cycle. GPx catalyzes the reduction of H_2O_2 and lipid peroxides arising from the earlier stages of lipid peroxidation in the cell, converting reduced glutathione (GSH) to its oxidized form (GSSG) in the process. Thus, a change in the activity of GPx may be an important indicator of the ability of an organism to manage oxidative stress. At first glance, the lack of GPx induction in the liver, and the minimal change in GPx activity in the brain during HEA exposure, might suggest goldfish have a relatively low capacity for managing fluctuations in H_2O_2 and LPO levels. However, while the specific activity of GPx decreases in the brain of most ammonia-sensitive mammals during acute hyperammonemia (Kosenko et al. 1997, 1998, 2003), goldfish maintain constitutive GPx activity in both organs throughout HEA. The maintenance of constitutive GPx activities in both the brain and liver, along with the transient increase in the brain at 24 h HEA, may limit oxidative stress in both organs to a manageable and physiologically tolerable level. This result may explain why lipid peroxidation did not increase in the brain during the first 24 h of HEA. Moreover, the maintenance of a high intracellular GSH pool via the glutathione reductase (GR)-dependent or independent recycling of intracellular GSSG may reduce the reliance on such flexibility (see below).

GR catalyzes the reduction of glutathione disulfide (GSSG) to GSH, which maintains a strong reducing environment in the cell, thereby increasing its basal antioxidant scavenging capacity. Ammonia induced a significant increase in GR activity in both the goldfish liver and brain, suggesting that following exposure to acute hyperammonemia, hepatic and nervous tissue respond by increasing the rate of GSH regeneration, thereby rendering them relatively more

effective at managing ammonia-induced ROS production through GPx-catalzyed and spontaneous reduction of H_2O_2 and LPOs. However, GR activity in the goldfish brain was stimulated at an earlier exposure period (24 h) and did not recover during HEA, indicating that a significantly greater degree of ammonia-induced oxidative stress is occurring in neural tissue. This improved capacity for GSSG reduction in neural tissue likely explains why GPx activity in the goldfish brain increased, albeit transiently, while hepatic GPx remained largely unaffected. Nevertheless, as a limitation of this analysis we need to acknowledge that the concentration of cellular GSH was not measured. However, intracellular GSH concentrations in the goldfish brain and liver were maintained during exposure to anoxia/reoxygenation (Lushchak et al. 2001), 2,4-dichlorophenoxyacetic acid (Matviishyn et al. 2014), and 1 mmol L^{-1} NH_4HCO_3 (Sinha et al. 2014). Moreover, lipid peroxidation appears to be a more specific indicator of ROS-mediated cytotoxicity in fish than a change in cellular GSH concentration (Rau et al. 2004). Nevertheless, our results indicate that goldfish upregulate certain antioxidant enzymes during HEA exposure to manage increased rates of ROS production in both the brain and liver.

The goldfish liver was characterized by constitutively higher activities of GPx and GR, approximately 2– 4 times more than in the brain. Moreover, hepatic CAT activity was ~3 times greater than in the brain. Similar findings have been observed for other lower vertebrates (Hermes-Lima and Storey, 1993; Perez-Campo et al. 1993; Hermes-Lima and Storey 1996; Joanisse and Storey 1996), effectively rendering the liver relatively more resistant to H_2O_2 overproduction. The higher constitutive expression of antioxidant enzymes in the liver compared to the brain may be due to the fact that hepatic tissue is an important site of numerous oxidative reactions, which leads to high steady-state levels of ROS production (Lushchak 2011), arising from high rates of cytochrome P450 (CYPs) and peroxisomal enzymes (Goksøyr and Förlin

1992; Orbea et al. 1999; Schlezinger and Stegeman 2001; Orbea et al. 2002; Valko et al. 2007). It is also important to note that the liver is the most important site of ammoniogenesis in goldfish under normal physiological conditions (Van Waarde 1983). As a result, high constitutive antioxidant enzyme would also limit oxidative cellular damage by making it relatively less vulnerable to increases in ROS and the accumulation of oxidized proteins and lipids in the cell. This may explain the relatively greater susceptibility of nervous tissue and the higher resistance of hepatic tissue to ammonia-induced oxidative damage in the goldfish and perhaps other fish species.

Collectively, these findings suggest that goldfish brains have a high capacity to withstand oxidative and nitrosative stress, and are more resilient to this homeostatic disturbance than mammalian brains. Few studies have addressed the antioxidant response to ammonia in the teleost brain. Unlike other studies on ammonia neurotoxicity, we observed the upregulation of several key antioxidant enzymes and the maintenance of most other enzymatic activities (CAT, GPx, and GR) in the ammonia- and anoxia-tolerant goldfish brain during exposure to high external ammonia. In ammonia-sensitive mammals, antioxidant defenses in the brain are overwhelmed by high rates of ROS production and become rapidly down-regulated following injection with high doses of ammonium salts (Kosenko et al. 1997; Kosenko et al. 1998; Kosenko et al. 1999). Similarly, in the brain of mudskipper (*B. boddarti*), significant decreases in the activity of certain antioxidant enzymes such as catalase and glutathione reductase were observed during HEA (Ching et al. 2009). Although the level of MDA and protein carbonyls in the goldfish brain were elevated at 72 h HEA, the upregulation of certain antioxidant enzymes (CAT, GPX, GR) and the maintenance of other enzymatic activities may have limited cellular oxidative damage and brain swelling to a level that is physiologically tolerable and reversible in

the goldfish brain. This adaptation has also been demonstrated in the brain of goldfish exposed to post-anoxic recovery, where rates of ROS production can be devastating (Lushchak et al. 2001; Bickler and Buck 2007). While the antioxidant response in the brain of most animals is modest (Friedman 2011), the adaptive response by antioxidant enzymes in the brain reported here may contribute to the extreme neural tolerance of ammonia and associated swelling in the goldfish (Wilkie et al. 2011, 2015).

CONCLUSIONS

This study implicates oxidative stress in the mechanisms of ammonia-induced brain swelling in teleosts, in which blood ammonia concentrations can fluctuate markedly following feeding, exercise, and exposure to environmental ammonia (Wang et al. 1994a; Wicks and Randall 2002; Eddy 2005). The production of reactive oxygen species (ROS) and swelling in the goldfish (*Carassius auratus*) brain during acute exposure to high environmental ammonia (HEA) are temperature-dependent processes. Although oxidative cellular damage in the goldfish brain is still significant during HEA at warmer temperatures, the overall increase in neural antioxidant enzyme activity and the maintenance of other antioxidant enzyme activities may control the extent of oxidative damage and associated brain swelling to a level that is physiologically manageable and reversible for the organ (Wilkie et al. 2015). This adaptive response by antioxidants in the goldfish brain likely explains the extreme neural tolerance to ammonia and hypoxia in this species and the closely related curcian carp (*Carassius carassius*). With adaptations to limit oxidative damage and brain swelling, goldfish have the capacity to inhabit, exploit, and thrive in a wide range of niches that are often ice-covered, marginal, eutrophic, and unhabitable for potential competitors and predators. In mammals, however, neural antioxidant defenses are modest (Kosenko et al. 1997; Kosenko et al. 1998; Kosenko et al. 1999; Friedman 2011), requiring precise control of internal ammonia concentrations through metabolically demanding pathways in order to prevent oxidative stress, brain swelling, and irreversible apoptotic/necrotic brain damage.

Chapter 3

An Integrative Approach to Studying the Mechanisms of Ammonia-Induced Neurotoxicity in Vertebrates

This study implicates oxidative stress in the mechanism of ammonia-induced brain swelling in fish, in which blood ammonia concentrations can fluctuate markedly following feeding, exercise, and exposure to environmental ammonia arising from anthropogenic inputs such as agricultural run-off, industrial waste, sewage effluent, or overcrowding in confined, aquaculture facilities (Wang et al. 1994a; Wicks and Randall 2002; Eddy 2005). This study shows that increases in internal ammonia lead to a temperature-dependent oxidative stress response, which was most pronounced in the brain. Unlike other studies on ammonia tolerance in fishes, the present study demonstrated a significant upregulation in the activity of key antioxidant enzymes in the goldfish brain. This high antioxidant capacity may be an adaptation underlying the extreme ammonia tolerance of the goldfish, thereby minimizing the extent of ROS-mediated damage occurring in the brain and liver during acute ammonia exposure. Indeed, the high antioxidant capacity in the goldfish, and the closely related crucian carp (*Carassius carassius*), is likely a key factor that protects these anoxia-tolerant fishes from oxidative damage following anoxia and reoxygenation, where rates of ROS production can be devastating (Lushchak et al. 2001; Bickler and Buck 2007). While oxidative stress is still significant in the normothermic goldfish brain during HEA, the overall increase in neural antioxidant enzyme activity and the maintenance of most other antioxidant enzyme activities may control the extent of oxidative brain damage, and brain swelling, to a level that is physiologically manageable and reversible (Wilkie et al. 2015).

Proposed Model of Ammonia-Induced Brain Swelling in Teleosts

Ammonia has been shown to affect numerous metabolic pathways in mammals (for reviews see Norenberg et al. 2007, 2009). Here, I propose a contemporary model of brain swelling in teleosts, in which oxidative stress mediates the downstream events of ammonia-

induced neurotoxicity leading to a disruption of cell volume homeostasis in astrocytes. A key event in this process is the overactivation of N-methyl-*D*-aspartate receptors (NMDArs; Fan and Szerb 1993; Hermengildo et al. 1996, 2000), located on the surface of neurons and astrocytes in the teleost brain. Direct inhibition of astrocytic Na^+-glutamate co-transporters EAAT-1 and EAAT-2 by ammonia may cause an accumulation of excitatory L-glutamate in the synaptic cleft following its release from pre-synaptic neurons, resulting in the overstimulation of post-synaptic NMDA receptors (Knecht et al. 1997; Chan et al. 2000; Butterworth 2001; Walsh et al. 2007).

Growing evidence indicates that NMDAr-mediated events occur in teleosts exposed to ammonia. Inhibition of NMDAr with MK801 (dizocilpine) prevented mortality in oriental weather loach (*M. anguillicaudatus*) exposed to ammonium acetate (Tsui et al. 2004). More recently, NMDAr inhibition with MK801 has been shown to attenuate brain swelling and increase survivability in rainbow trout during HEA exposure (Wilkie et al. 2011; unpublished observations P.Q.H. Pham-Ho, M.P. Wilkie). The reversible potentiation of NMDAr currents has also been demonstrated in isolated goldfish brain slices following acute exposure to ammonia (Wilkie et al. 2011).

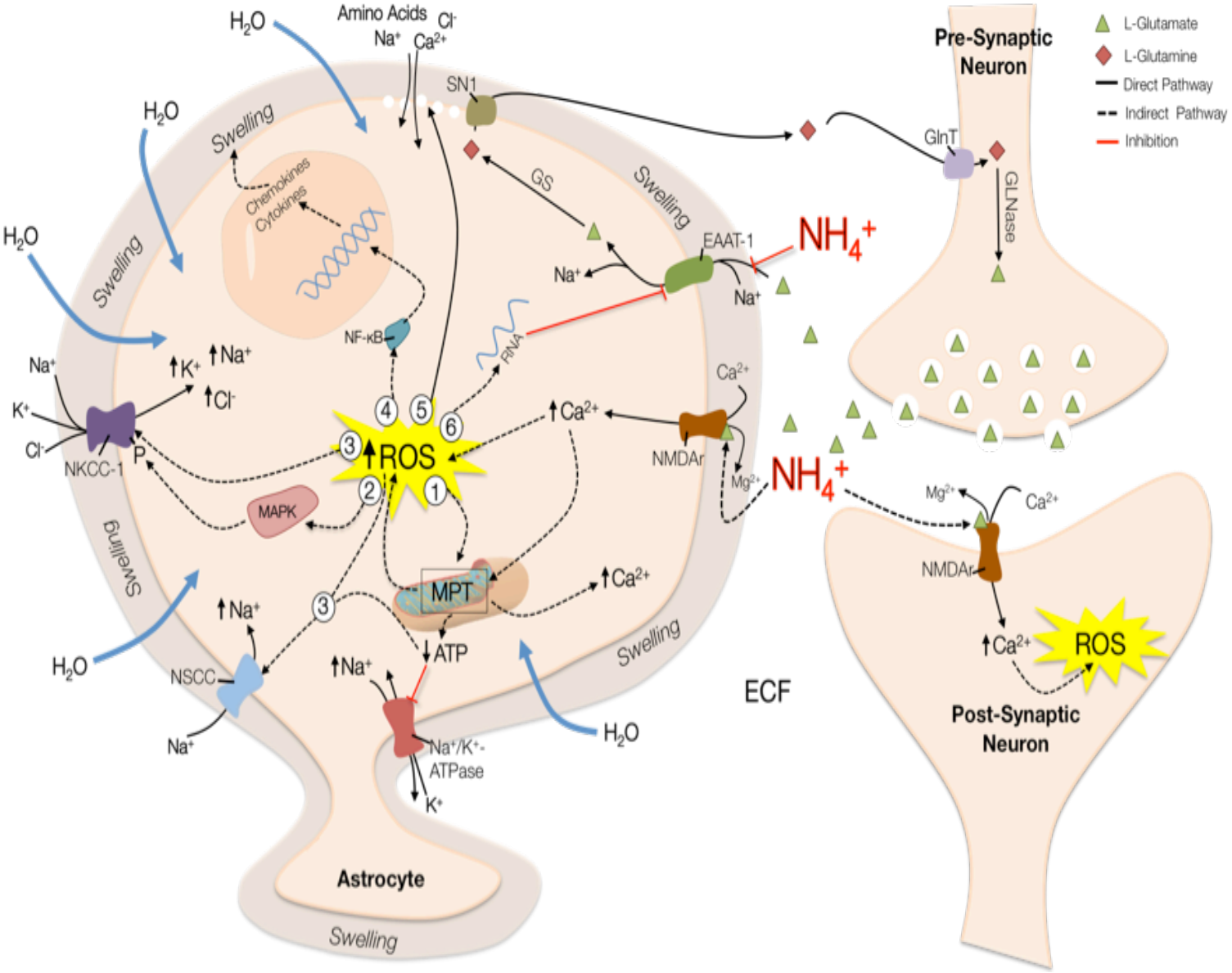
Overactivation of the NMDArs facilitates the entry of sodium (Na^+) and large amounts of Ca^{2+} into post-synaptic neurons and associated astrocytes (Schliess et al. 2002; Walsh et al. 2007). Increased intracellular Ca^{2+} concentrations can stimulate the production of reactive oxygen species (ROS) in both neurons and astrocytes through effects on superoxide ($O_2\bullet^-$) generating enzymes in the mitochondrial respiratory chain (Figure 1.3; Kowaltowski et al. 2001; Rama Rao et al. 2003, 2005). Ca^{2+} can also promote ROS generation by activating several cytosolic Ca^{2+}-dependent enzymes, such as constitutive nitric oxide synthase (cNOS) and NADPH oxidase (NOX), liberating NO• and $O_2\bullet^-$ respectively (Reinehr et al. 2007, 2009). ROS

and ammonia may also induce the mitochondrial permeability transition (MPT) in astrocytes, contributing to the deterioration of the mitochondrial membrane potential ($\Delta\Psi_m$), impaired oxidative phosphorylation, and the further production of ROS (for reviews see Kowaltowski et al. 2001; Rama Rao et al. 2003, 2005; Norenberg et al. 2009). Although studies in fish are few, the present study showed that a significant degree of oxidative cellular damage occurs in the goldfish brain during exposure to HEA. Moreover, HEA-induced oxidative stress has also been reported in the brain of mudskipper (*B. boddarti*; Ching et al. 2009).

However, the precise mechanisms by which reactive oxygen species (ROS) cause swelling in the teleost brain still remain poorly understood. Several possibilities exist that individually or collectively may result in defective ionic and cell volume regulation in the teleost brain, and these should be the focus of future research in this field. Indeed, significant increases in intracellular osmolarity, particularly $[Na^+]_{intracellular}$, have been observed in goldfish brain following acute exposure to HEA (Phillip Q.H. Pham-Ho, unpublished observations). When overproduced, ROS can oxidize key lipids, proteins, and nucleic acids involved in maintaining ionic and osmotic gradients across membranes, leading to a loss of membrane integrity and astrocyte swelling (Figure 3.1).

Ammonia-induced oxidative stress may bring about direct oxidative injury to mitochondrial and cellular membrane lipids, leading to permeabilization and altered bioenergetics, which is critical in the maintenance of ionic and cell volume homeostasis (Figure 3.1). As already discussed, one major consequence of ROS production in cells is the induction of mitochondrial permeability transition (MPT; for reviews see Kowaltowski et al. 2001; Rama Rao et al. 2003, 2005; Norenberg et al. 2004, 2007, 2009). As a result, mitochondrial membrane potential diminishes causing impaired oxidative phosphorylation, ATP synthesis, and

Figure 3.1. Proposed model of ammonia-induced astrocyte swelling in teleost brains: role for reactive oxygen species (ROS). Ammonia overstimulates NMDAr leading to an influx of calcium (Ca^{2+}) ions into astrocytes and neurons. Increased intracellular Ca^{2+} can stimulate ROS production through activation of Ca^{2+}-dependent enzymes in the cytosol (not shown) and mitochondrial uncoupling. The ROS produced may mediate several downstream events leading to a disruption in ionic and osmotic gradients, and astrocyte swelling: (1) ROS can induce mitochondrial permeability transition (MPT), leading to bioenergetic failure and further ROS production, resulting in the failure of ion-motive pumps to maintain transmembrane ion gradients; (2) ROS may activate mitogen-activated protein kinases (MAPKs) that phosphorylate (activate) NKCC-1, leading to a rapid influx of Na^+, K^+, and Cl^- ions; (3) ROS may also directly oxidize and activate certain ion transport systems (i.e. NSCCs), further exacerbating the loss of ion homeostasis; (4) ROS may activate nuclear factor-kappaB (NF-κB), leading to the transcription and expression of various chemokines and cytokines, which mediate cell swelling; (5) ROS can also directly oxidize membrane lipids leading to increased permeabilization and loss of membrane stability; (6) Lastly, ROS may oxidize RNA involved in gene expression and the synthesis of proteins involved in ion homeostasis. Water follows the influx of ions through osmosis, culminating in unregulated astrocyte swelling. EAAT-1, excitatory amino acids transporter 1; NKCC-1, Na^+-K^+-$2Cl^-$ co-transporter 1; NSCC, non-selective cation channel; GLNase, glutaminase; SN1, Na^+/glutamine co-transporter; NMDAr, *N*-methyl-D-aspartate receptor; ATP, adenosine triphosphate; GS, glutamine synthetase; NH_4^+, ammonium; GlnT, neuronal glutamine transporter.


H2O
Amino Acids
Cl-
Na+
Ca2+
SN1
Swelling
Chemokines
Cytokines
GS
EAAT-1
NH4+
Na+
NF-κB
RNA
K+
Cl-
NKCC-1
P
ROS
MAPK
Ca2+
NMDAr
Mg2+
MPT
ATP
NSCC
Na+/K+-
ATPase
Astrocyte
ECF
Pre-Synaptic
Neuron
GlnT
GLNase
Post-Synaptic
Neuron
L-Glutamate
L-Glutamine
Direct Pathway
Indirect Pathway
Inhibition

bioenergetic failure. Maintaining cell volume homeostasis is an energy-dependent process due to the operation of ion-motive pumps (ie. Na^+/K^+-ATPases), exchangers, and the extrusion of osmotically-active amino acids (Kimelberg et al. 1993; Kimelberg 1995; Kimelberg and Mongin 1998). Researchers have shown that energy metabolism is closely related to cell volume regulation in mammalian astrocytes *in vitro* (Olson et al. 1986; Olson and Evers 1992). Therefore, a ROS-induced disturbance in bioenergetics, conceivably via the induction of the MPT, may contribute to a loss of ion homeostasis and cell swelling (Figure 3.1).

While the failure of energy-dependent transporters to effectively extrude osmotically active solutes from the cytosol is a likely consequence of ammonia-induced ROS production, augmented ion influx may also be possible. ROS may participate in signaling cascades involved in regulating the activity of key ion transport systems required for cell volume homeostasis in astrocytes. For instance, one known consequence of oxidative stress is the activation of mitogen-activated protein kinases (MAPKs; Guyton et al. 1996; Kyriakis and Ayruch 1996; Lo et al. 1996; Aikawa et al. 1997; Suzuki et al. 1997; Czaja et al. 2003). These well-known serine/threonine kinases phosphorylate, and subsequently activate numerous enzymes, transcription factors, structural proteins, and other intracellular signaling proteins involved in regulating a number of cellular responses to extracellular stimuli (Remacle et al. 1995; Sen and Packer 1996; Suzuki et al. 1997; Czaja et al. 2003). Evidence suggests that these signaling proteins are also stimulated by cell stress, including free radicals (for reviews see Pearson et al. 2001; English and Cobb 2002). The activation of MAPKs in ammonia-exposed mammalian astrocytes has also been demonstrated *in vitro* (Jayakumar et al. 2006a). Subsequent treatment with various antioxidants mitigated such activation *in vitro*, suggesting that oxidative stress was responsible for the observed MAPK phosphorylation (Jayakumar et al. 2006a). Moreover, pre-

treatment with specific kinase inhibitors resulted in a marked reduction in astrocyte swelling following ammonia exposure, indicating that MAPK activation is a key step in the mechanism of ammonia-induced astrocyte swelling (Jayakumar et al. 2006a). MAPK activation (phosphorylation) has not yet been studied in ammonia-exposed teleost brains, but given the significant degree of oxidative stress observed in the goldfish brain in this study, it would be informative to study the MAPK response to ammonia in fish. While the mechanisms through which MAPK phosphorylation mediates ammonia-induced cell swelling are still not understood, the phosphorylation and activation of key proteins involved in ion transport is a likely possibility.

Na^+-K^+-$2Cl^-$ (NKCC) cotransporters are a key class of membrane transport proteins that actively transports Na^+, K^+, and Cl^- into and out of a wide variety of cells, including astrocytes (Tas et al. 1986, 1987; Walz 1992; Haas 1994; Plotkin et al. 1997; Yan et al. 2001). Activation of astrocytic NKCC-1 has been implicated in brain swelling in a variety of neurological disorders (Chen and Sun 2005). Cultured rat astrocytes treated with ammonia (NH_4Cl, 5 mM) demonstrate significantly greater NKCC-1 expression and activity, and inhibition of such activity with bumetanide effectively attenuated ammonia-induced astrocytic swelling (Jayakumar et al. 2008). Interestingly, Jayakumar et al. (2008) showed that such astrocytes demonstrated significant increases in the degree of oxidation/nitration of NKCC-1 and phosphorylated NKCC-1. Treatment with various antioxidants (dimethylthiourea, Mn(III) tetrakis (4-benzoic acid) porphyrin, CAT, α-tocopherol, tempol) decreased the phosphorylation and activity of NKCC-1, indicating that oxidative stress was responsible for phosphorylation and activation of this transporter in ammonia-exposed astrocytes, thereby providing a key link between oxidative stress, MAPK activation, and cell swelling in the mammalian brain during acute

hyperammonemia (Figure 3.1).

Non-selective cation channels (NSCC) may represent another key target for ROS-mediated disturbances in cell volume homeostasis. Although largely dormant in healthy cells, NSCCs may be activated under severe oxidative stress, facilitating large influxes of Na^+ and Ca^+ ions, followed by water and cell swelling (Barros et al. 2001; Simon et al. 2002). NSCCs are ubiquitous in mammals, yet most function in spontaneous membrane depolarization and neuronal firing by facilitating the entry of various types of cations into the cell (Kim et al. 2007; Blythe et al. 2009; Mrejuru et al. 2011). However, given the functional relationship between these transport systems and oxidative stress, it is likely that they also play a role in mediating ammonia-induced swelling in vertebrate brains (Figure 3.1).

Interestingly, a well-known known consequence of oxidative stress and MAPK phosphorylation is the activation of the ubiquitous, rapid-response transcription factor, nuclear factor-kappaB (NF-κB; for a review see Norenberg et al. 2007). NF-κB regulates cellular immune and inflammatory responses by stimulating the expression of numerous chemokines, cytokines, cell adhesion molecules (CAMs), growth factors, and immunoreceptors (Baldwin 1996; Ahn et al. 2007). Cultured astrocytes treated with ammonia (5 mM, NH_4Cl) demonstrate a significant increase in NF-κB nuclear translocation (Sinke et al. 2008), while antioxidants (SOD, Vitamin E) and MAPK inhibitors all block such activation. Inhibition of NF-κB activation by treatment with NF-κB inhibitors (BAY11-7082 and SN-50) attenuated ammonia-induced astrocyte swelling *in vitro* (Sinke et al. 2008). Ammonia-induced brain swelling was also completely attenuated in transgenic mice lacking functional NF-κB *in vivo* (Jayakumar et al. 2011), suggesting that NF-κB activation by ROS and/or MAPK is a critical factor in mediating ammonia-induced astrocyte swelling in mammals (Figure 3.1). While these studies implicate

NF-κB in cell swelling mechanisms in cultured astrocytes as a consequence of oxidative stress, the role of NF-κB in the brain edema associated with ammonia toxicity is not known.

Although data sets are limited, recent evidence suggests that ammonia also induces a rapid oxidation of astrocytic and neuronal RNA *in vitro* and *in vivo* (Görg et al. 2008; Haussinger and Görg 2010). Although this is a more recently recognized consequence of ammonia toxicity in vertebrates, RNA oxidation may affect gene expression and the synthesis of signaling and transport proteins involved in maintaining ionic and osmotic homeostasis in astrocytes, further exacerbating astrocytic swelling during acute hyperammonemia. Interestingly, mRNA coding for EAAT-1 becomes oxidized during acute ammonia toxicity *in vitro*, contributing to the further aggrevation of glutamate excitotoxicity through the inhibition of glutamate synaptic uptake (Görg et al. 2008; Figure 3.1). Further research on the oxidation of nucleic acids during ammonia exposure could yield insights into alternative mechanisms of ammonia-induced brain swelling and may provide another link between ROS production and the mechanisms of cell swelling in vertebrates.

Numerous studies implicate the induction of oxidative stress as a critical signaling factor mediating many of the downstream events leading to cerebral/astrocytic swelling in vertebrates. In this model, events triggered by ROS represent the principal means through which ammonia causes a disruption in cell volume homeostasis, including activation/induction of the MPT, MAPKs, NF-κB, and various ion transport systems, culminating in bioenergetic failure and loss of ion homeostasis. Oxidation of astrocytic RNA has more recently emerged as a potential consequence of ammonia-induced oxidative stress and may also contribute to the swelling characteristics of astrocytes exposed to ammonia.

An Integrative Model of Ammonia-Induced Brain Swelling in Vertebrates

The proposed signaling events through which ammonia-induced oxidative stress leads to astrocyte swelling in teleosts are illustrated in Figure 3.1. It is important to emphasize that while the discussion thus far has implied a linear process, complex interactions among reactive oxygen species (ROS) and their downstream targets exist. The MPT, MAPK, and NF-κB are all capable of generating additional ROS, resulting in a self-amplifying positive feedback loop and amplification of the oxidative stress originally induced by ammonia (not shown; Norenberg et al. 2007).

In this model, ammonium ions (NH_4^+) overstimulate NMDArs leading to an influx of Ca^{2+} and Na^+ ions into astrocytes and neurons. Increased intracellular Ca^{2+} can stimulate reactive oxygen species (ROS) production through activation of Ca^{2+}-dependent enzymes in the cytosol, such as constitutive nitric oxide synthase (cNOS) and phospholipase A_2 (PLA2), liberating NO and $O_2\bullet^-$, respectively. High intracellular Ca^{2+} triggers oxidative stress through mitochondrial Ca^+ uncoupling and the induction of MPT.

The ROS produced as a result of ammonia-induced excitotoxicity mediates several downstream events leading to a disruption in ionic and osmotic gradients, and astrocyte swelling. In addition to high intracellular Ca^{2+}, high levels of ROS production can also induce mitochondrial permeability transition (MPT), leading to bioenergetic failure. As a result, ion-motive pumps (ie. Na^+/K^+-ATPases) lose their ability to maintain transmembrane ion gradients, causing net membrane depolarization and increased intracellular osmolarity as Na^+ ions move down their electrochemical gradient into the astrocyte. ROS also activate mitogen-activated protein kinases (MAPKs) that phosphorylate, and subsequently activate, NKCC-1 co-transporters located along the astrocytic membrane. Such activation leads to an influx of Na^+, K^+,

and Cl^- ions into the astrocyte, increasing intracellular osmolarity further. Additionally, certain non-selective cation channels (NSCCs) present on the astrocytic membrane become activated by the high degree of oxidative stress, further exacerbating the loss of ion homeostasis and net increase in intracellular osmolarity.

Although less understood, ROS appears to activate nuclear factor-kappaB (NF-κB), leading to the transcription and expression of various chemokines and cytokines mediating a variety of inflammatory responses and swelling in the astrocyte. ROS also directly oxidizes membrane lipids leading to increased permeabilization of organelle and plasma membranes, loss of astrocytic membrane integrity, and increased intracellular osmolarity as ions moved down their electrochemical gradients into the cytosol. Lastly, ROS may oxidize RNA involved in gene expression and the synthesis of transport and signaling proteins involved in maintaining ion homeostasis in the astrocyte. Oxidation of these RNA transcripts can cause altered expression of certain transporters and signaling molecules, causing osmotic and ionic gradients across astrocytic membranes to be altered, further exacerbating the increase in intracellular osmolarity duing ammonia-induced oxidative stress in the vertebrate brain. Consequently, the increase in intracellular osmolarity following the loss of astrocytic membrane integrity causes increased osmotic forces leading to astrocyte swelling.

An Integrative Perspective of Ammonia Toxicity, Encephalopathy, and Tolerance

a. Clinical Implications and Perspectives

Brain swelling is a characteristic feature of several hyperammonemic syndromes in humans, such as acute liver failure (ALF), fulminant hepatic failure, Reye's syndrome, and congenital urea cycle pathologies (Felipo and Butterworth 2002). The brain swelling observed in patients presenting with various liver insufficiencies is referred to clinically as hepatic

encephalopathy (HE; Blei and Cordoba 2001). Such brain swelling is accompanied by increases in intracranial pressure, brain herniation, and is the most frequent cause of death in patients exhibiting HE (Felipo and Butterworth 2002; Norenberg et al. 2007). Currently, the only effective treatment for the brain swelling associated with HE is an emergency liver transplantation (Hoofnagle et al. 1995; Norenberg et al. 2007).

While the molecular basis for ammonia-induced brain swelling remains elusive, a better understanding has emerged from a multitude of mammalian studies. The majority of current research directed to finding therapeutic options for HE has focused on identifying the detailed, molecular mechanisms underlying the pathogenesis of ammonia toxicity in the mammalian brain (for reviews see Norenberg et al. 2007, 2009). However, complex interactive pathways exist and identifying the best target for therapy can be very difficult. Alternatively, unraveling the adaptive responses of neurons and astrocytes to ammonia in ammonia-tolerant vertebrates may be a very valuable tool in the pursuit of new, neuroprotective strategies (Bickler 2004). While it may be difficult to directly translate results from the comparative physiology laboratory to the clinic, advancements in our understanding of how cells adapt to stressors will provide insights that may eventually lead to treatments.

Examples of such translation to clinical medicine include the alpha-stat hypothesis of pH balance during cardiopulmonary bypass and the use of therapeutic hypothermia (TH) in cardiac surgery, trauma, neurological disorders, and cancer (Bigelow et al. 1950; Reeves 1972; Bickler 2004). According to the alpha-stat hypothesis, intracellular pH changes with temperature, such that it remains at or near the pH of neutrality (Reeves 1972). This is achieved by appropriate temperature-induced changes in the pK of the imidazole group of histidine residues of intracellular proteins. The idea that the degree of dissociation (termed ‘alpha’) of imidazole

remains constant, despite changes in temperature is known as the alpha-stat hypothesis. Conventionally, the pH-stat hypothesis argues that intracellular pH should be kept constant despite changes in body temperature. However, the alpha-stat hypothesis corrects for the patients actual body temperature, thereby maintaining the net charge on intracellular proteins and protein function despite significant changes in body temperature. This has significant implications for clinical practice (e.g. management of hypothermia (20°C) during cardiopulmonary bypass).

Along with its known anoxia-tolerance, the present study showed that goldfish (*Carassius auratus*) exhibit a significantly high neural tolerance to ammonia, withstanding brain T_{Amm} concentrations of ~ 6-7 mmol Kg^{-1} WM (Figure 2.2; Wilkie et al. 2011). These concentrations of brain T_{Amm} greatly exceed those known to cause morbidity and death in mammals (Felipo and Butterworth 2002), suggesting that goldfish neurons and/or glial cells have evolved mechanism(s) of limiting ammonia-induced toxicity to the CNS. Therefore, unraveling the adaptive response of the goldfish to ammonia-induced neurotoxicity may prove extremely useful in the development of new, neuroprotective strategies to HE and related neurological disorders in the biomedical field.

Collectively, the findings of the present study suggest that goldfish brains have a high capacity to undergo oxidative stress, and are more resilient to this homeostatic disturbance than mammalian brains. Unlike other studies on ammonia neurotoxicity, we observed the upregulation of several key antioxidant enzymes and the maintenance of other enzymatic activities in the goldfish brain during acute hyperammonemia. In ammonia-sensitive mammals, antioxidant defenses in the brain are modest and become rapidly down-regulated following exposure to ammonium salts (Kosenko et al. 1997; Kosenko et al. 1998; Kosenko et al. 1999). Although oxidative stress and swelling in the goldfish brain were still significant, the relative

increase in antioxidant capacity observed here likely limited cellular oxidative damage and swelling to a level that is physiologically tolerable and reversible for the goldfish brain (Wilkie et al. 2015). This adaptation has also been demonstrated in the brain of goldfish exposed to post-anoxic recovery, where rates of ROS production can be devastating (Lushchak et al. 2001; Bickler and Buck 2007).

Consequently, antioxidant therapy may prove to be an important neuroprotective strategy against ammonia-induced brain swelling in humans. Currently, one antioxidant demonstrating success in clinical trials for stroke therapy is Ebselen (Minnerup et al. 2012). Ebselen, an organoselenium compound, mimicks glutathione peroxidase (GPx) activity and protects cellular components from ROS-mediated damage (Azad and Tomar 2014). Studies have demonstrated a decrease in the extent of infarction, delayed neuronal death, and diminished oxidative cellular damage following focal brain ischemia in rat brains (He et al. 2007). Ebselen has already demonstrated beneficial outcomes in initial clinical stroke trials (Minnerup et al. 2012). Given the role of antioxidant enzymes in controlling post-anoxic and ammonia-induced oxidative stress in the goldfish, health care professionals should consider Ebselen as a promising neuroprotective agent for clinical trials against ammonia-induced brain swelling in the biomedical field. This drug could also be a very useful tool to further dissect the neurophysiological responses of not only the goldfish, but also other more sensitive fish species, to environmental stressors capable of inducing ROS such as hyperammonemia, anoxia/hypoxia, and thermal shock.

Therapeutic hypothermia (TH) is currently one of the most important methods of neuroprotection in the biomedical field (Gonzalez-Ibarra et al. 2011). TH has been demonstrated to be a strong neuroprotective measure against traumatic brain injury (TBI), cardiopulmonary resuscitation, stroke, and various other neurological disorders in mammals (Busto et al. 1987;

Busto et al. 1989; Meden et al. 1994; Kammersgaard et al. 2002; for review see Polderman 2004; Gonzalez-Ibarra et al. 2011). The results of the present study demonstrates the overproduction of ROS and brain swelling in teleosts acutely exposed to HEA, and that this response was temperature-dependent. Indeed, hypothermia has also been shown to diminish brain swelling and oxidative stress in mammalian models of ALF (Rose et al. 2000; Zwingmann et al. 2004; Jiang et al. 2009). If as the limited dataset above suggests, ROS production and brain swelling following acute hyperamonemia are temperature-dependent processes, then it is likely that TH may be an effective neuroprotective strategy against HE and related neurological disorders in humans. Some evidence to date in fact suggests the use of TH in clinical trials against ammonia-induced brain edema in ALF patients (Stravitz et al. 2008). The present findings may help to further explain why TH is effective in managing encephalopathy in mammals.

b. Ecological Implications and Perspectives

Hyperammonemia can be a daily challenge to aquatic life. Low millimolar (mmol L^{-1}) concentrations of ammonia may enter aquatic ecosystems routinely due to anthropogenic inputs, including agricultural run-off, industrial effluent, spills, and decomposition of biological waste (Randall and Tsui 2002). Furthermore, the deamination of amino acids following feeding, vigorous swimming, and prolonged periods of starvation can cause endogenous ammonia to accumulate to toxic levels in the blood and tissues of fish (Mommsen and Walsh 1992; Wang et al. 1994a; Wood 2001; Wicks and Randall 2002; Ortega et al. 2005; Randall and Tsui 2002). The effect of environmental ammonia in fish can be devastating, causing ammonia excretion (J_{Amm}) via the gills to be inhibited leading to both the accumulation of endogenous ammonia and uptake of exogenous ammonia. Although fish are more tolerant to ammonia than mammals, their relative resistance to ammonia's toxic effects varies considerably among different fish species

(Ip et al. 2001; Randall and Tsui 2002; Eddy 2005; Sinha et al. 2014).

Certain aquatic vertebrates are capable of enzymatically-converting ammonia to less noxious compounds such as urea, glutamine, and other free amino acids. However, only a few teleosts are ureotelic (e.g., *O. beta*; *Mugilogobius abei; Alcolapia grahami*), excreting significant amounts of accumulated ammonia to urea during exposure to HEA (Randall et al. 1989; Wood et al. 1989; Walsh et al. 1990; Wood et al. 1994; Iwata et al. 2000; Barimo and Walsh 2006). Earlier studies demonstrate the absence of urea in the blood and brain of ammonia-exposed goldfish (Wilkie et al. 2011). Nor do they express the OUC enzymes required for ureogenesis, indicating that urea synthesis does not play a role in ammonia detoxification in this species (Felskie et al. 1998). For teleosts, the synthesis of one mol of urea requires the hydrolysis of 5 mol of ATP (Ip and Chew 2010). Owing to this high energetic cost, there would likely have been little selective pressure to adopt ureogenesis as a single major strategy against ammonia toxicity in most fish species (Ip and Chew 2010). As expected, exposure of goldfish to HEA resulted in pronounced 5–6 fold increases in brain glutamine concentrations (Figure 2.3). However, the role of glutamine synthesis in ammonia detoxification is currently under debate, and may in fact exacerbate ammonia toxicity (for a review see Albrecht and Norenberg 2006).

Moreover, many ammonotelic, tropical fishes are capable of volatilizing significant amounts of ammonia during aerial exposure (Rozemeijer and Plaut 1993; Frick and Wright 2002; Tsui et al. 2002), limiting further ammonia accumulation in body tissues. Mudskippers (*Periophthalmodon schlosseri, Boleophthalmus boddaerti)* and other tropical air-breathing fishes also prevent the accumulation of endogenous ammonia by reducing the rates of protein and amino acid catabolism (Ip et al. 2001; Lim et al. 2001; Ip and Chew 2010). Additionally, certain teleosts are capable of augmenting rates of ammonia excretion by upregulating Na^+/NH_4^+

exchange complexes via Rh glycoproteins located along the gill epithelium (Wright and Wood 2009).

The extremely anoxia- and ammonia-tolerant goldfish (*Carassius auratus*), on the other hand, has evolved strategies for dealing with ammonia tolerance at the cellular/subcellular level. Rather than preventing ammonia accumulation in the brain, the goldfish exhibits a high neural tolerance of ammonia (Figure 2.2; Wilkie et al. 2011). One strategy for managing ammonia-induced toxicity to the goldfish brain is a relative resistance to NMDAr-mediated excitotoxicity. Wilkie et al. (2011) showed that goldfish survival time was largely unaffected by the NMDAr antagonist, MK801, during HEA exposure. Moreover, NMDAr NR1 subunit abundance was significantly reduced in ammonia-exposed goldfish brain, suggesting that goldfish minimize the toxic effects of ammonia on the brain by downregulation of NMDAr and avoidance of glutamate excitotoxicity (Wilkie et al. 2011). However, exposure of isolated brain slices of goldfish telencephelon to ammonia reversibly potentiated NMDAr currents, indicating that the goldfish brain may not be completely resistant to NMDAr-mediated excitotoxicity during HEA exposure (Wilkie et al. 2011).

One major consequence of NMDAr overactivation is the induction of oxidative stress (Norenberg et al. 2007, 2009). The present study showed that a significant degree of oxidative cellular damage to proteins and lipids occurs in the goldfish brain during HEA exposure. However, the present study demonstrates a significant upregulation in the activity of key antioxidant enzymes in the goldfish brain and the maintenance of other antioxidant enzyme activities during exposure to HEA. Although oxidative stress and swelling in the goldfish brain is still significant, the relative increase in antioxidant capacity in the goldfish brain may have limited cellular oxidative damage and swelling to a level that is physiologically tolerable and

reversible for the goldfish brain. This adaptation has also been demonstrated in the brain of goldfish exposed to post-anoxic recovery, where rates of ROS production can be devastating (Lushchak et al. 2001; Bickler and Buck 2007).

Permitting a manageable degree of oxidative stress and brain swelling may be an adaptive response to transient fluctuations in internal ammonia arising from environmental and/or endogenous sources. Unlike in mammals, teleost brains occupy only a small portion of the cranium (Cserr and Bundgaard 1984), with the majority of the cranial cavity being filled with perimeningeal fluid (PMF) and adipose tissue (Wang et al. 1995; Kotrschal et al. 1998). Therefore, brain swelling in teleosts may occur with little risk of herniation and associated brain damage, as demonstrated by the rapid and complete recovery of crucian carp (*Carassius carassius*) and goldfish from acute hyperammonemia (Wilkie et al. 2015). The upregulation of antioxidant defenses in these fishes likely limits oxidative stress, preventing irreversible necrotic/apoptotic cell death, and thereby allowing reversible, physiological increases in brain water content and volume (Wilkie et al. 2015). This adaptive response by antioxidants in the goldfish brain likely minimized the selective pressure to use ATP-demanding pathways such as the OUC to detoxify ammonia. With adaptations to minimize oxidative damage, goldfish and crucian carp would have the capacity to inhabit, exploit, and thrive in a wide range of niches that are often ice-covered, marginal, eutrophic, and which are unhabitable for potential competitors and predators.

Future Directions

The present study demonstrated the temperature-sensitive induction of oxidative stress in the goldfish brain and liver during exposure to HEA. However, the downstream events by which ammonia-induced oxidative stress leads to a disruption in ionic and cell volume homeostasis

(described above) have only been described for mammals. These targets (namely, NKCC-1 co-transporters, NSCCs, MPT, NF-κB, and RNA) provide a powerful link between the oxidative stress and brain swelling observed in this study and, therefore, should form the basis of future research on ammonia tolerance and brain swelling in teleosts. Of particular interest are the mitochondrial responses to acute ammonia toxicity in teleosts, specifically the induction of the MPT. The MPT has been strongly implicated in the production of ROS, as well as mediating ROS-induced cell swelling in numerous *in vitro* and *in vivo* mammalian studies (for reviews see Kowaltowski et al. 2001; Rama Rao et al. 2003, 2005; Norenberg et al. 2004). Understanding the downstream events following the ammonia-induced production of ROS in the anoxia- and ammonia-tolerant goldfish brain could provide further insights into the fundamental mechanisms of ammonia tolerance and brain swelling in aquatic vertebrates. Additionally, while numerous studies exist on the acute and chronic effects of ammonia toxicity in various fish species (for reviews see Arillo et al. 1981; Walsh et al. 2007; Ip and Chew 2010), very little is known about the potential for behavioural responses to ammonia toxicity in fish. Given the particular sensitivity of neural tissue to ammonia's toxic effects and the role of the CNS in regulating behaviour, significant behavioural deficits could occur in teleosts exposed to ammonia or anoxia/hypoxia. Indeed, the behavioural deficits observed in patients with hepatic encephalopathy (HE) are characteristic and have been extensively documented (Blei and Cordoba 2001).

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