AMMONIA TRANSPORT IN FRESHWATER RAINBOW TROUT

MOLECULAR PHYSIOLOGICAL CHARACTERIZATION OF AMMONIA TRANSPORT IN FRESHWATER RAINBOW TROUT

By

C. MICHELE NAWATA, B.Sc., M.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

© Copyright by C. Michele Nawata, December, 2009

DOCTOR OF PHILOSOPHY (2009)

McMaster University

(Biology)

Hamilton, Ontario

TITLE:	Molecular physiological characterization of ammonia transport in freshwater rainbow trout
AUTHOR:	C. Michele Nawata, M.Sc. (University of the Ryukyus, Okinawa, Japan)
SUPERVISOR:	Dr. C. M. Wood
NUMBER OF PAGES:	xxi, 219

ABSTRACT

Ammonia excretion from the freshwater fish gill is thought to occur mainly via passive diffusion of NH₃ aided by a favourable plasma-to-water ammonia gradient sustained by a pH gradient formed by an acidified gill boundary layer. Rhesus (Rh) proteins are the newest members of the ammonia transporter superfamily. In this thesis research, ten rainbow trout Rh cDNA sequences were cloned and characterized. Rhcg2 mRNA and H⁺-ATPase mRNA and activity levels were upregulated in the trout gill pavement cells in response to experimentally elevated plasma ammonia, concurrent with enhanced ammonia excretion. Controversially, Rh proteins are thought to transport CO_2 . However, Rh mRNA levels in most tissues of hypercapnia-exposed trout remained stable suggesting that trout Rh proteins likely do not conduct CO2. Xenopus oocytes expressing trout Rh proteins facilitated the bi-directional transport of methylamine, an ammonia analogue. Methylamine transport was inhibited by ammonia and sensitive to a pH gradient and the concentration of the protonated species. Use of the scanning ion electrode technique (SIET) indicated that trout Rh proteins have an ammonia affinity within the physiological range, which is greater than that for methylamine, and they transport ammonia more rapidly than methylamine. A model of ammonia excretion in the trout gill pavement cell is proposed wherein ammonia enters via basolateral Rhbg and exits via apical Rhcg2, binding to these channels as NH4⁺ but transiting as NH3. In the gill boundary layer, NH₃ combines with an H⁺ ion released from H⁺-ATPase and/or Na⁺/H⁺ exchange, forming NH₄⁺. As low-affinity, high-capacity ammonia transporters, Rh proteins in the trout gill would exploit the favourable pH gradient formed by the acidic boundary layer to facilitate rapid ammonia efflux when plasma ammonia levels are elevated. Basal plasma ammonia levels are likely maintained by simple passive NH_3 diffusion with a smaller role for Rh proteins under these conditions.

ACKNOWLEDGEMENTS

This work would not have been possible without the excellent mentorship and supervision of Chris Wood, to whom I am immensely grateful. My committee members, Pat Wright, Mike O'Donnell and Grant McClellend were integral in this process and they saw me through to the end. Jonathan Wilson has been an important collaborator from the beginning and Tommy Tsui and Carrie Hung provided the intellectual and moral support that I needed to stay focused and motivated in the early stages. Lastly, I acknowledge my colleagues and lab-mates (past and present) who supplied me with company and laughs, and all the special people in my life who have supported me from near and afar.

PREFACE

This thesis is organized in the "sandwich thesis" format approved by McMaster University. Chapter 1 provides a general introduction for the thesis research. Chapters 2 through 4 were each published as primary articles prior to completion of this thesis work and Chapter 5 represents a manuscript accepted for publication. Chapter 6 discusses the findings of the preceeding chapters as well as the overall implications of the thesis work.

Thesis organization and format:

CHAPTER 1: GENERAL INTRODUCTION

CHAPTER 2: AMMONIA EXCRETION IN RAINBOW TROUT (Oncorhynchus mykiss): EVIDENCE FOR Rh GLYCOPROTEIN AND H⁺-ATPase INVOLVEMENT

Authors: Nawata, C. M., Hung, C. C. Y., Tsui, T. K. N., Wilson, J. M.,

Wright, P. A. and Wood, C. M.

Journal: Physiological Genomics 31, 463-474 (2007).

Comments: C. M. Nawata conducted this study and wrote the manuscript under the supervision of C. M. Wood. J. M. Wilson provided primer sequences for the initial cDNA cloning. C. C. Y. Hung provided technical advice on molecular biological techniques. T. K. N. Tsui provided technical advice on gill cell separation. P. A. Wright provided critical input into the manuscript.

CHAPTER 3: THE EFFECTS OF CO₂ AND EXTERNAL BUFFERING ON AMMONIA EXCRETION AND Rh GLYCOPROTEIN mRNA EXPRESSION IN RAINBOW TROUT

- Authors: Nawata, C. M. and Wood, C. M.
- Journal: Journal of Experimental Biology 211, 3226-3236 (2008).
- Comments: C. M. Nawata conducted this study and wrote the manuscript under

the supervision of C. M. Wood.

CHAPTER 4: mRNA ANALYSIS OF THE PHYSIOLOGICAL RESPONSES TO AMMONIA INFUSION IN RAINBOW TROUT

- Authors: Nawata, C. M. and Wood, C. M.
- Journal: Journal of Comparative Physiology B 179, 799-810 (2009).

Comments: C. M. Nawata conducted this study and wrote the manuscript under the supervision of C. M. Wood.

CHAPTER 5: FUNCTIONAL CHARACTERIZATION OF RHESUS GLYCOPROTEINS FROM AN AMMONIOTELIC TELEOST, THE RAINBOW TROUT, USING OOCYTE EXPRESSION AND SIET ANALYSIS

Authors: Nawata, C. M., Wood, C. M., and O'Donnell, M. J.

Journal: Journal of Experimental Biology (in press)

Comments: C. M. Nawata conducted this study and wrote most of the manuscript under the supervision of C. M. Wood. In addition to providing critical instruction on SIET, M. J. O'Donnell generated one portion of the SIET data and wrote the Materials and Methods for the SIET analysis.

CHAPTER 6: GENERAL DISCUSSION

TABLE OF CONTENTS

DESCRIPTIVE NOTE ii
ABSTRACT iii
ACKNOWLEDGEMENTS v
PREFACEvi
TABLE OF CONTENTS ix
LIST OF TABLES xiv
LIST OF FIGURES xv
LIST OF ABBREVIATIONS xx
CHAPTER 1. INTRODUCTION 1
1.1 Preface.21.2 Background on ammonia transport in freshwater fish.21.2.1 NH3 or NH4 ⁺ ?31.2.2 Passive diffusion and the acid-trapping theory.41.2.2.1 Carbonic anhydrase.51.2.3 The apparent linkage to sodium uptake.51.2.4 Evidence against sodium involvement.61.3 Alternative ammonia transport mechanisms.61.3.1 Na ⁺ /K ⁺ -ATPase (NKA).71.3.2 Rhesus (Rh) proteins.71.3.2.1 Tissue distribution in mammals.81.3.2.2 Function based on structure.91.3.2.3 NH3, NH4 ⁺ or both? Electrogenic or electroneutral?91.3.2.5 The CO2 debate.111.3.2.6 Rh proteins in fish.121.4 Hypotheses and objectives.13
1.5 Overview of experimental chapters 1.5.1 Chapter 2
1.6 Summary

CHAPTER 2. AMMONIA EXCRETION IN RAINBOW TROUT (<i>Oncorhynchus mykiss</i>): EVIDENCE FOR Rh GLYCOPROTEIN AND H ⁺ -ATPase
INVOLVEMENT
2.1 Abstract
2.2 Introduction20
2.3 Materials and methods
2.3.1 Animals
2.3.2 High external ammonia (HEA) experiment23
2.3.3 Sequencing and characterization of rainbow trout Rh cDNAs
2.3.3.1 Tissue sampling
2.3.3.2 RNA extraction and reverse-transcriptase PCR
amplification
2.3.3.3 Identification of Rh orthologues
2.3.3.4 Phylogenetic, amino acid sequence identity.
hydropathy and glycosylation analysis
2.3.3.5 Tissue distribution (PCR)
2.3.3.6 Tissue expression (aPCR)
2.3.3.7 Pavement cell and mitochondria-rich cell
expression
2.3.4 Enzyme/transporter expression in the gill
2.3.5 Enzyme activity in the gill
2.3.5.1 ATPase activity
2.3.5.2 Carbonic anhydrase
2.3.6 Statistical analysis
2.4 Results
2.4.1 High external ammonia experiment
2.4.2 Identification of Rh orthologues in trout
2.4.3 Tissue distribution and expression of Rh
2.4.4 Enzyme/transporter expression and activity in the gill
2.5 Discussion
2.6 Tables and figures
2.7 References
CHAPTER 3. THE EFFECTS OF CO ₂ AND EXTERNAL BUFFERING ON AMMONIA EXCRETION AND RHESUS GLYCOPROTEIN mRNA EXPRESSION IN RAINBOW TROUT
3.1 Abstract
3.2 Introduction
3.3 Materials and methods
3.3.1 Animals
3.3.2 Experimental conditions73

	3.3.2.1 Effect of external hypercapnia	. 73
	3.3.2.2 Effect of Hepes buffering	73
	3.3.2.3 Effect of high environmental ammonia (HEA)	73
	3.3.3 Analyses	74
	3.3.3.1 Tissue sampling	75
	3.3.3.2 Total RNA isolation and reverse-transcriptase	
	PCR amplification	75
	3.3.3.3 mRNA expression	76
	3.3.3.4 Plasma cortisol	77
	3.3.3.5 Statistical analysis	77
3.4 F	Results	
	3.4.1 Identification of Rhbg2	78
	3.4.2 Tissue distribution and expression of Rhbg2	79
	3.4.3 External hypercapnia	79
	3.4.4 External hypercapnia with Hepes buffering	80
	3.4.5 External normocapnia with Hepes buffering	80
	3.4.6 High environmental ammonia	81
3.5 I	Discussion	82
3.6 7	Tables and Figures	. 90
3.7 F	References	101
CHAPTER	4. mRNA ANALYSIS OF THE PHYSIOLOGICAL RESPON	SES
		100
TO AMMC	DNIA INFUSION IN RAINBOW TROUT	108
TO AMMO 4.1 /	DNIA INFUSION IN RAINBOW TROUT Abstract	108
4.1 <i>A</i> 4.2 I	Abstract	108 109
4.1 A 4.2 I 4.3 M	DNIA INFUSION IN RAINBOW TROUT Abstract Introduction Materials and methods	108 109 110
4.1 A 4.2 I 4.3 N	Abstract Abstract Introduction	108 109 110
4.1 A 4.2 I 4.3 N	Abstract. Abstract. ntroduction. Materials and methods 4.3.1 Experimental animals. 4.3.2 Infusions and analyses.	108 109 110 113 113
4.1 A 4.2 I 4.3 N	Abstract. Abstract. ntroduction. Materials and methods 4.3.1 Experimental animals. 4.3.2 Infusions and analyses. 4.3.3 Tissue sampling.	108 109 110 113 113 115
4.1 A 4.2 I 4.3 N	Abstract. Abstract. ntroduction. Materials and methods 4.3.1 Experimental animals. 4.3.2 Infusions and analyses. 4.3.3 Tissue sampling. 4.3.4 RNA extraction and cDNA synthesis.	108 109 110 113 113 115 116
4.1 A 4.2 I 4.3 N	Abstract. Abstract. ntroduction. Materials and methods 4.3.1 Experimental animals. 4.3.2 Infusions and analyses. 4.3.3 Tissue sampling. 4.3.4 RNA extraction and cDNA synthesis. 4.3.5 Real-time quantitative PCR (aPCR).	108 109 110 113 113 115 116 116
4.1 4 4.2 I 4.3 N	Abstract. Abstract. ntroduction. Materials and methods 4.3.1 Experimental animals. 4.3.2 Infusions and analyses. 4.3.3 Tissue sampling. 4.3.4 RNA extraction and cDNA synthesis. 4.3.5 Real-time quantitative PCR (qPCR). 4.3.6 Statistical analysis.	108 109 113 113 113 115 116 116 117
4.1 A 4.2 I 4.3 M	Abstract. Abstract. ntroduction. Materials and methods 4.3.1 Experimental animals. 4.3.2 Infusions and analyses. 4.3.3 Tissue sampling. 4.3.4 RNA extraction and cDNA synthesis. 4.3.5 Real-time quantitative PCR (qPCR). 4.3.6 Statistical analysis.	108 109 110 113 113 115 116 116 117
4.1 A 4.2 I 4.3 N 4.4 F	Abstract. Abstract. ntroduction. Materials and methods 4.3.1 Experimental animals. 4.3.2 Infusions and analyses. 4.3.3 Tissue sampling. 4.3.4 RNA extraction and cDNA synthesis. 4.3.5 Real-time quantitative PCR (qPCR). 4.3.6 Statistical analysis. Results 4.4.1 Ammonia and cortisol responses.	108 109 110 113 113 115 116 116 117 117
4.1 A 4.2 I 4.3 N 4.4 F	Abstract. Abstract. ntroduction. Materials and methods 4.3.1 Experimental animals. 4.3.2 Infusions and analyses. 4.3.3 Tissue sampling. 4.3.4 RNA extraction and cDNA synthesis. 4.3.5 Real-time quantitative PCR (qPCR). 4.3.6 Statistical analysis. Results 4.4.1 Ammonia and cortisol responses. 4.4.2 Expression of Rh genes.	108 109 110 113 113 115 116 116 117 117 118
4.1 A 4.2 I 4.3 N 4.4 F	Abstract. Abstract. ntroduction. Materials and methods 4.3.1 Experimental animals. 4.3.2 Infusions and analyses. 4.3.3 Tissue sampling. 4.3.4 RNA extraction and cDNA synthesis. 4.3.5 Real-time quantitative PCR (qPCR). 4.3.6 Statistical analysis. Results 4.4.1 Ammonia and cortisol responses. 4.4.2 Expression of Rh genes. 4.4.3 Expression of other transport genes in the gills.	108 109 110 113 113 115 116 116 117 117 118 119
4.1 A 4.2 I 4.3 N 4.4 F 4.5 I	Abstract. Abstract. ntroduction. Materials and methods 4.3.1 Experimental animals. 4.3.2 Infusions and analyses. 4.3.3 Tissue sampling. 4.3.4 RNA extraction and cDNA synthesis. 4.3.5 Real-time quantitative PCR (qPCR). 4.3.6 Statistical analysis. Results 4.4.1 Ammonia and cortisol responses. 4.4.3 Expression of Rh genes. 4.4.3 Expression of other transport genes in the gills.	108 109 110 113 113 115 116 116 117 117 117 118 119
4.1 4 4.2 I 4.3 N 4.4 F 4.5 I	Abstract. Abstract. ntroduction. Materials and methods 4.3.1 Experimental animals. 4.3.2 Infusions and analyses. 4.3.3 Tissue sampling. 4.3.4 RNA extraction and cDNA synthesis. 4.3.5 Real-time quantitative PCR (qPCR). 4.3.6 Statistical analysis. Results 4.4.1 Ammonia and cortisol responses. 4.4.3 Expression of Rh genes. 4.4.3 Expression of other transport genes in the gills. Discussion 4.5.1 Overview.	108 109 110 113 113 115 116 116 117 117 117 117 119 120
4.1 A 4.2 I 4.3 N 4.4 F 4.5 I	Abstract. Abstract. Introduction. Materials and methods 4.3.1 Experimental animals. 4.3.2 Infusions and analyses. 4.3.3 Tissue sampling. 4.3.4 RNA extraction and cDNA synthesis. 4.3.5 Real-time quantitative PCR (qPCR). 4.3.6 Statistical analysis. Results 4.4.1 Ammonia and cortisol responses. 4.4.3 Expression of Rh genes. 4.4.3 Expression of other transport genes in the gills. Discussion 4.5.1 Overview. 4.5.2 The influence of surgical procedures and infusion.	108 109 110 113 113 115 116 116 117 117 117 118 119 120 121
4.1 4 4.2 I 4.3 N 4.4 F 4.5 I	Abstract. Abstract. Introduction. Materials and methods 4.3.1 Experimental animals. 4.3.2 Infusions and analyses. 4.3.3 Tissue sampling. 4.3.4 RNA extraction and cDNA synthesis. 4.3.5 Real-time quantitative PCR (qPCR). 4.3.6 Statistical analysis. Results 4.4.1 Ammonia and cortisol responses. 4.4.3 Expression of Rh genes. 4.4.3 Expression of other transport genes in the gills. Discussion 4.5.1 Overview. 4.5.2 The influence of surgical procedures and infusion. 4.5.3 Responses to internal ammonia loading.	108 109 110 113 113 115 116 116 117 117 117 117 117 117 120 121 124
4.1 4 4.2 I 4.3 N 4.4 F 4.5 I	Abstract. Materials and methods 4.3.1 Experimental animals. 4.3.2 Infusions and analyses. 4.3.3 Tissue sampling. 4.3.4 RNA extraction and cDNA synthesis. 4.3.5 Real-time quantitative PCR (qPCR). 4.3.6 Statistical analysis. Results 4.4.1 Ammonia and cortisol responses. 4.4.3 Expression of Rh genes. 4.4.3 Expression of other transport genes in the gills. Discussion 4.5.1 Overview. 4.5.2 The influence of surgical procedures and infusion. 4.5.3 Responses to internal ammonia loading. 4.5.4 Does cortisol play a role in Rh gene expression and	108 109 110 113 113 115 116 116 117 117 117 117 117 117 117 1120 120 124
4.1 <i>A</i> 4.2 I 4.3 N 4.4 F 4.5 I	Abstract. Introduction. Materials and methods 4.3.1 Experimental animals. 4.3.2 Infusions and analyses. 4.3.3 Tissue sampling. 4.3.4 RNA extraction and cDNA synthesis. 4.3.5 Real-time quantitative PCR (qPCR). 4.3.6 Statistical analysis. Results 4.4.1 Ammonia and cortisol responses. 4.4.3 Expression of Rh genes. 4.4.3 Expression of other transport genes in the gills. Discussion 4.5.1 Overview. 4.5.2 The influence of surgical procedures and infusion. 4.5.3 Responses to internal ammonia loading. 4.5.4 Does cortisol play a role in Rh gene expression and ammonia excretion?	108 109 110 113 113 115 116 116 116 117 117 117 117 117 118 120 124 128

4.6 Figures4.7 References	131 138
CHAPTER 5. FUNCTIONAL CHARACTERIZATION OF RHESUS GLYCOPROTEINS FROM AN AMMONIOTELIC TELEOST, THE BAINBOW TROUT, USING ODCYTE EXPRESSION AND SIET	
ANALYSIS	144
5.1 Abstract	
5.2 Introduction	146
5.3 Materials and methods	
5.3.1 Reagents and solutions	149
5.3.2 Plasmid constructs and cRNA synthesis	149
5.3.3 Preparation of oocytes	150
5.3.4 Injection of oocytes	150
5.3.5 [¹⁴ C]methylamine studies	151
5.3.6 Methylamine kinetics	152
5.3.7 NH ₄ Cl inhibition	152
5.3.8 Measurements of ammonia uptake using the	
scanning ion electrode technique (SIET)	153
5.3.9 Data analysis	
5.4 Results	
5.4.1 Methylamine uptake	155
5.4.2 Kinetics of methylamine uptake	156
5.4.3 pH-sensitive transport	157
5.4.4 Efflux of methylamine	158
5.4.5 Inhibition by NH ₄ Cl	159
5.4.6 Ammonia uptake measured by SIET	160
5.4.7 Kinetics of ammonia uptake measured by SIET	160
5.5 Discussion	161
5.6 Tables and figures	174
5.7 References	
CHAPTER 6. DISCUSSION	189
6 1 Profess	103
6.2 Many Rh genes are present in the rainhow trout	195
6.3 Trout Rh mRNA levels change in response to both HEA and	
ammonia infusion	10/
6.4 Other trout gill proteins also respond to elevated plasma ammoni	a 105
6.4.1 Importance of H ⁺ -ATPase and an acidified cill	u195
boundary laver	195
6.4.2 NHE2 and NKA may be involved in the ammonia excre	etion
mechanism.	196

6.4.3 Carbonic anhydrase (CA2) is downregulated	
during HEA 19	7
6.5 Trout Rh mRNA levels do not change in response to	
high external CO ₂ 19	8
6.6 Trout Rh proteins bind NH ₄ ⁺ but transport NH ₃ 19	9
6.7 Other studies on the involvement of Rh proteins in ammonia	
transport in fish19	9
6.7.1 More evidence for Na ⁺ involvement in ammonia	
excretion	0
6.7.2 The linkage between Rh proteins and H^+ -ATPase	
in zebrafish larvae20	1
6.7.3 Rh proteins in the early life stages of fish 20	1
6.7.4 Amphibious air-breathing fish20	1
6.7.5 Marine elasmobranch20	2
6.8 Overall implications of work 20	3
6.9 Future directions	4
6.9.1 Localization and distribution if trout Rh proteins	4
6.9.2 A role for trout skin in ammonia excretion?20	4
6.9.3 Signaling mechanism of trout Rh genes	5
REFERENCES (for Chapters 1 and 6)	7

LIST OF TABLES

Table 2.1	Primers used for cloning, PCR, and qPCR analysis
Table 2.2	Accession number and size of each Rh cDNA identified44
CHAPTER 3	
Table 3.1	Primer list
CHAPTER 5	
Table 5.1	Primer list for cloning174
Table 5.2	Protonated and unprotonated fractions of $K_{\rm m}$ for methylamine and $K_{\rm i}$ values for ammonia as an inhibitor of methylamine uptake at pH 7.5 and 8.5

LIST OF FIGURES

Figure 2.1	The effect of 48 hours of exposure to 1.5 mmol L ⁻¹ NH ₄ HCO ₃ on net ammonia flux (J_{Amm}) 45
Figure 2.2	The effect of 48 hours of exposure to 1.5 mmol L ⁻¹ NH ₄ HCO ₃ on water and plasma total ammonia (T_{Amm}) 46
Figure 2.3	An unrooted phylogenetic tree of Rh homologue sequences in fish
Figure 2.4	Amino acid sequence alignments of fish Rh homologues48
Figure 2.5	Hydropathy profile of rainbow trout Rhbg 49
Figure 2.6	Tissue distribution of Rh glycoproteins as determined by PCR in rainbow trout exposed to 0 mmol L^{-1} NH ₄ HCO ₃ and 1.5 mmol L^{-1} NH ₄ HCO ₃
Figure 2.7	mRNA expression of Rhbg relative to β -actin in the blood and the unperfused and perfused brain, kidney and liver of rainbow trout exposed to 0 mmol L ⁻¹ NH ₄ HCO ₃ and 1.5 mmol L ⁻¹ NH ₄ HCO ₃ for 48 hours
Figure 2.8	mRNA expression of Rhbg, Rhcg1, and Rhcg2 relative to EF-1 α expression in the gills of rainbow trout exposed to 1.5 mmol L ⁻¹ NH ₄ HCO ₃ for 12 and 48 hours
Figure 2.9	mRNA expression of Rhbg, Rhcg1, and Rhcg2 relative to β -actin in the skin of rainbow trout exposed to 1.5 mmol L ⁻¹ NH ₄ HCO ₃ for 12 and 48 hours
Figure 2.10	mRNA expression of Rhcg1 relative to β -actin in the brain and liver of rainbow trout exposed to 1.5 mmol L ⁻¹ NH ₄ HCO ₃ for 12 and 48 hours
Figure 2.11	mRNA expression of Rhbg, Rhcg1, and Rhcg2 relative to β -actin in pavement cells and mitochondria-rich cells isolated from the gills of rainbow trout exposed to 1.5 mmol L ⁻¹ NH ₄ HCO ₃ for 12 and 48 hours

Figure 2.12	mRNA expression of Rh30-like2 , Rh30-like3, and Rhag relative to β -actin in the RBCs from rainbow trout exposed to 1.5 mmol L ⁻¹ NH ₄ HCO ₃ for 12 and 48 hours
Figure 2.13	mRNA expression relative to EF-1 α of H ⁺ -ATPase, carbonic anhydrase (CA2), and Na ⁺ /K ⁺ -ATPase α -subunit isoforms and the enzyme activities of H ⁺ -ATPase, carbonic anhydrase, and Na ⁺ /K ⁺ -ATPase in the gills of rainbow trout exposed to 1.5 mmol L ⁻¹ NH ₄ HCO ₃ for 12 and 48 hours
Figure 2.14	mRNA expression relative to β -actin of carbonic anhydrase, H ⁺ -ATPase, NHE2 and Na ⁺ /K ⁺ -ATPase (α 1a) in pavement cells and mitochondria-rich cells
Figure 2.15	Schematic diagram of a gill cell showing Rhcg2-mediated NH ₃ transport coupled to active H ⁺ secretion 59

Figure 3.1	Sequence alignment of Rhbg1, Rhbg2a, and Rhbg2c, the 5'-untranslated region of the longest Rhbg2 variant, and a schematic representation of the mRNA variants identified in Rhbg2	91
Figure 3.2	Tissue distribution of Rhbg2 in rainbow trout exposed to control conditions (100% air for 12 hours) as determined by reverse transcriptase PCR) 2
Figure 3.3	Relative abundance of 5'-untranslated region variants $(v1,2,3,4)$ in the gill under control conditions (12-hours exposure to 100% air) and skin during control conditions and after 12 hours of exposure to hypercapnia $(1\% \text{ CO}_2 \text{ in air})$ in 10 mM Hepes-buffered water	93
Figure 3.4	The effect of 12-hour exposures of rainbow trout to: hypercapnia (1% CO_2 in air) in unbuffered water, hypercapnia in 10 mM Hepes-buffered water, and normocapnia (100% air) in Hepes-buffered water on the net ammonia flux (L_{rec})	24
	the net annioma nux (JAmm)	/ Т

Figure 3.5	Plasma total ammonia after 12 hours of exposure of rainbow trout to 1% CO ₂ in air (CO ₂), 1% CO ₂ in air with 10 mM Hepes buffered-water (CO ₂ + Hepes), 100% air with 10 mM Hepes-buffered water (Air + Hepes), and high environmental ammonia (HEA; 1.5 mmoL NH ₄ HCO ₃)
Figure 3.6	Gill Rh mRNA expression in rainbow trout after 12 hours of exposure to 1% CO ₂ in air (CO ₂), 1% CO ₂ in air with 10 mM Hepes buffered-water (CO ₂ + Hepes), 100% air with 10 mM Hepes-buffered water (Air + Hepes), and high environmental ammonia (HEA; 1.5 mmoL NH ₄ HCO ₃)
Figure 3.7	Skin Rh mRNA expression in rainbow trout after 12 hours of exposure to 1% CO ₂ in air (CO ₂), 1% CO ₂ in air with 10 mM Hepes buffered-water (CO ₂ + Hepes), 100% air with 10 mM Hepes-buffered water (Air + Hepes), and high environmental ammonia (HEA; 1.5 mmoL NH ₄ HCO ₃)97
Figure 3.8	Erythrocyte Rh mRNA expression in the rainbow trout exposed to 12 hours of to 1% CO ₂ in air (CO ₂), 1% CO ₂ in air with 10 mM Hepes buffered-water (CO ₂ + Hepes), and 100% air with 10 mM Hepes-buffered water (Air + Hepes)
Figure 3.9	The effect of 12-hour exposure to 1% CO ₂ in air (CO ₂), 1% CO ₂ in air with 10 mM Hepes buffered-water (CO ₂ + Hepes), and 100% air with 10 mM Hepes-buffered water (Air + Hepes) on the mRNA expression of carbonic anhydrase (CA2), H ⁺ -ATPase, NHE2, and Na ⁺ /K ⁺ -ATPase α -1a (NKA) in the gills of rainbow trout
Figure 3.10	Plasma cortisol levels after 12 hours of exposure to 1% CO ₂ in air (CO ₂), 1% CO ₂ in air with 10 mM Hepes buffered-water (CO ₂ + Hepes), 100% air with 10 mM Hepes-buffered water (Air + Hepes), and high environmental ammonia (HEA; 1.5 mmoL NH ₄ HCO3) 100

Figure 4.1	The ammonia excretion rates (J_{Amm}) in rainbow trout infused with 140 mmol L ⁻¹ NaCl or 140 mmol L ⁻¹ NH ₄ HCO ₃ over a course of 12 hours
Figure 4.2	Plasma total ammonia (T_{Amm}) in rainbow trout infused with 140 mmol L ⁻¹ NaCl or 140 mmol L ⁻¹ NH ₄ HCO ₃ over a course of 12 hours
Figure 4.3	Plasma cortisol levels during 12 hours of infusion with 140 mmol L^{-1} NaCl or 140 mmol L^{-1} NH ₄ HCO ₃ 133
Figure 4.4	Gill Rh mRNA expression in rainbow trout during 12 hours of infusion with 140 mmol L ⁻¹ NaCl or 140 mmol L ⁻¹ NH ₄ HCO ₃ 134
Figure 4.5	Erythrocyte Rhag mRNA expression in rainbow trout after 12 hours of infusion with 140 mmol L^{-1} NaCl or 140 mmol L^{-1} NH ₄ HCO ₃
Figure 4.6	Rh mRNA expression in the brain and skin of rainbow trout after 12 hours of infusion with 140 mmol L^{-1} NaCl or 140 mmol L^{-1} NH ₄ HCO ₃
Figure 4.7	The effect of 12 hours of infusion with 140 mmol L ⁻¹ NaCl or 140 mmol L ⁻¹ NH ₄ HCO ₃ on the mRNA expression of carbonic anhydrase (CA2), H ⁺ -ATPase, NHE2, and Na ⁺ /K ⁺ -ATPase α -1a (NKA) in the gills of rainbow trout

Figure 5.1	Rh-facilitated methylamine uptake176
Figure 5.2	Methylamine uptake kinetics at pH 7.5 177
Figure 5.3	Methylamine uptake kinetics at pH 8.5 178
Figure 5.4	The effect of external pH on methylamine uptake179

Figure 5.5	The effect of intracellular acidification on methylamine uptake	180
Figure 5.6	Methylamine efflux	181
Figure 5.7	Inhibition of methylamine uptake with NH ₄ Cl	182
Figure 5.7	Ammonia uptake	183
Figure 5.9	Kinetics of ammonia uptake	184

Figure 6.1	Proposed model of ammonia excretion in the pavement	
	cell of the freshwater rainbow trout gill	206

LIST OF ABBREVIATIONS

Amt	ammonium transporter of plants and bacteria
bp	base pair
CA2	cytoplasmic carbonic anhydrase
CDS	coding region
HEA	high external / environmental ammonia
Hepes	N-[2-hydroxethyl] piperazine-N'-[2-ethane sulfonic acid]
$J_{\rm Amm}$	ammonia excretion rate
J_{\max}	maximum rate of transport
Ki	dissociation constant for inhibitor binding
K _m	Michaelis-Menten binding constant
Мер	methylammonium / ammonium permease
MR cell	mitochondria-rich cell
MS222	ethyl 3-aminobenzoate methanesulfonic acid
NHE2	Na^+/H^+ exchanger 2
NKA	Na ⁺ /K ⁺ -ATPase
ORF	open reading frame
PCR	polymerase chain reaction
pK _a	acid dissociation constant
P _{NH3}	partial pressure of NH ₃
qPCR	quantitative polymerase chain reaction
Rh	Rhesus

RBC	red blood cell / erythrocyte
RT-PCR	reverse-transcriptase polymerase chain reaction
SIET	scanning ion electrode technique
$T_{\rm Amm}$	total ammonia (sum of NH_3 and NH_4^+)
uORF	untranslated open reading frame
UTR	untranslated region

INTRODUCTION

1.1 Preface

This thesis presents a study of the ammonia transport mechanisms in the freshwater rainbow trout, with particular emphasis on the role of Rhesus (Rh) proteins in the gill. Over the course of the four-year period during which this doctoral thesis research was carried out, there was an upsurge of research in the area of ammonia transport both in mammals and in fish. This was due to the very recent addition of the Rh proteins to the ammonia transporter superfamily. Consequently, during the same time period as the papers representing Chapters 2 (Nawata et al., 2007), 3 (Nawata and Wood, 2008), 4 (Nawata and Wood, 2009), and 5 (Nawata et al., 2009, in press) of this thesis were sequentially published, a number of very relevant studies have been published by other groups. Several reviews on ammonia transport in fish have also been published recently which highlight the main findings detailed in Chapters 2, 3, and 4 (Perry et al., 2009; Weihrauch et al., 2009; Wright and Wood, 2009). This Introduction will therefore provide a brief background on ammonia transport in fish through to the first characterizations of fish Rh proteins. Much of the content within this Introduction has been introduced and discussed in the following chapters and therefore the reader is forewarned of some overlap.

1.2 Background on ammonia transport in freshwater fish

As the major end product of nitrogen metabolism, ammonia must be excreted in order to avoid toxic accumulation, and this is accomplished mainly via the gills in freshwater fish (Smith, 1929). The interest in ammonia excretion and handling at the gill likely stems from early observations that elevated water ammonia levels were associated with various problems such as gill damage, impaired ammonia excretion, toxicity, and reduced growth and survival (e.g. Brockway, 1950; Fromm and Gillette, 1968; Larmoyeux and Piper, 1973; Smart, 1976). Waste decomposition and high stocking density contribute to high ammonia levels in aquaculture systems (Hargreaves, 1998) while agricultural and industrial run-offs raise levels in aquatic environments (Jordan and Weller, 1996). Understanding the strategies which aquatic organisms use to survive under conditions of high environmental ammonia (HEA) is important for establishing environmental and aquacultural guidelines (Wilkie, 1997; Wood, 1993). While the studies on ammonia excretion in fish are voluminous (see reviews by Walsh, 1998; Wilkie, 2002; Wood, 1993), the mechanism(s) involved remain unclear. Most of the proposed mechanisms for freshwater fish involve passive diffusion of NH₃ or apical Na⁺/NH₄⁺ exchange, and are discussed in more detail in the sections below.

1.2.1 NH₃ or NH₄⁺?

Total ammonia in aqueous solutions is the sum of the gaseous (NH₃) and ionic (NH₄⁺) components. Throughout this thesis, the term ammonia or T_{Amm} will be used to represent the sum of NH₃ and NH₄⁺, while the chemical symbols will be used to specifically denote the unprotonated NH₃ and the protonated NH₄⁺ species. As a weak base with a p K_a between 9 and 10, ammonia exists mainly as NH₄⁺ (> 95%) in fish plasma (Cameron and Heisler, 1983). As the freshwater gill epithelium is relatively impermeable to cations, diffusion of NH₄⁺ is thought to be minimal and the majority of

ammonia likely diffuses out of the gill as NH₃ (Evans *et al.*, 2005; Wilkie 1997, 2002). The evidence for this is based on the plasma-to-water NH₃ partial pressure (P_{NH3}) gradients, which correlate well with the ammonia excretion rates (J_{Amm}). J_{Amm} is reduced in the presence of unfavourable P_{NH3} gradients created by elevated water ammonia (Cameron and Heisler, 1983; Wilson *et al.*, 1994) or high water pH (Wilson *et al.*, 1998; Wright and Wood, 1985). Stimulation of J_{Amm} on the other hand, occurs in the presence of favourable gradients created by high plasma ammonia levels (Avella and Bornancin, 1989; Salama, *et al.*, 1999; Wilson *et al.*, 1994) or low water pH (Wright and Wood, 1985).

1.2.2 Passive diffusion and the acid-trapping theory

The most widely accepted model of ammonia transport in the freshwater gill involves the diffusion of NH₃ across the gill into an acidic gill boundary layer wherein NH₃ is protonated to NH₄⁺ (Wilkie, 1997, 2002) and this sustains the $P_{\rm NH3}$ diffusion gradient. This boundary layer may be acidified by the hydration of expired CO₂, which produces HCO₃⁻ and H⁺ (Wright *et al.*, 1986, 1989), or by H⁺ ions released either from an apical H⁺-ATPase or Na⁺/H⁺ exchange system (Lin and Randall, 1990; Lin *et al.*, 1994). The favourable $P_{\rm NH3}$ gradient created by this acidified layer is especially advantageous to fish living in poorly buffered fresh water.

1.2.2.1 Carbonic anhydrase

Carbonic anhydrase present on the gill surface (Rahim *et al.*, 1988; Wright *et al.*, 1986) may be responsible for catalyzing the hydration reaction of expired CO_2 that acidifies the gill boundary layer, but this remains a controversial issue (Henry and Heming, 1998). Cytoplasmic carbonic anhydrase (CA2) in the gill cells however, is well studied and is thought to be important for maintaining acid-base homeostasis (Henry and Heming, 1988; Perry, 1986; Perry and Gilmour, 2006). The H⁺ ions that are produced from the hydration of CO_2 in the gill cells likely fuel the H⁺-ATPase or exit *via* an apical NHE (Edwards *et al.*, 2005).

1.2.3 The apparent linkage to sodium uptake

In 1938, Krogh proposed that Na^+/NH_4^+ exchange took place on the apical gill epithelium of goldfish (*Carassius auratus*). Indirect evidence of this came later in another study on goldfish, which showed that HEA inhibited ammonia excretion as well as Na⁺ uptake, while injection of ammonia had the opposite effect and stimulated Na⁺ uptake (Maetz and Garcia Romeu, 1964). This stimulation of Na⁺ uptake with ammonia loading was confirmed in later studies (Salama et al., 1999; Wilson et al., 1994). Several other studies have supported the idea that NH₄⁺ could replace H⁺ on a Na⁺/H⁺ exchanger (NHE) after observations that J_{Amm} decreased upon treatment with the sodium-proton blocker, amiloride (e.g. Kirschner *et al.*, 1973; Lin and Randall, 1991; McGeer and Eddy, 1998; Wright and Wood, 1985; Yesaki and Iwama, 1992).

1.2.4 Evidence against sodium involvement

Theoretical considerations suggest that NHEs present on the apical gill membrane of freshwater fish may function in Na⁺ uptake, but only within a very narrow range of environmental parameters including high external pH or Na⁺ (Parks et al., 2008). Therefore, most workers believe that Na⁺ uptake likely occurs via an epithelial Na⁺ channel energized by apical membrane H⁺-ATPase (Marshall, 2002; Potts, 1994). The reduction of J_{Amm} that was observed in studies that used amiloride may have been caused not by the inhibition of Na⁺ uptake via an NHE, but by amiloride's other known action in also blocking Na⁺ channels. This would hyperpolarize the apical membrane potential and thereby reduce H⁺ pumping by the H⁺-ATPase (Potts, 1994) and concomitantly reduce the acid-trapping ability. In support of this notion, some studies have shown that increasing the external Na⁺ had no effect on J_{Amm} (Kerstetter *et al.*, 1970; Salama *et al.*, 1999). Similarly removing Na⁺ from the external medium (DeVooys, 1968; Kerstetter et al., 1970; Wilson et al., 1994) or reducing Na⁺ uptake with acetazolamide treatment, also had no effect on JAmm (Kerstetter et al., 1970). Furthermore, during HEA, amiloride almost completely blocked Na⁺ influx but J_{Amm} continued, albeit at a reduced level (Wilson et al., 1994).

1.3 Alternative ammonia transport mechanisms

While it remains unclear whether or not Na⁺ is directly involved in the ammonia excretion process, it is also not certain how much NH₃ actually diffuses across the gill membranes since the lipid-to-water partition coefficient for NH₃ is only within the range

McMaster - Biology

of 0.04 and 0.08 (Evans and Cameron, 1986). Water-filled channels, if present, would facilitate a much faster rate of NH₃ diffusion (Wood, 1993). Moreover, it appears that diffusion of NH₃ alone cannot account for all of the ammonia excreted from the gill. Heisler (1990) proposed that above a threshold plasma ammonia level of 200 μ mol L⁻¹, diffusion is replaced by a carrier-mediated mechanism. Evidence for a carrier-mediated transport has been demonstrated in a cultured trout gill cell preparation where J_{Amm} was correlated with a basolateral-to-apical membrane NH₄⁺ electrochemical gradient (Kelly and Wood, 2001), but how well this mirrors the *in vivo* situation is unknown.

1.3.1 Na⁺/K⁺-ATPase (NKA)

Since NH_4^+ can substitute for K^+ in the Na^+/K^+ -ATPase (NKA) (Towle and Holleland, 1987), basolateral uptake of ammonia by this route is possible. Salama *et al.* (1999) however, reported that increasing amounts of NH_4^+ failed to activate the NKA of the rainbow trout gill and instead proposed that most of the ammonia diffused into the gill basolaterally as NH_3 while the rest entered via a Na^+/H^+ (NH_4^+)-ATPase, as proposed earlier by Balm *et al.* (1988).

1.3.2 Rhesus (Rh) proteins

Ammonia transport has also been studied extensively in organisms that utilize ammonia as a nitrogen source (Kleiner, 1981). It was only recently however, that the genes and corresponding proteins for ammonia transporters were identified. The first ammonia transporter genes were characterized in yeast (Mep) and plants (Amt) (Marini *et*

7

al., 1994; Ninnemann *et al.*, 1994). Shortly thereafter, based on sequence similarity, mammalian homologues to the Mep/Amt proteins were identified as the Rhesus (Rh) blood group antigens, normally recognized for their role in transfusion-incompatible immune reactions (Marini *et al.*, 1997). While the function of the unglycosylated Rh30 (30 kDa predicted molecular weight) proteins RhCE and RhD is unknown, the glycoslyated Rh50 (50 kDa predicted molecular weight) proteins are believed to function as ammonia transporters (Zidi-Yahiaoui *et al.*, 2009). Three mammalian Rh glycoproteins have been identified to date: RhAG/Rhag, RhBG/Rhbg, and RhCG/Rhcg (Huang and Peng, 2005; Huang, 2008). The terminologies RhAG, RhBG and RhCG are dedicated to human tissues while Rhag, Rhbg, and Rhcg are reserved for non-human tissues.

1.3.2.1 Tissue distribution in mammals

RhAG/Rhag is expressed predominantly on erythrocytes (RBCs) while RhBG/Rhbg and RhCG/Rhcg are broadly distributed in tissues including the brain, kidney, liver, ovary, skin, and testes (Huang, 2008; Liu *et al.*, 2000, 2001). The Rh proteins are expressed in an opposite and polarized orientation along the gastro-intestinal tract of mice with Rhcg oriented apically and Rhbg situated basolaterally (Handlogten *et al.*, 2005). In the kidney of the mouse and rat however, while Rhbg appears to be exclusively basolateral in orientation, Rhcg is situated both apically and basolaterally (Kim *et al.*, 2009; Seshadri *et al.*, 2006a),

8

1.3.2.2 Function based on structure

Results from homology modeling and site-directed mutagenesis indicate that Rh proteins are structurally and functionally similar to the ammonium transporter (AmtB) of Escherichia coli (Callebaut et al., 2006; Conroy et al., 2005; Marini et al., 2006; Zidi-Yahiaoui et al., 2009). The X-ray structure of AmtB reveals that this trimeric membranespanning complex functions as an NH₃ channel (Khademi et al., 2004, Zheng et al., 2004). Binding and deprotonation of NH4⁺ occurs in an external vestibule before NH3 passes through the channel and reprotonates upon exiting. The recently solved X-ray structure of the Rh50 protein from Nitrosomonas europaea confirms similarities in the channel, but some differences in the external vestibules of AmtB and Rh proteins (Li et al., 2007; Lupo et al., 2007). The lack of a conserved NH4⁺ binding site on the Rh proteins suggests that the relative affinity of Rh proteins for NH_4^+ may be lower than that of AmtB. This may reflect the difference in ammonia transport requirements. NH4⁺ binding may be essential for bacteria in order to capture ammonia from very low environmental concentrations whereas this feature may be less important in the mammalian renal system, where ammonia is present in high millimolar concentrations (Zidi-Yahiaoui et al., 2009).

1.3.2.3 NH_3 , NH_4^+ or both? Electrogenic or electroneutral?

In 2000, Marini *et al.* reported that RhAG and RhCG expressed in Mep-deficient yeast mediated the uptake of ammonia as well as the efflux of the ammonium analogue, methylamine. This was followed subsequently by numerous functional studies involving

the heterologous expression of the Rh proteins in cells or cell preparations. All three mammalian Rh glycoproteins have been shown to transport ammonia or its analogue, methylamine but considerable disagreement exists as to whether the species transported is NH₃ or NH₄⁺. Electroneutral NH₄⁺/H⁺ exchangers, NH₃ channels, electroneutral NH₃ transporters, NH₄⁺ exporters, NH₄⁺/NH₃ transporters, and electrogenic NH₄⁺ transporters have all been proposed (for summary, see Javelle *et al.*, 2007). The discrepancies in these results may simply reflect differences in experimental methodology. Furthermore, because many of these studies used *Xenopus* oocyte expression systems, interpretation of results is complicated by non-specific endogenous NH₃/NH₄⁺ transporter activation in these oocytes (Boldt *et al.*, 2003; Burckhardt and Fromter, 1992; Cougnon *et al.*, 1996). Also, it has been argued that the commonly used radiolabeled analogue for ammonia, [¹⁴C]methylamine is not a perfect tracer because transport affinities for methylamine and ammonia differ (Bakouh *et al.*, 2006) and also because the pK_a for methylamine is higher than that for ammonia (10.6 vs. 9.3).

1.3.2.4 The functional role of Rh proteins

It is unclear what function RhBG/Rhbg has in renal metabolism since it appears to have no role under basal conditions or during chronic metabolic acidosis (Weiner and Hamm, 2006). Chronic metabolic acidosis which normally increases renal ammonia excretion, resulted in no change in Rhbg protein expression in rats (Seshadri *et al.* 2006a). Similarly, Rhbg knock-out mice had normal acid-base balance and the response to chronic acid loading was also unaffected (Chambrey *et al.*, 2005). Additionally, it was reported recently that RhBG is not expressed in detectable levels in the human kidney (Brown *et al.*, 2009). Rhcg however, does seem to be involved during chronic metabolic acidosis. Increased total cellular Rhcg expression and changes in subcellular distribution of Rhcg were observed under these conditions (Seshadri *et al.*, 2006b). More recently, knock-down studies in mice have confirmed that Rhcg is essential to renal ammonia excretion (Biver *et al.*, 2008; Lee *et al.*, 2009).

1.3.2.5 The CO_2 debate

An ongoing controversy regarding Rh protein function is that they may also transport CO₂. The green alga, *Chlamydomonas reinhardtii* possesses both Amt and Rh 50 (Rh1) genes. Soupene *et al.* (2002) showed that *C. reinhardtii* grows rapidly in the presence of CO₂ and that the Rh1 protein was highly expressed under high CO₂ conditions. Furthermore, knockdown of Rh1 with RNA interference impaired this high rate of growth in the presence of elevated CO₂ (Soupene *et al.*, 2004). More recently, Endeward *et al.* (2006) reported that RBCs lacking RhAG (Rh_{null}) had reduced CO₂ permeability and followed this with a study that demonstrated the transit of both NH₃ and CO₂ through the RhAG protein (Endeward *et al.*, 2007). RhCG-expressing *Xenopus* ooctyes were also shown to have increased CO₂ permeability (Bakouh *et al.*, 2006). To further support the CO₂ transport function of Rh proteins, Li *et al.*, (2007) described in a possible CO₂ binding site at the COOH-terminal end, in their structural study of the Rh50 protein of *N. europaea*. On the opposite side of the debate, Ripoche *et al.* (2006) presented evidence indicating that Rh_{null} ghost cell preparations had no change in CO₂ permeability. Also, alterations in CO₂ concentrations did not affect the transcription levels of Rh1 in *N. europaea* (Weidinger *et al.*, 2007). Similarly, there was no change in growth rate or yield in Rh1 knockout mutants of *N. europaea* exposed to varying levels of CO₂ (Cherif-Zahar et al., 2007).

1.3.2.6 Rh proteins in fish

Fish Rh genes were first identified in medaka (*Oryzias latipes*) (Kitano and Saitou, 2000) and subsequently in several other fish species (Huang and Peng, 2005). The first comprehensive characterization of Rh proteins in fish was carried out in the pufferfish (*Takifugu rubripes*) (Nakada *et al.*, 2007a). Rhag, Rhbg, Rhcg1, and Rhcg2 were localized to specific regions of cells in the gill epithelium and these proteins, when expressed in *Xenopus* oocytes, enhanced the transport of the ammonia analogue, methylamine. No experimental manipulations were performed, but a hypothetical model for ammonia excretion was presented. Specifically, it was proposed that Rhag in the pillar cells function in conjunction with basolateral Rhbg and apical Rhcg2 in the gill pavement cells to facilitate ammonia excretion out of the gill when plasma ammonia levels are high. Additionally, in mitochondria-rich (MR) gill cells, an apical Rhcg1 and basolateral Na⁺/K⁺-ATPase could cooperate as an auxiliary route of excretion to maintain low plasma ammonia levels. This study was followed by another in the mangrove killifish (*Kryptolebias marmoratus*) wherein Rh cDNA sequences were identified in the

gill as well as in other tissues (Hung *et al.*, 2007). Experimental exposure of these fish to HEA induced changes in mRNA levels of Rhcg2 in the gills, Rhcg1 in the skin, and Rhbg in the liver and muscle.

1.4 Hypotheses and objectives

Although there is clear evidence that Rh proteins are present in fish (Kitano and Saitou, 2000; Huang and Peng, 2005; Hung *et al.*, 2007; Nakada *et al.*, 2007a), studies that directly associate Rh proteins with enhanced ammonia excretion at the gill are lacking. Similarly, although there is some evidence that CO₂ also passes through the Rh channels, no physiological studies have been performed to test this hypothesis in fish. Finally, although several functional studies have been carried out on heterologously expressed Rh proteins (see Javelle *et al.*, 2007) most have relied solely on the ammonia analogue, methylamine, to characterize transport properties and none have been able to directly measure the movement of ammonia. With this background in mind, this thesis used molecular and physiological approaches to test the following five hypotheses, with a particular emphasis on the gills:

1) A wide range of Rh proteins are present and differentially expressed in the tissues of rainbow trout.

2) The mRNA expression levels of these trout Rh proteins will respond to both external and internal ammonia loading.

13

3) The mRNA expression levels of other trout gill proteins thought to be involved in ammonia excretion will also respond to ammonia loading, revealing functional synergies.

4) The mRNA expression levels of trout Rh proteins will respond to an elevation in environmental CO₂ tension.

5) The transport characteristics of trout Rh proteins, when evaluated in a heterologous expression system, will fit a model whereby the protonated form (NH_4^+) is initially bound and deprotonated, allowing only the unprotonated form (NH_3) to pass through the channel.

1.5 Overview of experimental chapters

1.5.1 Chapter 2

The focus of this chapter was to test hypothesis 1 and 3, and the external ammonia loading part of hypothesis 2. Specifically, seven full-length Rh cDNA sequences (Rhag, Rhbg-a, Rhbg-b, Rhcg1-a, Rhcg1-b, Rh30-like2, and Rh30-like3) were cloned and characterized in the trout gill and other tissues. Exposure to 48 hours of HEA revealed that while ammonia excretion was initially blocked, it recovered over time. The recovery of excretion against an apparent gradient was correlated with increases in the expression of Rhcg2 mRNA and H⁺-ATPase mRNA and activity in the gill at 12 hours. These changes were localized to the gill pavement cells, and intracellular carbonic anhydrase mRNA expression and activity was down-regulated in these same cells. While all of the
genes analyzed except Rhcg1 and NHE2 responded, Rhcg2 and H^+ -ATPase were most sensitive to HEA. Therefore the principal conclusion was that the pavement cells were instrumental in the ammonia excretion mechanism with Rhcg2 and H^+ -ATPase functioning in a cooperative manner to facilitate the removal of NH_4^+ from the gill during HEA.

1.5.2 Chapter 3

In this chapter, hypothesis 4 was tested. Three full-length Rhbg2 variants (Rhbg2a, Rhbg2b, and Rhbg2c) were also identified. mRNA levels of Rh proteins were assessed in the tissues of trout exposed to external hypercapnic conditions. External hypercapnia lowered the water pH resulting in increased ammonia excretion and lower plasma ammonia levels, however no changes in Rh mRNA levels were noted in the gill. Addition of Hepes prevented acidification of the external water during hypercapnia and normocapnia but resulted in reduced ammonia excretion, increased plasma ammonia, and a downregulation of Rhcg2 in the gill and upregulation of Rhcg2 in the skin. These changes in Rh mRNA expression appeared to reflect a response to high plasma ammonia rather than a response to high CO₂. Rhag mRNA levels in the RBCs decreased during normocapnia and Hepes exposure, but increased during hypercapnia and Hepes exposure and therefore it is possible that Rhag responded to both CO₂ and ammonia. Overall, however, hypercapnia itself did not result in any significant changes in Rh mRNA levels and therefore a role for Rh proteins in CO₂ transport was not supported.

1.5.3 Chapter 4

Hypotheses 2 and 3 were tested in this chapter. Trout were infused with ammonia so as to raise plasma ammonia levels without inducing HEA and the mRNA levels of Rh proteins and other transporters and enzymes were assessed. Ammonia infusion resulted in a rapid elevation of plasma ammonia with a concurrent increase in ammonia excretion. It was observed in this study that the control saline infusion as well as the surgical procedure itself also induced changes in gene expression. In general however, since mRNA expression levels of the gill Rh proteins (Rhbg, Rhcg1, and Rhcg2) responded to elevated plasma ammonia, and the mRNA of other gill proteins (NHE2, NKA and H⁺-ATPase) responded as well, the pattern of gene expression in response to ammonia infusion was similar to that observed after HEA exposure.

1.5.4 Chapter 5

This final experimental chapter tested hypothesis 5. Trout Rh proteins were expressed in *Xenopus* oocytes and the transport of methylamine was measured using $[^{14}C]$ methylamine while the transport of ammonia was tracked using the scanning ion electrode technique (SIET). Trout Rh proteins facilitated the saturable and bi-directional flux of methylamine across the oocyte membrane. The transport rates were sensitive to a pH gradient with higher rates observed when H⁺ gradients were steeper. Faster transport rates also occurred when the concentration of the protonated species was increased. The principal conclusion was that trout Rh proteins are low-affinity, high-capacity ammonia transporters that bind NH₄⁺ but conduct NH₃ through the channel in a manner responsive

to pH gradients. When plasma ammonia levels are elevated, Rh proteins would thereby facilitate rapid ammonia efflux from the gill by exploiting the favourable pH gradient formed by the acidified gill boundary layer.

1.6 Summary

Overall, the four experimental chapters of this thesis (Nawata *et al.*, 2007; Nawata and Wood, 2008; Nawata and Wood, 2009; Nawata *et al.*, submitted), as well as related studies to which I contributed (Hung *et al.*, 2007, 2008; Tsui *et al.*, 2009), have provided considerable evidence that Rh proteins play a key role in ammonia excretion across the gills of teleost fish. Together with the work of other groups (e.g. Braun *et al.*, 2009; Nakada *et al.*, 2007a,b; Shih *et al.*, 2008), this research has opened up a new paradigm in an area of comparative physiology that has been controversial for more than three quarters of a century (Smith, 1929; Krogh, 1938; Wright and Wood, 2009).

CHAPTER 2

AMMONIA EXCRETION IN RAINBOW TROUT (*Oncorhynchus mykiss*): EVIDENCE FOR Rh GLYCOPROTEIN AND H⁺-ATPase INVOLVEMENT

2.1 ABSTRACT

Branchial ammonia transport in freshwater teleosts is not well understood. Most studies conclude that NH_3 diffuses out of the gill and becomes protonated to NH_4^+ in an acidified gill boundary layer. Rhesus (Rh) proteins are new members of the ammonia transporter superfamily and rainbow trout possess genes encoding for Rh30-like1 and Rhcg2. We identified seven additional full-length trout Rh cDNA sequences: one Rhag and two each of Rhbg, Rhcg1 and Rh30-like. The mRNA expression of Rhbg, Rhcg1, and Rhcg2 was examined in trout tissues (blood, brain, eye, gill, heart, intestine, kidney, liver, muscle, skin, spleen) exposed to high external ammonia (HEA; 1.5 mmol L⁻¹ NH₄HCO₃, pH 7.95, 15°C). Rhbg was expressed in all tissues, Rhcg1 was expressed in brain, gill, liver and skin, and Rhcg2 was expressed in gill and skin. Brain Rhbg and Rhcg1 were downregulated, blood Rh30-like and Rhag were downregulated, and skin Rhbg and Rhcg2 were upregulated with HEA. After an initial uptake of ammonia into the fish during HEA, excretion was re-established, coinciding with upregulations of gill Rh mRNA in the pavement cell fraction: Rhcg2 at 12 and 48 hours, and Rhbg at 48 hours. NHE2 expression remained unchanged, but upregulated H⁺-ATPase (V-type, Bsubunit) and downregulated carbonic anhydrase (CA2) expression and activity were noted in the gill and again expression changes occurred in pavement cells, and not in mitochondria-rich cells. Together, these results indicate Rh glycoprotein involvement in ammonia transport and excretion in the rainbow trout while underscoring the significance of gill boundary layer acidification by H⁺-ATPase.

2.2 INTRODUCTION

The current model of ammonia excretion from the freshwater teleost gill links NH₃ diffusion to CO₂ hydration (Wilkie, 2002). Expired CO₂ is converted to HCO₃⁻ and H⁺ by carbonic anhydrase (CA) in the gill mucus and the resulting acidified gill boundary layer then traps NH₃ to NH₄⁺ (Wright *et al.*, 1986, 1989). In addition, this layer could also be acidified by H⁺ extruded from an apical vacuolar H⁺-ATPase (Lin and Randall, 1990, Lin *et al.*, 1995). However it is acidified, this boundary layer creates a microenvironment for a favourable transbranchial (blood-to-water) P_{NH3} gradient that facilitates ammonia excretion. This is especially advantageous during exposure to high external ammonia (HEA), which reverses the normally positive ammonia gradient. Indeed, abolishing this layer with HEPES buffer during HEA reduces J_{Amm} (Wilson *et al.*, 1994).

Despite the dominance of this NH_3 diffusion-trapping theory, many past studies have supported the possibility of apical Na^+/NH_4^+ exchange (e.g. Kirschner *et al.*, 1973; Wright and Wood, 1985). It has been argued however, that freshwater cannot provide the Na^+ gradients necessary to drive this exchange (Wilkie, 2002). Likewise, basolateral entry of NH_4^+ into the gill via an Na^+/NH_4^+ exchanger (NHE) is unlikely since the electromotive force for Na^+ is directed into the gill cytosol and NH_4^+ cannot outcompete the much more abundant Na^+ in the plasma for access to the NHEs (Wilkie, 2002). Also, no evidence exists to support basolateral ammonia transport via NH_4^+ substitution for K^+ on the Na^+/K^+ -ATPase in the freshwater gill, although there are reports of this occurring in seawater fish (Mallery, 1993; Randall *et al.*, 1999).

Ammonia transport has been studied for decades in microorganisms but the molecular identity and function of potential transporters involved were not elucidated until recently. The genes and corresponding proteins were first identified as methylammonium permease (Mep) in yeast (Marini et al., 1994) and the ammonium transporter (Amt) in plants (Ninnemann et al., 1994). Based on sequence similarity, mammalian homologues to the Mep/Amt proteins were identified as the Rh blood grouprelated proteins: Rh30 and the glycoproteins RhAG/Rhag, RhBG/Rhbg, and RhCG/Rhcg (Huang and Liu, 2001). The terminologies RhAG, RhBG and RhCG refer to human tissue while Rhag, Rhbg, and Rhcg indicate non-human tissue (Weiner and Hamm, 2006). Rh30 and RhAG/Rhag are restricted to erythrocytes (RBCs) whereas RhBG/Rhbg and RhCG/Rhcg are expressed in various other tissues (Huang and Liu, 2001; Liu et al., 2000, 2001). The three glycoproteins conduct ammonia when expressed in heterologous systems, although disagreement exists as to whether NH₃ or NH₄⁺ is transported (Javelle et al., 2007). The non-glycosylated Rh30 proteins form part of the Rh complex with RhAG on the RBC membrane, but their function is unknown (Westhoff, 2007; Westhoff and Wylie, 2006).

Rh proteins have been identified in different fish species (Huang and Peng, 2005; Hung *et al.*, 2007; Nakada *et al.*, 2007) and functionally, it has been shown that pufferfish *Takifugu rubripes* Rh glycoproteins, like their mammalian counterparts, mediate the transport of the ammonium analogue, methylammonium, when expressed in *Xenopus* oocytes (Nakada *et al.*, 2007). Recently we identified three Rh cDNA sequences in the unexposed and HEA-exposed mangrove killifish *Kryptolebias marmoratus* (Hung *et al.*,

2007). HEA exposure resulted in upregulated Rh mRNA expression in the gill, liver, muscle, and skin. Two rainbow trout Rh genes (Rh30-like1 and Rhcg2) were identified in a previous study (Huang and Peng, 2005), but their tissue expression and potential involvement in ammonia transport have not been investigated.

With this background in mind, the objectives of this study were to identify and sequence Rh orthologues in the rainbow trout, to examine their expression pattern in different trout tissues under control conditions and when exposed to HEA, and to quantify the HEA-exposed gill expression of Rh orthologues as well as other proteins thought to be involved in the ammonia transport process.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Rainbow trout, [*Oncorhynchus mykiss*, (Walbaum); 178-260g], obtained from Humber Springs Trout Hatchery, Ontario, Canada were used for all experiments. The fish were held in dechlorinated Hamilton tapwater (moderately hard: $[Na^+]=0.6$ mequiv L^{-1} , $[CI^-]=0.8$ mequiv L^{-1} , $[Ca^{++}]=0.8$ mequiv L^{-1} , $[Mg^{++}]=0.3$ mequiv L^{-1} , $[K^+]=0.05$ mequiv L^{-1} ; titration alkalinity 2.1 mequiv L^{-1} ; pH ~8.0; hardness ~140 mg L^{-1} as CaCO₃ equivalents; temperature 12-16° C) and food was withheld for at least one week prior to experimentation to stabilize the endogenous fraction of nitrogenous waste excretion (Fromm, 1963). All fish were transferred to individual, darkened acrylic flux boxes supplied with aerated, continuously flowing dechlorinated tap water at $15\pm0.5^{\circ}C$ and allowed to recover overnight prior to experimentation. All procedures used were approved by the McMaster University Animal Research Ethics Board and are in accordance with the Guidelines of the Canadian Council on Animal Care.

2.3.2 High external ammonia (HEA) experiment

Six fish were exposed to a nominal total ammonia (T_{Amm}) concentration of 1.5 mmol L⁻¹ NH₄HCO₃ (pH 7.95±0.05, 15.0±0.5°C) for 12 or 48 hours. Fluxes were conducted during which time the water flow was stopped and the box volume was set to 4 L. For the 48-hour flux, a period of flow-through flushing was performed every 12 hours to prevent toxic build-up of ammonia. Control fish (six each) were treated identically for 12 and 48 hours without the addition of ammonia.

Water samples (10 ml) were taken at 12-hour intervals, frozen at -20° C and later assayed in triplicate for T_{Amm} using a modified salicylate-hypochlorite method (Verdouw *et al.*, 1978). After the flux periods, the fish were anaesthetized with MS222 (~0.1 g L⁻¹; Sigma, St. Louis, MO) and caudal blood was drawn into a heparinized syringe. Samples were centrifuged to separate plasma from RBCs (2 min, 20,000 g), immediately frozen in liquid nitrogen, stored at -70° C, and later analyzed enzymatically for plasma T_{Amm} (Raichem, Hemagen Diagnostics, Inc., San Diego, CA). Net flux rates of total ammonia (J_{Amm} , µmol kg⁻¹h⁻¹) were calculated as:

$$J_{\text{Amm}} = (T_{\text{Ammi}} - T_{\text{Ammf}}) \times V/(t \times M)$$

where *i* and *f* refer to initial and final concentration (μ mol L⁻¹), *V* is water volume (L) in the box, *t* is the time elapsed (h) and *M* is the fish mass (kg). A negative J_{Amm} indicates a

net excretion of ammonia to the water. A positive J_{Amm} indicates a net uptake of ammonia into the fish.

2.3.3 Sequencing and characterization of rainbow trout Rh cDNAs

2.3.3.1 Tissue sampling

Gill and blood samples for cloning purposes were removed from fish that were exposed to $1.5 \text{ mmol } \text{L}^{-1} \text{ NH}_4\text{HCO}_3$ for 24 hours. Control samples for all subsequent analyses were collected from fish that were held in individual flux boxes with flowing dechlorinated tap water for 24 hours. Blood samples were collected (as described above) immediately after anaesthetization and prior to tissue collection.

Preliminary studies indicated Rhbg expression on RBCs, so all analyses subsequent to cloning were performed on saline-perfused tissues to minimize the contribution of RBC mRNA to tissue levels. Samples of brain, eye, gill, heart, intestine, kidney, liver, muscle, skin, and spleen were removed after the fish were anaesthetized, placed on ice, and perfused free of blood with chilled, heparinized (50 i.u. ml⁻¹ lithium heparin; Sigma) Cortland saline (Wolf, 1963). Perfusions were performed by cannulation of the bulbus arteriosus with a 23-gauge butterfly needle attached to a peristaltic pump and the ventricle was severed immediately after initiation of perfusion to allow for drainage. Tissue samples were immediately frozen in liquid nitrogen and stored at –70°C until analysis.

2.3.3.2 RNA extraction and reverse-transcriptase PCR amplification

Total RNA was extracted from blood and tissues using Trizol (Invitrogen, Burlington, ON, Canada), quantified spectrophotometrically and electrophoresed on 1% agarose gels stained with ethidium bromide to verify integrity. First strand cDNA was synthesized from 1 μ g total RNA using an oligo(dT₁₇) primer and Superscript II reverse transcriptase (Invitrogen). Samples were stored at -70° C.

2.3.3.3 Identification of Rh orthologues

Partial Rh30-like, Rhag, Rhbg and Rhcg1 sequences were obtained from cDNA of HEA-exposed blood or gill samples using primers (Table 2.1) designed from the trout Rh30-like1 sequence and pufferfish Rhag, Rhbg and Rhcg2 sequences (GenBank: AY207445, AY618933, AY116074, AY116076 respectively). Polymerase chain reactions (PCR) were carried out in a PTC-200 MJ Research thermocycler with Taq DNA polymerase (Invitrogen) at 94°C (2 min), followed by 45 cycles of 94°C (30 s), 52°C (30 s) and 72°C (1 min). Products were electrophoresed on ethidium bromidestained 1% agarose gels and bands of appropriate size were excised and extracted using the Qiaquick gel extraction kit (Qiagen Inc., Mississauga, ON, Canada). Purified gel products were ligated to a pGEM-T easy vector (Promega, Fisher Scientific, Nepean, ON, Canada), transformed into heat-shock competent Escherichia coli (XL-Blue, Stratagene, Mississauga, ON, Canada), then grown on ampicillin LB agar plates. Colonies containing the ligated product were inoculated into liquid media and grown overnight. Plasmids were harvested using GeneJet Plasmid Miniprep Kit (Fermentas Canada Inc.,

Burlington, ON, Canada) and sequencing was performed on an ABI 3100 Gene Analyzer at the MOBIX Lab, McMaster University, Hamilton, ON, Canada. Primers designed specific to the Rh orthologues (Table 2.1) were used to obtain full-length cDNA sequences by 5' and 3' RACE (Smart RACE cDNA amplification kit, BD Bioscience Clontech, Mississauga, ON, Canada). Several clones of the 3' and 5' ends were sequenced in both directions and full-length transcripts were determined by majority-rule consensus using BioEdit (Hall, 1999). Sequences have been deposited into GenBank (Table 2.2).

2.3.3.4 Phylogenetic, amino acid sequence identity, hydropathy and glycosylation analysis

Phylogenetic analyses were conducted using ClustalW (Thompson *et al.*, 1994) and MEGA 3.1 software (Kumar *et al.*, 2004) using the neighbour-joining method with support for each node using 500 bootstrap replicates. Amino acid sequence analyses were performed using BioEdit (Hall, 1999), hydropathy profiles were determined using EMBOSS (Rice *et al.*, 2000) and SPLIT 4.0 (Juretic *et al.*, 2002), and N-glycosylation sites were predicted using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.3.3.5 Tissue distribution (PCR)

Following the methods described above, total RNA and cDNA were prepared from the RBCs and saline-perfused tissues collected from the initial HEA experiment.

Distribution of Rh orthologues in control and HEA-exposed (48-hour) tissues was determined using orthologue-specific primers (Table 2.1) with the PCR protocol described earlier. Products were electrophoresed on ethidium bromide-stained 1.5% agarose gels.

2.3.3.6 Tissue expression (qPCR)

Rh expression in tissues exposed to HEA for 12 and 48 hours was compared to control tissues by quantitative real-time PCR (qPCR) using the cDNA prepared above and orthologue-specific primers (Table 2.1). Primer specificity was confirmed in preliminary experiments and qPCR analyses were performed on an Mx3000P QPCR System (Stratagene, Cedar Creek, TX). Reactions (20 µl) containing 4 µl of DNaseItreated (Invitrogen) cDNA, 4 pmoles of each primer, 10 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 0.8 µl of ROX (1:10 dilution) were performed at 50°C (2 min), 95°C (2 min), followed by 40 cycles of 95°C (15 s) and 60°C (30 s). Meltcurve analysis confirmed production of a unique product and gel electrophoresis verified presence of a single band. A non-reversed-transcribed sample controlled for possible genomic DNA contamination. Elongation factor-1a (EF-1a; GenBank AF498320) for gill and β -actin (GenBank AF157514) for other tissues had the most stable expression across samples and were used as endogenous standards to calculate relative mRNA expression by the standard curve method. Standard curves were generated by serial dilution of a random mixture of control samples. To discriminate between erythroid and non-erythroid Rhbg mRNA expression, unperfused samples of brain, kidney and liver

were analyzed and compared to samples which had been perfused free of blood, using the same methods as those described earlier.

2.3.3.7 Pavement cell and mitochondria-rich cell expression

Pavement and mitochondria-rich (MR) cells were isolated from gill epithelial cells using a technique modified from Galvez *et al.* (2002). Whole branchial baskets were removed from control and HEA-exposed fish and gill filaments were trypsindigested, passed through 96 µm filters, treated with RBC lysis buffer and layered onto a discontinuous Percoll density gradient. Pavement cells were harvested from the 1.03-1.05 g ml⁻¹ interface and MR cells were harvested from the 1.05-1.09 g ml⁻¹ interface. Total RNA and cDNA were processed as described above from both cell fractions and the mRNA expression of Rh was measured relative to β-actin.

2.3.4 Enzyme / transporter expression in the gill

Following the qPCR protocol outlined earlier, expression of H⁺-ATPase, (V-type, B subunit; GenBank AF14002), carbonic anhydrase (cytoplasmic, CA2; GenBank AY514870), four isoforms of Na⁺/K⁺-ATPase (α 1a, α 1b, α 1c, α 3; GenBank: AY319391, AY319390, AY319387, AY319388), and NHE2 (GenBank EF446605) was quantified in control and HEA-exposed whole gill samples relative to EF-1 α , and in control and HEAexposed pavement and MR cells relative to β -actin. Expression of Na⁺/K⁺-ATPase isoforms α 1a and α 1b were quantified in the gill cell fractions, but as the results were similar, only the results of α 1a are reported here.

2.3.5 Enzyme activity in the gill

Enzyme assays were performed in triplicate on homogenates of gill tissues that were perfused free of blood, extracted, and frozen from the previous HEA experiment. Protein assays were performed using Bradford Reagent (Sigma) and BSA standards.

2.3.5.1 ATPase activity

Protocols for Na⁺/K⁺-ATPase (McCormick, 1993) and H⁺-ATPase (Lin and Randall, 1993) assays were modified and adapted to measure activity levels in control and HEA-exposed fish gills. Activities are reported as control activity (no inhibitor) minus inhibitor-treated activity [ouabain for Na⁺/K⁺-ATPase and sodium azide with N-ethymaleimide (NEM) for V-type H⁺-ATPase], measured at 340 nm in a kinetic microplate reader (SpectraMAX Plus, Molecular Devices, Menlo Park, CA) at 15 s intervals for 30 min. We obtained the same results for H⁺-ATPase activity using 10 μ M bafilomycin as an inhibitor, but are only reporting results obtained using NEM.

2.3.5.2 Carbonic anhydrase

Carbonic anhydrase activity was determined using the electrometric ΔpH method (Henry, 1991). Briefly, samples were assayed in a pH 7.4 buffer (225 mmol mannitol, 75 mmol sucrose, 10 mmol Tris) held at 4°C and the reaction was initiated with 200 µl of CO₂-saturated water. The reaction velocity was measured, using hydrogen and reference electrodes (Kwik-Tip, World Precision Instruments, Sarasota, FL) attached to an

Accumet XL15 pH meter (Fisher Scientific), over a 0.15 pH change and the activity reported as the observed rate minus the uncatalyzed rate.

2.3.6 Statistical analysis

All data are presented as means \pm s.e.m. (*n*, number of fish). Values at each time point were compared to control values using one-way analysis of variance (ANOVA) with Fisher's Least Significant Difference post-hoc test. Student's *t*-test was used for comparisons of T_{Amm} and J_{Amm} values and for comparisons between unperfused and perfused tissues. Significance was set at α =0.05.

2.4 RESULTS

2.4.1 High external ammonia experiment

Exposure to HEA initially resulted in net uptake of ammonia from the water into the fish during the first 12 hours. Thereafter, net excretion of ammonia occurred and by 48 hours, J_{Amm} exceeded the 12 and 48-hour control rates, which remained unchanged (Fig. 2.1). Plasma T_{Amm} levels in HEA-exposed fish were significantly higher than the control value (Fig. 2.2) but they remained lower than water levels throughout the time course. There is however, a noticeable upward trend towards 48 hours approaching a toxic level (Lumsden *et al.*, 1993), but no signs of stress were visible in these fish.

2.4.2 Identification of Rh orthologues in trout

Seven Rh orthologue cDNA sequences were identified in this study. Phylogenetic analysis (Fig. 2.3) showed that two sequences with different coding regions (CDS), isolated from HEA-exposed RBCs, fell into a cluster of fish sequences described as Rh30-like (designated Rh30-like2 and Rh30-like3). Another sequence, also isolated from RBCs, fell into the Rhag cluster (designated Rhag). Two sequences with identical CDS but different 3'-untranslated regions (3'-UTR) were cloned from HEA-exposed gill and these fell into the Rhbg cluster (designated Rhbg-a and Rhbg-b). A further two sequences that differ only in the 3'-UTRs were identified in the HEA-exposed gill and these fell into subgroup 1 in the Rhcg cluster (designated Rhcg1-a and Rhcg1-b). Amino acid sequence analysis revealed that the Rh30-like2 sequence is 98.8% identical to the previously identified Rh30-like1 sequence and all Rh30-like sequences are 44.5-96.4% similar to their counterparts in other fish. Rhag is 78.5-83.7%, Rhbg is 76.1-80.6 % and Rhcg1 is 75.8-82.7% identical to their respective homologues in other fish (Fig. 2.4). The CDS of the identified sequences vary in length from 1050-1463 nucleotides (Table 2.2) and hydrophobicity analyses of all sequences revealed 12 predicted transmembrane domains (Fig. 2.5). Rhag, Rhbg and Rhcg1 have predicted N-glycosylation sites in the extracellular domain at Asp45, Asp48 and Asp60 respectively. No potential glycosylation sites were identified in the Rh30-like sequences.

2.4.3 Tissue distribution and expression of Rh

PCR analysis of the various trout tissues revealed the presence of Rhbg in all control and HEA-exposed tissues examined, including the blood (Fig. 2.6). Since Rhbg was expressed in RBCs, it was important to ensure that expression in tissues nominally cleared of blood was not due to residual RBCs. Taking into consideration mRNA copy number, Rhbg was always higher in the unperfused tissues due to contribution from the blood. The contribution of Rhbg mRNA from the blood however, was small compared to that of β -actin and therefore the removal of blood in perfused tissues produced a more profound reduction in the relative amount of β -actin. On average, perfusion of brain, kidney, and liver reduced the Rhbg mRNA copy number by 1.7-fold, but β-actin copy number was reduced by 6-fold. While this large reduction in β -actin expression represents a substantial elimination of contaminating blood, the smaller reduction in Rhbg indicates that Rhbg expression was originating from the tissues. As a result, Rhbg mRNA expression was always higher in the perfused tissues when normalized to β -actin (Fig. 2.7). Interestingly, Rhbg expression decreased significantly at 48 hours of HEA in the perfused brain (Fig. 2.7), did not change in the HEA-exposed gill (Fig. 2.8), but increased by almost 200-fold at 48 hours HEA in the skin (Fig. 2.9). Of particular note also, is the increased Rhbg expression in the pavement cell fraction that is devoid of contaminating RBCs after lysis treatment (see below and Fig. 2.11*A*).

Rhcg1 was clearly detected in the gill and skin by PCR in both control and HEA conditions and there was some evidence of expression in the brain and liver during HEA as well (Fig. 2.6). Analysis by qPCR confirmed that Rhcg1 mRNA was expressed in

control and HEA conditions in brain, gill, liver and skin but the only significant change noted was a decrease in expression at 48 hours of HEA in the brain (Fig. 2.10), parallel to that seen with Rhbg in the brain at this time (cf. Fig. 2.7).

Although PCR analysis revealed Rhcg2 expression in the gill only (Fig. 2.6), qPCR analysis also detected Rhcg2 in the skin and its expression increased about 9-fold with 48 hours HEA (Fig. 2.9). In the HEA-exposed gill, Rhcg2 expression increased by 6.2-fold at 12 hours and remained elevated by 4.2-fold at 48 hours (Fig. 2.8).

Gill cell fractionation was performed to ascertain the cellular location of these changes in expression. In the pavement cell fraction, there was no change in Rhcg1 expression but there was a 4.3-fold increase in Rhbg expression at 48 hours of HEA and an 8.1 and 10.8-fold increase in Rhcg2 expression at 12 and 48 hours of HEA respectively (Fig. 2.11*A*). No significant changes in Rh mRNA expression were noted in the MR cell fraction, though Rhcg1 expression tended to fall (Fig. 2.11*B*).

With the Rh30-like1 and Rh30-like2 sequences being nearly identical, only the expression of Rh30-like2 and Rh30-like3 were analyzed. The expression of these as well as Rhag decreased significantly to less than half the control value in RBCs exposed to 12 and 48 hours of HEA (Fig. 2.12)

2.4.4 Enzyme / transporter expression and activity in the gill

The expression of H^+ -ATPase in the HEA-exposed gill increased by 2-fold at 12 and 48 hours (Fig. 2.13*A*) and H^+ -ATPase enzyme activity increased significantly by 1.4-fold at 48 hours HEA (Fig. 2.13*D*). CA2 expression decreased significantly at 48 hours to

McMaster - Biology

half of the control value (Fig. 2.13*B*) and this corresponded to a 1.7-fold decrease in CA enzyme activity at 48 hours HEA (Fig. 2.13*E*). Expression of four α -isoforms of Na⁺/K⁺-ATPase in the gill did not change significantly with HEA exposure (Fig. 2.13*C*) and Na⁺/K⁺-ATPase enzyme activity also remained unchanged (Fig. 2.13*F*). Similarly, NHE2 expression did not change significantly with control, 12 and 48 hours HEA values of 1.00 ± 0.21 , 1.82 ± 0.41 , and 0.72 ± 0.09 , respectively (*n*=6).

In the pavement cells, there was no change in NHE2 expression but there was a 8.3-fold decrease in CA2 expression at 48 hours, a 3-fold increase in H^+ -ATPase expression at 12 hours, and a 5.3-fold increase in Na⁺/K⁺-ATPase expression at 48 hours HEA (Fig. 2.14*A*). In MR cells, Na⁺/K⁺-ATPase was significantly downregulated by 5.6-fold at 48 hours HEA while CA2, H⁺-ATPase, and NHE2 expression remained unchanged (Fig 2.14*B*).

2.5 DISCUSSION

Marini *et al.* (2000) first reported that RhAG and RhCG rescued the growth of Mep-deficient yeast and that RhAG mediated efflux of methylammonium. Since then, the role of Rh proteins as potential ammonia transporters has become an active area of investigation. Previous work by Huang and Peng (2005) identified an Rh30-like1 and Rhcg2 gene in the rainbow trout, but the physiological significance of these genes has not been explored. In the present study, we identified additional cDNA sequences encoding for Rh30-like, Rhag, Rhbg, and Rhcg1 and examined their potential involvement in ammonia transport. Rh expression was studied exclusively at the mRNA expression

level in the present investigation. An important next step in future studies will be to quantify expression at the protein level, once suitable antibodies have been developed for use in rainbow trout.

All sequences identified in this study have 12 predicted transmembrane domains, a highly conserved characteristic observed in the Rh protein family (Huang and Liu, 2001). The Rh30-like1 and Rh30-like2 sequences are identical, with the exception of a single amino acid deletion and four amino acid substitutions in Rh30-like1 (Fig. 2.4). Rh30-like2 and Rh30-like3 differ only at the 3' end due to a 73 base pair (bp) deletion in Rh30-like2. We also identified a partial Rh30-like4 sequence with a more varied CDS as well as two variants of Rhag lacking the 12 predicted transmembrane spanning domains typical of the other Rh family members.

These multiple Rh30-like and Rhag sequences are likely the result of polyploidization events that occurred in the lineage leading to modern ray-finned fishes (Wittbrodt *et al.*, 1998). Salmonids have further undergone a recent (25-100 million years ago) tetraploidization event (Allendorf and Thorgaard, 1984), resulting in duplicated genes. Indeed, multiple Wilms' tumor suppressor (Brunelli *et al.*, 2001) and glutamine synthetase (Murray *et al.*, 2003) genes in trout are likely the result of both ancient and recent duplication events. It is possible therefore, that additional (yet unidentified) Rh genes will be characterized in the future.

The cDNA sequences of Rhbg-a and Rhbg-b are identical with the exception of a 474 bp deletion in the 3'-UTR of Rhbg-b. Rhcg1-a and Rhcg1-b are also identical, except for a 299 bp deletion in the 3'-UTR of Rhcg1-b. Presumably the two Rhbg

sequences are RNA splice variants from the same gene and this is likely the case for the two Rhcg1 sequences. Although we cannot speculate about the function of the Rhbg and Rhcg1 variants, it is reasonable to conclude that they are differentially expressed given that 3'-UTRs are important for post-transcriptional regulation (Mazumder *et al.*, 2003). Furthermore, preliminary results using primers designed specifically to Rhbg-a suggest that the Rhbg expression changes that we report here in the brain and skin (Figs. 2.7 and 2.9) likely reflect changes in Rhbg-b.

Both Rhbg and Rhcg1 are very similar (over 75%) to their counterparts in other fish species, but there is more variability between the Rh30-like sequences (Fig. 2.4). The Rh30-like sequences fall into a cluster of homologues related to the Rh30 proteins (Fig. 2.3) that were first described in humans (Avent and Reid, 2000). There is about 60% amino acid similarity between trout Rhbg/Rhcg and human RhBG/RhCG, but trout Rh30-like sequences are less than 31% similar to human Rh30 (not shown). This agrees well with Huang and Peng (2005) who showed that Rh30 is more functionally divergent across species than the other Rh genes. Rh30 proteins do not directly transport ammonia but their membrane expression depends on the presence of RhAG (Westhoff, 2007). In the present study, the mRNA expression of Rh30-like and Rhag followed the same pattern of down-regulation in HEA-exposed RBCs (Fig. 2.12) suggesting that these proteins could also be co-expressed together on the trout RBC membrane. Whether Rhag is additionally expressed in pillar cells of the trout gill, as they are in the pufferfish (Nakada *et al.*, 2007), remains to be determined.

Exposure to 1.5 mmol L⁻¹ NH₄HCO₃ for 48 hours resulted in an initial net uptake of ammonia into the fish with subsequent recovery of excretion (Fig. 2.1). This is consistent with earlier investigations that reported the same phenomenon in both freshwater and seawater fish exposed to high (up to 1 mmol L⁻¹) external concentrations of NH₄Cl or (NH₄)₂SO₄ (Cameron, 1986; Cameron and Heisler, 1983; Claiborne and Evans, 1988; Wilson and Taylor, 1992). These studies hypothesized that an active NH₄⁺ extrusion mechanism is induced to counteract the inward diffusion of NH₃, but active NH₄⁺ exchange has not yet been demonstrated in the freshwater teleost gill, leaving NH₃ diffusion as the main route of excretion (Salama *et al.*, 1999; Wilkie, 2002).

The lipid solubility of NH₃ is however only moderate at best (Evans and Cameron, 1986; Wood, 1993) and a protein channel would greatly facilitate NH₃ passage even under routine conditions. This is particularly advantageous when potentially toxic concentrations of ammonia need to be transported out quickly. In HEA conditions, fish need to counter the influx of ammonia as well as eliminate endogenously produced ammonia. This became apparent at 48 hours of HEA when plasma T_{Amm} approached water T_{Amm} (Fig. 2.2). Recovery of excretion after 12 hours and the increased J_{Amm} at 48 hours (Fig. 2.1) was accompanied by the simultaneous upregulation of Rhcg2 at 12 and 48 hours (Fig. 2.8). This suggests that Rhcg2 is upregulated in response to HEA, resulting in enhanced ammonia excretion.

Although Rhcg2 was upregulated during HEA, all three Rh mRNAs (Rhbg, Rhcg1, and Rhcg2) were expressed in the whole gill and gill cell fractions during control and HEA conditions (Fig. 2.8 and 2.11). Analysis of the gill cell fractions revealed that

Rhcg2 upregulation at 12 and 48 hours was accompanied by Rhbg upregulation at 48 hours and that these changes were occurring in the pavement cells and not in MR cells (Fig. 2.11). Taken together, this suggests that while both cell types may be involved in routine ammonia transport via Rh glycoproteins during control and HEA conditions, pavement cells respond to HEA with an upregulation of Rhcg2, restoring excretion and elevating rates above basal levels. Rhbg may be involved in the maintenance or further regulation of this process. These ideas still need to be validated with trout-specific Rh protein studies which will reveal whether changes in Rh protein levels or subcellular distribution occur as they do in rat renal cells during metabolic acidosis (Seshadri *et al.*, 2006).

In the gill of the pufferfish, localization of Rh mRNAs and proteins suggests that at least under basal conditions, Rhcg1 is expressed apically and is confined to the MR cells while Rhcg2 and Rhbg are restricted to pavement cells in the apical and basolateral regions respectively (Nakada *et al.*, 2007). In the gills of the rainbow trout, on the other hand, our findings, show that the trout Rh mRNAs are expressed in both the MR and pavement cells under control conditions, but respond differentially under HEA: Rhbg and Rhcg2 expression increases in pavement cells only, while Rhcg1 expression does not change in pavement cells but tends to fall in MR cells. Whether this discrepancy in findings is due to species differences and whether or not the same polarized localization of Rh glycoproteins occurs in the trout gill awaits further investigation.

Previous studies demonstrated that expired water passing over the gills of freshwater fish is acidified by up to 1.5 pH units, depending on initial water pH and

McMaster - Biology

buffer capacity (Lin and Randall, 1990; Playle and Wood, 1989; Wright *et al.*, 1989). This acidified gill boundary layer traps excreted NH₃ to NH₄⁺ (Randall and Wright, 1987; Wilson *et al.*, 1994), but whether the acidification is due to H⁺ arising from CO₂ hydration or is a result of H⁺ extruded by H⁺-ATPase, is unknown (Wilkie, 2002). In our study, an increase in H⁺-ATPase expression and activity (Figs. 2.13*A* and *D*) accompanied the Rhcg2 upregulation and recovery of ammonia excretion during HEA, suggesting that H⁺-ATPase is responsible for the acidification. Moreover, this upregulation of H⁺-ATPase occurred in the pavement cells (Fig. 2.14), indicating a possible functional coupling with Rhcg2 which has also been hypothesized to occur in the intercalated cells of the rat nephron (Eladari *et al.*, 2002).

The CA-catalyzed hydration of CO₂ normally fuels the H⁺-ATPase with H⁺ in the trout gill (Lin and Randall, 1991), so we expected to see an increase in CA expression and or activity in conjunction with the upregulated H⁺-ATPase in HEA conditions. Instead CA expression and activity were significantly decreased at 48 hours (Figs. 2.13*B* and *E*), a response that again appeared specific to the pavement cells (Fig. 2.14), in agreement with one study that reported inhibition of CA activity by HEA in trout (ArasHisar *et al.*, 2004). Downregulation or lowered CA activity suggests that CO₂ is no longer the main source of H⁺ for the H⁺-ATPase pump. Under ammonia-loaded conditions, NH₄⁺ may be the major supplier of H⁺. This correlates with the present model of the ammonium transporter (AmtB) of *E. coli* which is structurally and functionally similar to Rh proteins (Conroy *et al.*, 2005; Marini *et al.*, 2006; Zidi-Yahiaoui *et al.*, 2006). AmtB is a trimeric membrane-spanning complex (Conroy *et al.*, 2004; Khademi

et al., 2004; Zheng *et al.*, 2004) functioning as an NH₃ channel. The NH₄⁺ is deprotonated before entering the channel so that it can pass through as NH₃ and reprotonate after exiting (Khademi *et al.*, 2004; Khademi and Stroud, 2006). Under HEA, a continuous and abundant supply of H⁺ stripped off from NH₄⁺ entering the Rh channel may be fuelling the H⁺-ATPase (Fig. 2.15), making elevated CA activity unnecessary.

Alternative routes of ammonia transport in the gill are via the NHE and Na^+/K^+ -ATPase. So far, only the giant mudskipper, Periophthalmodon schlosseri, is documented to actively excrete NH_4^+ by a basolateral Na^+/K^+ -ATPase and an apical NHE during HEA (Randall et al., 1999). The lack of evidence for ammonia transport via Na⁺/K⁺-ATPase in the freshwater fish gill may be related to higher Na⁺/K⁺-ATPase activity levels in seawater fish (Wilkie, 2002) however, not all seawater species display high enzyme activity (Marshall and Bryson, 1998). In our study, HEA did not result in changes in Na^+/K^+ -ATPase at the transcript (Fig. 2.13C) or activity level (Fig. 2.13F) in whole gill samples. Analysis of mRNA expression in the gill cell fractions however, revealed upregulation in the pavement cells and downregulation in the MR cells (Fig. 2.14). These opposing cell-specific differences may explain the absence of change in expression and activity in the whole gill. Therefore, we cannot rule-out the potential for basolateral NH4⁺ transport via this route until more cell-specific studies are performed. No changes in NHE2 mRNA expression were seen in whole gill or the gill cell fractions (Fig. 1.14), so a role for this transporter during HEA is not apparent. NH_4^+ transport through the gill remains a possibility, but a mechanism has yet to be characterized.

Results from this study revealed a wide tissue expression of Rh mRNAs with a noted downregulation of Rhbg and Rhcg1 occurring in the brain with HEA exposure (Figs. 2.7 and 2.10). Since trout brain cells have only a limited capacity to detoxify ammonia (Mommsen and Walsh, 1992), we could hypothesize that this downregulation of Rh was a protective response to prevent ammonia over-loading into the cells. Downregulation of Rhbg did not occur in the mangrove killifish brain in response to similar HEA conditions (Hung *et al.*, 2007) and this may reflect species differences: the mangrove killifish is very ammonia-tolerant (Frick and Wright, 2002) compared to the trout (Mommsen and Walsh, 1992). Interestingly, Rh glycoproteins were not detected in the pufferfish brain (Nakada et al., 2007).

During HEA, Rhbg and Rhcg2 were upregulated in the skin (Fig. 2.9). This was surprising as the skin under normal conditions, contributes very little to total ammonia excretion in freshwater teleosts in comparison to seawater species (Wood, 1993). Upregulation of Rhcg1 and Rhcg2 was also observed in the mangrove killifish skin exposed to HEA or air (Hung *et al.*, 2007). This species however, is known to volatilize ammonia via the skin during air exposure (Litwiller *et al.*, 2006). Whether the trout skin also assumes an excretory role during HEA is not clear, but Rh proteins appear to play some role in this tissue during HEA.

An important controversy regarding the function of Rh proteins is whether or not they transport CO₂. *Chlamydomonas reinhardtii* is a green algae with Amt and Rh (Rh1) genes. When Rh1 is inhibited, ammonium uptake is unaffected but the growth increase that normally occurs in high CO₂ is impaired. Elevating the CO₂ content of the growth

McMaster - Biology

medium increases Rh1 expression (Kustu and Inwood, 2006; Soupene *et al.*, 2004). Also, RBCs lacking RhAG (Rh_{null}) have reduced CO₂ permeability (Endeward *et al.*, 2006) and RhCG-expressing oocytes have increased CO₂ permeability (Bakouh *et al.*, 2006). Contrary to this however, Ripoche *et al.* (2006) reported no change in CO₂ permeability in Rh_{null} ghost cell preparations. Future studies should include investigations into the potential CO₂ transport via Rh proteins.

In summary, we identified seven full-length Rh cDNA sequences in the rainbow trout and evaluated the gene expression of these as well as Rhcg2 at the transcript level in the rainbow trout challenged with HEA. Rh mRNAs were expressed in many tissues and changes in expression upon HEA exposure were noted in some. In the gill, the main site of ammonia excretion, Rhcg2 expression as well as H⁺-ATPase expression and activity were upregulated during HEA and this coincided with the net recovery of ammonia excretion. CA expression and activity were significantly downregulated at 48 hours of HEA suggesting that CA is no longer fuelling the H⁺-ATPase pump at this point. Furthermore, pavement cells and not MR cells appear to be instrumental in the regulation of ammonia transport during HEA since clear expression changes in Rh and enzymes were occurring in this cell type only. The contribution of Rh glycoproteins and H⁺-ATPase to enhanced ammonia excretion across the gill during HEA has been highlighted, adding to our current knowledge and understanding of branchial ammonia transport in the freshwater rainbow trout.

2.6 TABLES AND FIGURES

Name	Application Sequence (5'-3')		Name	Application	Sequence (5'-3')	
Rh30-like	cloning-forward	ccagacattcgtaaaggtgcag	Rhag	qPCR-forward	ctggcggccaatgatgttg	
Rh30-like	cloning-reverse	catagctacccacggcaaatg	Rhag	qPCR-reverse	atggcgaagaggtcagagtg	
Rhag	cloning-forward	caacgcagacttcagcac	Rhbg	qPCR-forward	cgacaacgacttttactaccgc	
Rhag	cloning-reverse	ccacaggtgtcctggatg	Rhbg	qPCR-reverse	gacgaagccctgcatgagag	
Rhbg	cloning-forward	catcctcatcatcctctttggc	Rhcg1	qPCR-forward	catectcagectcatacatge	
Rhbg	cloning-reverse	cagaacatccacaggtagacg	Rhcg1	qPCR-reverse	tgaatgacagacggagccaatc	
Rhcg1	cloning-forward	gggattcaacttcctgatcg	Rhcg2	qPCR-forward	cctcttcggagtcttcatc	
Rhcg1	cloning-reverse	accaaaggtggagatgatgc	Rhcg2	qPCR-reverse	ctatgtcgctggtgatgttg	
Rh30-like	3' RACE	gggacattccggtttcgcgtagaagg	CA	qPCR-forward	gccagtctcccattgacatc	
Rh30-like	5' RACE	gcagctctcacaggcagtttctctcttc	CA	qPCR-reverse	cctgtacgtccctgaaatgg	
Rhag	3' RACE	ccgatcatggcgaagaggtcagagtgg	HATP	qPCR-forward	tcagccttggttgtgagatg	
Rhag	5' RACE	gacaatttcacagcactggccatcctgg	HATP	qPCR-reverse	caacattggtgggaaacagg	
Rhbg	3' RACE	ggtccagtacgacgatgagacagacgccaag	NHE	qPCR-forward	ttgttgagggtacggaggtg	
Rhbg	5' RACE	ctctattccgacatggatcttgcccccgtgc	NHE	qPCR-reverse	tacccagccacaagtatcc	
Rhcg1	3' RACE	cggtgctaggcaaggtgagcccagtc	NKA1a	qPCR-forward	ttgacctggatgaccacaag	
Rhcg1	5' RACE	gactgggctcaccttgcctagcaccg	NKA1a	qPCR-reverse	ggateteettageeegaac	
Rhbg	PCR-forward	cgacaacgacttttactaccgc	NKA1b	qPCR-forward	tataagctggtggcgacctc	
Rhbg	PCR-reverse	agaacacgagccaccatcag	NKA1b	qPCR-reverse	ggtcatccaggtcaacttcc	
Rhcg1	PCR-forward	catectcagectcatacatge	NKA1c	qPCR-forward	aggccatgaagaaggagagg	
Rhcg1	PCR-reverse	gtttctgtccagcaggcgtc	NKA1c	qPCR-reverse	taaggtcggtgccgtatttg	
Rhcg2	PCR-forward	gcacactgttcctgtggatg	NKA3	qPCR-forward	gcaaatcttctcccagcaag	
Rhcg2	PCR-reverse	cagcaggatctccccaga	NKA3	qPCR-reverse	tcctgcaacactcctctatgg	
Rh30-like2	qPCR-forward	gctgctgcactgatatcaac	β-actin	qPCR-forward	actgggacgacatggagaag	
Rh30-like3	qPCR-forward	attgcccatgggctttcac	β-actin	qPCR-reverse	aggcgtatagggacaacacg	
Rh30-like2,3	qPCR-reverse	cgcccgttgtgagatcatc	EF1a	qPCR-forward	ggaaagtcaaccaccacag	
			EF1a	qPCR-reverse	gataccacgctccctctcag	

Table 2.1 Primers used for cloning, PCR, and qPCR analysis

Orthologue	GenBank accession number	5'-UTR	Coding region	3'-UTR
Rh30-like2	EF062577	83	1263	265
Rh30-like3	EF062578	83	1050	551
Rhag	EF667352	140	1305	358
Rhbg-a	EF051113	188	1386	1080
Rhbg-b	EF051114	188	1386	607
Rhcg1-a	DQ431244	73	1463	562
Rhcg1-b	EF051115	73	1463	215

Table 2.2 Accession number and size of each Rh cDNA identified



Figure 2.1 The effect of 48 hours of exposure to 1.5 mmol L⁻¹ NH₄ HCO₃ on net ammonia flux (J_{Amm}). Negative values indicate excretion; positive values represent uptake. Asterisks represent values significantly different from the 12 and 48-hour controls (0 mmolL⁻¹ NH₄HCO₃). Control values are not significantly different from each other (P<0.05). Data are means ± s.e.m. (n=6-12).



Figure 2.2 The effect of 48 hours of exposure to 1.5 mmol L⁻¹ NH₄HCO₃ on water and plasma total ammonia (T_{Amm}). Asterisks represent plasma values significantly different from water values (P<0.05). Data are means ± s.e.m. (n=6-12).



Figure 2.3 An unrooted phylogenetic tree of Rh homologue sequences in fish. Numbers represent bootstrap values from 500 replicates. Accession numbers appear in parentheses. Asterisks indicate sequences identified in this study.

Rh30-like

	Green pufferfish Pufferfish Salmon Rainbow trout Rainbow trout* Rainbow trout*	MAPOYACSLESSIAPLILLOGGF NAPOYACSLESSIAPLILLOGGF NAPOYACSLESSIAPLILLOGGF MAPOYACSLERIPPLICTOGF NAPOYACSLERIPPLICTIOGGF NAPOYACSLERIPPLICTIOGGF	IVVFAFVDIETHSFNTDLT IAIVVFIVEIDTHTDLT IVVFIFVDIEQNVQTKQHA IVVFIVIDIEQNVQTKQHA IVVFIVIDIEQNVQTKQHA IVVFIVIDIEQNVQTKQHA	S-NFYREFODVHVIVFLGFO R-DFYRFFODVOVIVFLGFO FTNYSEFODVHVVVILGFO FNNYSEFODVHVVVILGFO FNNYSEFODVHVVVILGFO FNNYSEFODVHVVVILGFO	SFLSTFLURYGFSGVGFNLLV SFLGSFLVRYGFSGIGFNLLV SFLATFLVRYSFSGAGFTLLV SFLATFLVRYSFSGAGFTLLV SFLATFLVRYSFSGAGFTLLV SFLATFLVRYSFSGAGFTLLV	A DATONIA TILING PES A VATONIA VILING PES	YHRG <mark>KISLNI</mark> RSLVVA YHRG <mark>KVSINI</mark> RSLVVA YHRGKIWINMRSLVVA YHRGNILINMRSLVVA YHRGNILINMRSLVVA YHRGNILINMRSLVVA	DMCTASPLISIGF PMCTASPLISIGA PMCTASSLIAIGA PMCTASSLIAIGA PMCTASSLIAIGA PMCTASSLIAIGA	129 126 130 130 130 130
	Green pufferfish Pufferfish Salmon Rainbow trout Rainbow trout* Rainbow trout*	VLGKTN FVOLTATALLEVIGFULN VLGKTN FVOLTITALLEVIGFULN VLGKTN FVOLTITALLET GFVLN VLGKTN FVOLTITALLET GFVLN VLGKTN FVOLTITALLET GFVLN VLGKTN FVOLTITALLET GFVLN	WILLISLEVOTINSIMOLH WILRSLEVOTINSIMOLH WILRSTERVOTINTIMILH WILOTFYNVOTINTIMILH WILOTFYNVOTINTIMILH WILOTFYNVOTINTIMILH	IFGSLFGUADTWYNTROS IFGPFGUATWYNDWRGC IFGAFGGWNSWYNTROGS IFGAFGGWNSWYNTROGS IFGAFGGWNSWYNTROGS IFGAFGGWNSWYNTROGS	ORFEKEKSGYESGIFSMAGS ROFEKESAIKSGIFSMAGS OOBEKEKIDRKTGIFSVMGI OOBEKEKIDRKTGIFSVMGI OOBEKEKIDRKTGIFSVMGI OOBEKEKIDRKTGIFSVMGI	TENNEWESENSULV PERIOSENESENSULV PERIOSENESENSULV PERIOSENSULVI PERIOSENSULVI PERIOSENSULVI PERIO	RTPER-HLEVVCGTY RTPVR-LLEVVLSTY DHLDRGRSLRAVISTY DHLDRGRSLRAVISTY DHLDRGRSLRAVISTY DHLDRGRSLRAVISTY	LALARGAVAAAAV LALARSAVAAAGF LALAVSAVAAGF LALAVSAVAAGF LALAVSAVAAGF LALAVSAVAAGF	257 254 260 260 260 260
	Green pufferfish Pufferfish Salmon Rainbow trout Rainbow trout* Rainbow trout*	AVULNPESUSUQLQSCIQAGOV TVLSNPRGLINDLQMBSCHAGOV SVLTSPQGUNUPHIQRCTAGOV SVLTSPQGUNUPHIQRCTAGOV SVLTSPQGUNUPHIQRCTAGOV SVLTSPQGUNUPHIQRCTAGOV	AVGVSASAVIOPIDAMTIGI ATGVCLTAVREPIDAMTIGI ATGVMSAVILEPIDAMATG ATGVMSAVILEPIDAMATG ATGVMSAVILEPIDAMATG ATGVMSAVILEPIDAMATG	SRAVLSAVGVOTLKVHMLLJ SRAVLSTAGFRYLKOPMLLJ ARAULSTLGSRYLKPLMLFJ ARAULSTLGSRYLKPLMLFJ ARAULSTLGSRYLKPLMLFJ ARALLSTLGSRYLK	ALQCQDTRNVLSTHGIPGLLG ARWFHDARDVLSTHAIPGLLG AFECHDTCGVLSVHGLPGILG AFECHDTCGVLSVHGLPGILG AFECHDTCGVLSVHGLPGLLG	TTAHLLLQLWDSEDHTM TVAHLLLQIQACDDFTT MVQLLLQIADSDDLTT MVQLLLQIADSDDLTT MVQLLLQIADSDDLTT	airfavehlotlfvtl Avrfavehlotllitl Avrfavehiotllitl Avrfavehiotllitl Avrfavehiotllitl - vrinkflvopwafti	TLSLLMGIITGIL ALSLSMGLVTGII SLSMILGIITGFL SLSMSLGLIT-GF SLSMSLGLITGFL SY	387 384 390 389 390 390 335
Rhag Privative of the state of	Green pufferfish Pufferfish Salmon Rainbow trout Rainbow trout* Rainbow trout*	LKWNSWRTPQDKKCFDDQAFWEFP LKWNSWRPPQDKKCFDDQAFWEFP LKCNSWRPPQNKKFFDDQAFWEFP LKCNSWRPPQNKKCFDDQAFWEFP LKCNSWRPPQNKKCFDDQAFWEFP -TCFSTLSAHHAVCI	HLAVRK 417 HLAVGK 414 HLGGKN 420 HQAGKR 419 HQAGKR 420 349						
	Rhag								
	Pufferfish Rainbow trout* Stickleback Zebrafish	PAYATIMRIK FPIJALILEMMUTU PAYSTIMRIK FPIJALILEMUTIN PAYATIMRIK FPIJALALEIVIII PAYSTIMRIK FPIJALILEIITIVI	FAVFVVYDDGRPSSDPHDPH FGVFVDYDDGRHGGHGAH FAVFVYYDDGR-SHGHDPH YALFVVYDTGD-DRGHGDH	DPHAGNHTQEGAPMDLYPM N – TTHHKEKKDPLTLYPM S – NATHHEEPRPMELYPM H – – EKSNQTESPMTLYPM	FQDVHVMIFIGFGFLMTFLKR FQDVHVMIFIGFGFLMTFLKR FQDVHVMIFIGFGFLMTFLKR FQDVHVMIFIGFGFLMTFLKR	IGFSSVGVNLLLAAFCI IGFSSVGINLLLAAFTI IGFSSVGINLLLAAFCI IGFTSVGVNLLLAAFCI	QWGL <mark>IMQ</mark> GE <mark>WH YED</mark> GK QWGL <mark>IMQ</mark> GIWH <mark>WED</mark> GK QWGL <mark>IMQ</mark> GIWH <mark>WED</mark> GK QWGL <mark>IMQNV</mark> WH <mark>WEG</mark> GK	IKINIFKIINA 1 IKVSIFKMINA 1 IKVTIFKIINA 1 IRVSIORMINA 1	.30 26 26 25
Definition of the second of	Pufferfish Rainbow trout* Stickleback Zebrafish	FSTATVLISFGAVLGKTSPVQLLIM FSTATVLISFGAVLGKTSPVQLLIM FSTATVLISFGAVLGKTSPVQLLIM FSTATVLISFGAVLGKTSPVQLLIM	TILEITIFSINEHLVATVIH TILEITIFSINEHLVAEILE TVLEISLFSINEHLVAQIFK TLLEITTFCINEYLVVEILG	ANDVGASMITHAFGAYFGLI ANDVGASMITHAFGAYFGL ANDVGASMITHAFGAYFGL VSDVGASMITHAFGAYFGL	VARVLYRGURNGHEKEGSV VARMLYRPALRNSHENDGSV VARVLYRGURNGHDNDGSV VARVLYRGURNGHDNDGSV	YHS <mark>DMFAMIGTVF</mark> LWME YHSDLFAMIGTVFLWME YHSDLFAMIGTVFLWME YHS <mark>HL</mark> FAMIGTV <mark>Y</mark> LWME	WPSFNSAIADPGFTQL WPSFNSAIADSGTDQL WPSFNSAIADPGLTQL WPSFNSAIA <mark>TPGDH</mark> QL	TAVINTYISLA 2 VAVINTYISLA 2 TAVINTYISLA 2 RAVVNTYISLA 2	60 56 56 55
Puttering in the second sec	Pufferfish A Rainbow trout* A Stickleback A Zebrafish A	C <mark>VLSAYAV</mark> SSLVE <mark>B</mark> KGKLDMVHIQN CVLSAYAVSSLVEBKGKLDMVHIQN CVLSAYATSSLVEBKGKLDMVHIQN CALAAYATSSLVEKKGKLDMVHIQN	ATLAGGVAVGTCADM <mark>NI GPF</mark> ATLAGGVAVGTCADMDI GPF ATLAGGVAVGTCADMAI GPT ATLAGGVAVGTCADM <mark>DI O</mark> PF	GAMLIGLVAGIISTUGFK GAMLIGPVAGIVSTUGFK GAMLIGPVAGIISTVGFK GAMLIGLVAGIISTVGFK	LTPILAS <mark>S</mark> LGIQDTCGVHNLHO LTPILASNLGIQDTCGVHNLHO LTPILASNLGIQDTCGVHNLHO LTPILAS <mark>N</mark> LGIQDTCGVHNLHO	SMPGILGG <mark>VAGIVAVA</mark> SMPGILGGUAGIVAVA SMPGILGGUAGIVAVA SMPGILGG <mark>V</mark> AGI <mark>LAV</mark> AI	igrkdegna smoaaala ,grkke-saamogaala ,grkeegdaamoaaala ,krkegvsptioaagla	SSLGFALVGG <mark>H 3</mark> SSLGFALVGG <mark>H 3</mark> SSLGFALVGG <mark>H 3</mark> SSLGFALVGG <mark>H 3</mark>	90 85 86 85
Build Description Description <thdescription< th=""> <thdescription< th=""> <thde< td=""><td>Pufferfish V Rainbow trout* I Stickleback I Zebrafish I</td><td>TGTINKTPLWGQPPDQNCYDDSTYW TGLINKTPPWGQPPDQNCPDDSTYW TGTINKTPFWGQPPDQNCYDDSTYW TGLINKTPFLGQPPDQNCPDDSTYW</td><td>evp-deeesgestahadhs Evpgedeegeeslahgdo- Evp-eeeeheslahgdohs Evp-eeeeheslahadhs Evp-eeeeteerlthgdis</td><td>KNKAEV 441 KAEA 434 KNKAEV 437 KNKAEA 436</td><td></td><td></td><td></td><td></td><td></td></thde<></thdescription<></thdescription<>	Pufferfish V Rainbow trout* I Stickleback I Zebrafish I	TGTINKTPLWGQPPDQNCYDDSTYW TGLINKTPPWGQPPDQNCPDDSTYW TGTINKTPFWGQPPDQNCYDDSTYW TGLINKTPFLGQPPDQNCPDDSTYW	evp-deeesgestahadhs Evpgedeegeeslahgdo- Evp-eeeeheslahgdohs Evp-eeeeheslahadhs Evp-eeeeteerlthgdis	KNKAEV 441 KAEA 434 KNKAEV 437 KNKAEA 436					
Green pufferfish Manbow Kilifish Manbow Kilifish Manbo	Rhbg								
Owner putfarfah Magrook kilifah Kangook kilifah	Green pufferfish Mangrove killifis Pufferfish Japanese rice fis Stickleback Rainbow trout* Zebrafish	MADVS SAMRER LEVVOT LEI MTDTS FMRER LETTOFILEI MTDARINGER LETTOFILEI MTDS FAMRER LETTOFILEI MTDVF MRER LETTOFILOI MTNS ARMER LEVTOFILOI - MRES MIRRE LEVI - MRES MIRRE LEVI	LIILFGATVQYDHDTDAKE- LIILFGYVYYDHDTDSKLS LIILFGTVYQYDHDTDSKLS LIILFGTVQYDHDTDAKE- LIILFGYTVQYDHDTDAKE- LIILFGTVQYDHDTDAKE- LIILFGTVQYDHDTAKE-	WHNQSHNDYEND NEHGNDSHKHADYEND WHNTSHQDYEND HHHGNHSESKSDIEND WHSKNHSDYEND WHKKIANGSSNIDND WHKKINS-TDFATNE	FYFRYESFQDVHVMIFTGFGFY FYFRYESFQDVHVMIFTGFGF FYFRYESFQDVHVMIFTGFGF YTRYESFQDVHVMIFTGFGF FYRYESFQDVHVMIFTGFGF FYRYESFQDVHVMIFTGFGF	LMTFLORYGFCSVGFNE LMTFLORYGFSSVGFNE LMTFLORYGFSSVGFNE LMTFLORYGFSSVGFNE LMTFLORYGFSSVGFNE LMTFLORYGFSSVGFNE	LIAAFSLQWATLMQGF LIAAFSLQWATLMQGF LIAAFSLQWATLMQGF LIAAFSLQWATLMQGF LIAAFSLQWATLMQGF LIAAFSLQWATLMQGF LIAAFSLQWATLMQGF	FHGXHCGKIHVGV IHGIHCGKIHIGV FHGXHCGKIHIGV FHGXHCGKIHIGV YHGXHCGKIHIGV YHGXHCGKIHVGI FHGXHFGKIHVGI	ES 122 ES 127 ES 122 ES 126 ILS 122 ES 125 ILS 122
<pre>Grand put for find manage strain for an and put for find put for an and put for</pre>	Green pufferfish Mangrove killifis Pufferfish Japanese rice fis Stickleback Rainbow trout* Zebrafish	MINADFCTGULISFGAVLGKT MINADFCTGULISFGAVLGKT MINADFCTGULISFGAVLGKT MINADFCTGULISFGAVLGKT MINADFCTGULISFGAVLGKT MINADFCTGULISFGAVLGKT MINADFCTGULISFGAVLGKT	SPIOLLUMANPEUTLFAUNE SPIOLLUMAIPEUTLFAUNE SPIOLLUMAIPEUTLFAUNE SPIOLLUMAIPEUTLFAUNE SPIOLLUMAIPEUTLFAUNE SPIOLLUMAIPEUTLFAUNE	TILLSILGT TILLSILGT TILLSILGT TILLSILGT TILLTLGT TILLTLGT TILLTLGT TILLSU TILSU TILLSU	PGAY PGLMYRT LYRPIDX PGAY PGLMYRT LYRPIDX PGAY FGLMYRT LYRPIDX PGAY FGLMYRT LYRPIDX PGAY FGLMYRT LYRPIDX PGAY FGLMYRT LYRPIDX PGAY FGLMYRT LYRPIDX	SKHRNSSVYHSDLFAMI SKHRNCSVYHSDLFAMI SKHRNSSVYHSDLFAMI SKHRNSSVYHSDLFAMI SKHRNSVYHSDLFAMI SKHRNSVYHSDLFAMI	GTITLIUMFWPSFNSAT GTITLIMFWPSFNSAT GTITLIMFWPSFNSAT GTITLIMFWPSFNSAY GTITLIMFWPSFNSAY GTITLIMFWPSFNSAY GTITLIMFWPSFNSAT	TAHGD QHRTAIN TEHGD QHRTAIN TAHGD QHRTAIN TEHGD QHRTAIN TAGGD QHRTAIN TAIGD QHRTAIN TQIGD QHRTAIN	TY 252 TY 257 TY 252 TY 256 TY 255 TY 255 TY 252
Green pufferfish Mangrove killifish Mangrove killif	Green pufferfish Mangrove killifis Pufferfish Japanese rice fis Stickleback Rainbow trout* Zebrafish	YSLAACTLATYGMSAVTSHDGK YSLAACTLSTYGMSALTAHDGK YSLAACTLSTYGMSALTAHDGK YSLAACTLSTYGMSALTAHDGK YSLAACTLSTYGMSALTAHDG YSLAACTLATGGSAVVAHDG YSLAACTLATGGSSLVNP2GK	LDMYHIQNAALAGGVAVG TA LDMYHIQNAALAGGVAAG TA LDMSTSKMLWPVALPMATA LDMYHIQNAALAGVAAGTA LDMYHIQNAALAGVAAGTA LDMYHIQNAALAGVAYG TA LDMYHIQNAALAGVAYG TA	GEMMLTPFGSMIVGFDAGI GEMMLTPFGSMIVGFDAGI GEMMLTPFGSMIVGFTAGI GEMMLTPFGSMIVGFDAGI GEMMLTPFGSMIVGFDAGI GEMMLTPFGSMIVGFDAGI GEMMLTPFGSMIVGFDAGI	ISVIERSISIIESKIRO SVIERSISIESE SVIERSISESE SVIERSISESE SVIERSISESE SVIERSISE SVIERS SVIERSISE SVIE SVIERS SVIE SVIE SVIE SVIE SVIE SVIE SVIE SVI	PCG <mark>UHNLHGMPGULGAI PCGUHNLHGMPGULGAI PCGUHNLHGMPGULGAI PCGUHNLHGMPGULGAI PCGUHNLHGMPGULGAI PCGUHNLHGMPGULGAI PCGUHNLHGMPG<mark>U</mark>LGAI</mark>	VGAVTATATATID VYGK VGAVTAŠVATKD VYG VGAVTALATTD VYG VGAVTALATTD VYG VGAVTATATATTD VYG VGAVTATATATKD VYG VGAVTATATASKD VYG	GMEDVFPAVADGS GLERVFPDVASGE GMADVFPAVADGS GLERVFPDVASGR GMRDVFPDVESGA GMADVFPDIHSGD GLDRVFMEAADNS	-I 381 -R 386 -V 381 -R 385 -I 381 -V 384 -V 384 -V 384
Rhog (subgroup 1) Green pufferfish Mangrove killifish Rainbow trout* Structure for the formation of the formation	Green pufferfish Mangrove killifis Pufferfish Japanese rice fis	DASKOGSVQATSLAITLGIALL h NANIQGSIQATSLAITLGIALL NATKOGSIQATSLAITLGIAVL h TASDOGSVQATSLAVILGVALF	GGLIVVFGTPPDT GGLIVGFILKLPMFGAPDDT GGLIVVFGTPPDT GGLIVGFILKLPIFGAPRDT	lcfedgviwevpeseaphej Icfedsiiwevpgee-hhei Icfedsviwevpgsespec Icfedslywevpgeeshei	AQLTTVRTEETEKISS 451 SOMTAVNTEEFEKINA 462 SELTSVKPEETEHINS 451 OQLTTVKTEESDKINS 462				
Green pufferfish Mangrove kilifish Mangrove kili	Rhcg (su	bgroup 1)							
Green pufforfish Margrove Killifish Margrove Killifish Margrove Killifish Margrove Killifish Margrove Killifish Margrove Killifish Margrove Killifish Margrove Killifish Pufforfish Margrove Killifish Div Strand a state of the s	Green pufferfish Mangrove killifis Pufferfish Rainbow trout* Zebrafish	MGCVQSPRNFCDRFRNTNVR h MGACQSFRIMCDRFRNTNR MGCVQSFRIMCDRFRNTVRI MGCIQSFRCCDRFRNTVRI MGNCFGSRGICDRFRNTNRI	SLPAVC <mark>TVWQIAMIILFG</mark> VF SLPAVC <mark>VVWQSAMIILFGV</mark> F SLPAVC <mark>TVWQIAMIILFGVF</mark> SLPAVC <mark>TVWQIAMIILFGIF</mark> SLPAVC <mark>TRWQVS</mark> MIILFG <mark>V</mark> F	iryneeadt Hwyeyrken Irydeeadh Hwyehrkann Irydeesd Hwyehrkann Irydeesd Hwiehrken Iryneesda Hwiehrtynn Yryneead Hwyytkrorn	ISSDIENDFYFRYPSFQDVHVI ISSDIENDFYFRYPSFQDVHVI ISSDIENDFYFRYPSFQDVHVI ISSDIENDFYFRYPSFQDVHVI ISSNIENDFYFRYPSFQDVHVI KISDIENDFYFRYPSFQDVHVI	MIFVGFGFLMTFL <mark>R</mark> RYS MIFVGFGFLMTFLRRYS MIFVGFGFLMTFLRRYS MIFVGFGFLMTFL <mark>R</mark> RYS MIFVGFGFLMTFL <mark>R</mark> RYS	FGAVGFNFLIAAFGLQ FGEVGFNFLIAAFGLQ FGAVGFNFLIAAFGLQ FGAVGFNFLIAAFGLQ FGAVGFNFLIAAFGLQ	WALLMQGWFHSLD WALLMQGWFHSLD WALLMQGWFHSLD WALLMQGWFHSLD WALLMQGWFSPLG	YTD 130 YTD 130 YTD 130 YTD 130 PMD 130 IDD 129
Green pufferfish Mangrove kilifish Rainbow trout* Green pufferfish Rainbow trout* Green pufferfish Barinbow trout* Green pufferfish Barinbow trout* Green pufferfish Drugstnovjen geografication of the state of the	Green pufferfish Mangrove killifis Pufferfish Rainbow trout* Zebrafish	GKIKIGIENFINADFCVACI h gkikigvespinadfcvaci gkikigvespinadfcvaci gkikigvestinadfcvaci gkikigrestinadfcvaci	JAYGAVLGKVSPVQLYVTTL JAYGALLGKVSPVQLYVTTL JAYGAVLGKVSPVQLYVTTL JAYGAVLGKVSPVQLYVTL JAYGAVLGKVSPVQLYVTL	FGITLEAVEETIILLILEA FGITLEAVEETIILLIEA FGITLEAVEETIILLIEA FGITLEAVEETIILSLIEA FGITLEAVEETIILSLIEA FGITLEAVEETIILRVINA	RDAGGSMVIHTFG <mark>C</mark> YYGLSIS RDAGGSMVIHTFGAYYGLSIS RDAGGSMVIHTFGGYYGLSIS RDAGGSMVIHTFGAYYGLSIS RDAGGSMVIHTFG <mark>A</mark> YYGLSIS	WMLYRPNIEOSSNLOG MMLYRPNIDOSSLOR MMLYRPNIDOSSNLOG MMLYRPNIDOSKHRIG RVLYRPNIN <mark>RS</mark> KHRIG	VYCSDVFAMIGTLFLW VYHSDVFAMIGTLFLW VYHSDVFAMIGTLFLW VYHSDVFAMIGTLFLW VYHSDVFAMIGTLFLW	MFWPSFNSAITDH MFWPSFNSAITDH MFWPSFNSAITDH MFWPSFNSAI <mark>SD</mark> H MFWPSFNSAI <mark>CN</mark> H	GDG 260 GDG 260 GDG 260 GDG 260 GDG 259
Green pufforfish Manfrove Kallifsh Pofforfish Pafforfish Pafforfish Pafforfish Standard Control (1997) Sebrafish Standard Control (1997) Sebrafish Standard Control (1997) Sebrafish Standard Control (1997) Sebrafish Standard Control (1997) Sebrafish Standard Control (1997) Sebrafish	Green pufferfish Mangrove killifis Pufferfish Rainbow trout* Zebrafish	OHRAAINTYIALASTVLTTVA b Ohraaintyislastvlttja Ohraaintyislastvlttyi Ohraaintyislastvlttyi Ohraaintyislastvlttyi	ISS <mark>LFOKH</mark> GKLDMVHIQNST VSSLFOKHGKLDMVHIQNST ISSLFOKHGKLDMVHIQNST ISSLSOKHGKLDMVHIQNNT ISS <mark>MFF</mark> KHGKLDMVHIQNST	LAGGVAVGTAAE FMLMPYG LAGGVAVGTAAE FMLMPYG LAGGVAVGTAAE FMLMPYG LAGGVAVGTAAE FMLMPYG LAGGVAVGTAAE FMLMPYG	SLIVGFCCGIISTLGYIYTP) SLIVGFCCGIVSTLGYYYTP) SLIVGFCCGIISTLGYIYTP) SLIVGFCCGIISTLGYIYTP) SLIVGFCCGIISTLGYIYTP	FYSKYLKIODUCGIHNI FUSKYLKIODYCGIHNI FYSKILKIODUCGIHNI FYSKILKIODUCGIHNI FYSKILKIODUCGIHNI	HAMPGUIGGIVGAINA HAMPGUIGGIVGAINA HAMPGUIGGIVGAINA HAMPGUIGGIVGAINA HAMPGUIGGIVGAINA	AAA <mark>NES</mark> VYG <mark>KE</mark> GL AAASENVYGHEGL AAASESVYGKEGL AAASKEVYGKEGL AAASKEVYGDIGL	VNT 390 INT 390 INT 390 TNT 390 KNI 389
	Green pufferfish Mangrove killifis Pufferfish Rainbow trout* Zebrafish	FDFVGPFKNMVFTXQGGHQA h FDFEGEFKYMVPTVQGGHQA FDFEGAFKNMVFTXQGGHQA FDFDGKWKDMLPTQQGGHQA FSIEGSNVTRLPTVQGGYQA	GLCVAICFGIGGGIAVGCII GLCVAICFGIGGGIAVGAI GLCVAICFGIGGGIAVGCII GLCVAICFGIGGGIAVGCII ALCVAICFGIGGGTAVGLVI	RLPINCDPADDNCSNDEPY RLPINCDPADDNCSDDEPY RLPINCDPADDNCSDDEPY RLPINCDADDNSCPDDIY RLPINCDPADERCSNDEMY	NEVPED NEVPED NEGIFHSKATRAAPQCDLVAA NE	se – II Ppilh In Himy E – Sv Ppilq In Hiff St – Hvc Fpirs, Sawy E – Nn Pptve In Himr E SII Ppvls In Himf	IKDV	475 RS 490 493 487 488	

Figure 2.4 Amino acid sequence alignments of fish Rh homologues. Accession numbers appear in parentheses. Asterisks indicate sequences identified in this study.



Amino acid residues

Figure 2.5 Hydropathy profile of rainbow trout Rhbg. Grey boxes indicate predicted transmembrane regions.



Figure 2.6 Tissue distribution of Rh glycoproteins as determined by PCR in rainbow trout exposed to (*A*) 0 mmol L⁻¹ NH₄HCO₃ and (*B*) 1.5 mmol L⁻¹ NH₄HCO₃. These gels show qualitative rather than quantitative expression. n=3 for each Rh orthologue and treatment.


Figure 2.7 mRNA expression of Rhbg relative to β -actin in the blood and the unperfused and perfused brain, kidney and liver of rainbow trout exposed to 0 mmol L⁻¹ NH₄HCO₃ (control) and 1.5 mmol L⁻¹ NH₄HCO₃ for 48 hours. Asterisk indicates significant difference between control and 48-hour perfused brain (*P*<0.05). Data are means ± s.e.m. (*n*=6).



Figure 2.8 mRNA expression of Rhbg, Rhcg1, and Rhcg2 relative to EF-1 α expression in the gills of rainbow trout exposed to 1.5 mmol L⁻¹ NH₄HCO₃ for 12 and 48 hours. Significant differences from the control are indicated by an asterisk (*P*<0.05). Data are means \pm s.e.m. (*n*=6).



Figure 2.9 mRNA expression of Rhbg, Rhcg1, and Rhcg2 relative to β -actin in the skin of rainbow trout exposed to 1.5 mmol L⁻¹ NH₄HCO₃ for 12 and 48 hours. Significant differences from the control are indicated by an asterisk (*P*<0.05). Data are means ± s.e.m. (*n*=6).



Figure 2.10 mRNA expression of Rhcg1 relative to β -actin in the brain and liver of rainbow trout exposed to 1.5 mmol L⁻¹ NH₄HCO₃ for 12 and 48 hours. Significant differences from the control are indicated by an asterisk (*P*<0.05). Data are means ± s.e.m. (*n*=6).



Figure 2.11 mRNA expression of Rhbg, Rhcg1, and Rhcg2 relative to β -actin in (*A*) pavement cells and (*B*) mitochondria-rich cells isolated from the gills of rainbow trout exposed to 1.5 mmol L⁻¹ NH₄HCO₃ for 12 and 48 hours. Significant differences from the control are indicated by an asterisk (*P*<0.05). Data are means ± s.e.m. (*n*=6).



Figure 2.12 mRNA expression of Rh30-like2, Rh30-like3, and Rhag relative to β -actin in the RBCs from rainbow trout exposed to 1.5 mmol L⁻¹ NH₄HCO₃ for 12 and 48 hours. Significant differences from the control are indicated by an asterisk (*P*<0.05). Data are means ± s.e.m. (*n*=6).



Figure 2.13 mRNA expression relative to EF-1 α of (*A*) H⁺-ATPase, (*B*) carbonic anhydrase (CA2), and (*C*) Na⁺/K⁺-ATPase α -subunit isoforms and the enzyme activities of (*D*) H⁺-ATPase, (*E*) carbonic anhydrase, and (*F*) Na⁺/K⁺-ATPase in the gills of rainbow trout exposed to 1.5 mmol L⁻¹ NH₄HCO₃ for 12 and 48 hours. Significant differences from the control are indicated by an asterisk (*P*<0.05). Data are means ± s.e.m. (*n*=6).



Enzyme / transporter expression

Figure 2.14 mRNA expression relative to β -actin of carbonic anhydrase, H⁺-ATPase, NHE2 and Na⁺/K⁺-ATPase (α 1a) in (*A*) pavement cells and (*B*) mitochondria-rich cells. Significant differences from the control are indicated by an asterisk (*P*<0.05). Data are means \pm s.e.m. (*n*=6).



Figure 2.15 Schematic diagram of a gill cell showing Rhcg2-mediated NH₃ transport coupled to active H^+ secretion. The result is a net excretion of NH₄⁺ across the apical gill gill epithelium.

2.7 REFERENCES

- Allendorf, F. W. and Thorgaard, G. H. (1984). Tetraploidy and the evolution of salmonid fishes. In *Evolutionary Genetics of Fishes* (ed. B. J. Turner), pp.1-46. New York: Plenum Press.
- ArasHisar, S., Hisar, O., Yanik, T. and Aras, S. M. (2004). Inhibitory effects of ammonia and urea on gill carbonic anhydrase enzyme activity of rainbow trout (Oncorhynchus mykiss). Env. Tox. Pharm. 17, 125-128.
- Avent, N. D. and Reid, M. E. (2000). The Rh blood group system: a review. Blood 95, 375-387.
- Bakouh, N., Benjelloun, F., Hulin, P., Brouillard, F., Edelman, A., Chérif-Zahar, B. and Planelles, G. (2006). The challenge of understanding ammonium homeostasis and the role of Rh glycoproteins. *Tranfus. Clin. Biol.* 13, 139-146.
- Brunelli, J. P., Robison, B. D. and Thorgaard, G. H. (2001). Ancient and recent duplications of the rainbow trout Wilms' tumor gene. *Genome* 44, 455-462.
- **Cameron, J. N.** (1986). Responses to reversed NH_3 and NH_4^+ gradients in a teleost (*Ictalurus punctatus*), an elasmobranch (*Raja erinacea*), and a crustacean (*Callinectes sapidus*): Evidence for NH_4^+/H^+ exchange in the teleost and the elasmobranch. J. Exp. Zool. 239, 183-195.
- **Cameron, J. N. and Heisler, N.** (1983). Studies of ammonia in the trout: physicochemical parameters, acid-base behaviour and respiratory clearance. *J. Exp. Biol.* 105, 107-125.
- Claiborne, J. B. and Evans, D. H. (1988). Ammonia and acid-base balance during high ammonia exposure in a marine teleost (*Myoxocephalus octodecimspinosus*). J. *Exp. Biol.* 140, 889-105.
- Conroy, M. J., Bullough, P. A., Merrick, M. and Avent, N. D. (2005). Modelling the human rhesus proteins: implications for structure and function. *Br. J. Haematol.* 131, 543-551.
- Conroy, M. J., Jamieson, S. J., Blakey, D., Kaufmann, T., Engel, A., Fotiadis, D., Merrick, M. and Bullough, P. A. (2004). Electron and atomic force microscopy of the trimeric ammonium transporter AmtB. *EMBO Reports* 5, 1153-1158.
- Eladari, D., Cheval, L., Quentin, F., Bertrand, O., Mouro, I., Cherif-Zahar, B., Cartron, J.-P., Paillard, M., Doucet, A. and Chambrey, R. (2002). Expression

of RhCG, a new putative NH_3/NH_4^+ transporter, along the rat nephron. J. Am. Soc. Nephrol. 13: 1999-2008.

- Endeward, V., Cartron, J.-P., Ripoche, G. and Gros, G. (2006). Red cell membrane CO₂ permeability in normal human blood and in blood deficient in various blood groups, and effect of DIDS. *Transfus. Clin. Biol.* 13, 123-127.
- Evans, D. H. and Cameron, J. N. (1986). Gill ammonia transport. J. Exp. Zool. 239, 17-23.
- Frick, N. T. and Wright, P. A. (2002). Nitrogen metabolism and excretion in the mangrove killifish, *Rivulus marmoratus*. I. The influence of environmental salinity and external ammonia. *J. Exp. Biol.* 205, 79-89.
- Fromm, P. O. (1963). Studies on renal and extra-renal excretion in a freshwater teleost, Salmo gairdneri. Comp. Biochem. Physiol. 10, 121-128.
- Galvez, F., Reid, S. D., Hawkings, G., Goss, G. G. (2002). Isolation and characterization of mitochondria-rich cell types from the gill of freshwater rainbow trout. *Am. J. Physiol.* 282, R658–668.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser.* 41, 95-98.
- Henry, R. P. (1991). Techniques for measuring carbonic anhydrase activity in vitro: the electrometric delta pH and pH stat assay. In: *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics* (eds. S. J. Dodgson, R. E. Tashian, G. Gros, N. D. Carter), pp. 119-126. New York: Plenum.
- Huang, C. -H. and Liu, P. Z. (2001). New insights into the Rh superfamily of genes and proteins in erythroid cells and nonerythroid tissues. *Blood Cells Mol. Dis.* 27, 90-101.
- Huang, C. -H. and Peng, J. (2005). Evolutionary conservation and diversification of Rh family genes and proteins. *Proc. Natl. Acad. Sci. USA* 102, 15512-15517.
- Hung, C. Y. C., Tsui, K. N. T., Wilson, J. M., Nawata, C. M., Wood, C. M. and Wright, P. A. (2007). Rhesus glycoprotein gene expression in the mangrove killifish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air. J. Exp. Biol. 210, 2419-2429.
- Javelle, A., Lupo, D., Li, X.-D., Merrick, M., Chami, M., Ripoche, P. and Winkler, F. K. (2007). Structural and mechanistic aspects of Amt/Rh proteins. J. Struct. Biol. 158, 472-481.

- Juretic, D., Zoranic, L. and Zucic, D. (2002). Basic charge clusters and prediction of membrane protein topology. J. Chem. Inf. Comput. Sci. 42, 620-632.
- Khademi, S., O'Connell III, J., Remis, J., Robles-Colmenares, Y., Mierke, L. J. W. and Stroud, R. M. (2004). Mechanism of ammonia transport by Amt/MEP/Rh: structure of AmtB at 1.35 Å. *Science* 305, 1587-1594.
- Khademi, S. and Stroud, R. M. (2006). The AmtB and the mechanism of ammonia gas conduction. *Physiology* 21, 419-429.
- Kirschner, L. B., Greenwald, L., Kerstetter, T. H. (1973). Effect of amiloride on sodium transport across body surfaces of fresh water animals. *Am. J. Physiol.* 224, 832-837.
- Kumar, S., Tamura, K. and Nei, M. (2004). MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* 5, 150-163.
- Kustu, S. and Inwood, W. (2006). Biological gas channels for NH₃ and CO₂: evidence that Rh (Rhesus) proteins are CO₂ channels. *Transfus. Clin. Biol.* 13, 103-110.
- Lin, H., Pfeiffer, D. C., Wayne, V. A., Pan, J. and Randall, D. J. (1994). Immunolocalization of H⁺-ATPase in the gill epithelia of rainbow trout. *J. Exp. Biol.* 195, 169-183.
- Lin, H. and Randall, D. J. (1990). The effect of varying water pH on the acidification of expired water in rainbow trout. J. Exp. Biol. 141, 14-160.
- Lin, H. and Randall, D. J. (1991). Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. J. Exp. Biol. 161, 119-134.
- Lin, H. and Randall, D. J. (1993). H⁺-ATPase activity in crude homogenates of fish gill tissue: inhibitor sensitivity and environmental and hormonal regulation. J. Exp. Biol. 180, 163-174.
- Litwiller, S. L., O'Donnell, M. J. and Wright, P. A. (2006). Rapid increase in the partial pressure of NH₃ on the cutaneous surface of air-exposed mangrove killifish, *Rivulus marmoratus. J. Exp. Biol.* 209, 1737-1745.
- Liu, Z., Chen, Y., Mo, R., Hui, C., Cheng, J. –F., Mohandas, N. and Huang, C. -H. (2000). Characterization of human RhCG and mouse Rhcg as novel nonerythroid Rh glycoprotein homologues predominantly expressed in kidney and testis. J. Biol. Chem. 275, 25641-25651.

- Liu, Z., Peng, J., Mo, R., Hui, C. and Huang, C. -H. (2001). Rh type B glycoprotein is a new member of the Rh superfamily and a putative ammonia transporter in mammals. J. Biol. Chem. 276, 1424-1438.
- Lumsden, J. S., Wright, P., Derksen, J., Byrne, P. J. and Ferguson, H. W. (1993). Paralysis in farmed Arctic char (*Salvelinus alpinus*) associated with ammonia toxicity. *Vet. Rec.* 133, 422-423.
- Mallery, C. H. (1993). A carrier enzyme basis for ammonium excretion in teleost gill. NH₄⁺-stimulated Na-dependent ATPase activity in *Opsanus beta*. *Comp. Biochem. Physiol.* 74A, 889-897.
- Marini, A. –M., Boeckstaens, M., Benjelloun, F., Chérif-Zahar, B. and André, B. (2006). Structural involvement in substrate recognition of an essential aspartate residue conserved in Mep/Amt and Rh-type ammonium transporters. *Curr. Genet.* 49, 364-374.
- Marini, A. –M., Matassi, G., Raynal, V., André, B., Cartron, J. –P. and Chérif-Zahar, B. (2000). The human Rhesus-associated RhAG protein and a kidney homologue promote ammonium transport in yeast. *Nat. Genet.* 26, 341-344.
- Marini, A. –M., Urrestarazu, A., Beauwens, R. and André, B. (1997). The Rh (Rhesus) blood group polypeptides are related to NH₄⁺ transporters. *Trends Biochem. Sci.* 22, 460-461.
- Marini, A. –M., Vissers, S., Urrestarazu, A. and André, B. (1994). Cloning and expression of the MEP1 gene encoding an ammonium transporter of *Saccharomyces cerevisiae*. *EMBO J.* 13, 3456-3463.
- Marshall, W. S. and Bryson, S. E. (1998). Transport mechanisms of seawater teleost chloride cells: an inclusive model of a multifunctional cell. *Comp. Biochem. Physiol.* 119A, 97-106.
- Mazumder, B., Seshadri, V. and Fox, P. L. (2003). Translational control by the 3'-UTR: the ends specify the means. *TRENDS Biochem. Sci.* 28, 91-98.
- McCormick, S. D. (1993). Methods for the nonlethal gill biopsy and measurements of Na⁺, K⁺-ATPase activity. *Can. J. Fish. Aquat. Sci.* 50, 656-658.
- Mommsen, T. P. and Walsh, P. J. (1992). Biochemical and environmental perspectives on nitrogen metabolism in fishes. *Experientia* 48, 583-593.
- Murray, B. W., Busby, E. R., Mommsen, T. P. and Wright, P. A. (2003). Evolution of

glutamine synthetase in vertebrates: multiple glutamine synthetase genes expressed in rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 206, 1511-1521.

- Nakada, T., Westhoff, C. M., Kato, A. and Hirose, S. (2007). Ammonia secretion from fish gill depends on a set of Rh proteins. *FASEB J.* 21, 1067-1074.
- Ninnemann, O., Jauniaux, J. –C., Frommer, W. B. (1994). Identification of a high affinity NH₄⁺ transporter from plants. *EMBO J.* 13, 3464-3471.
- Playle, R. C. and Wood, C. W. (1989). Water chemistry changes in the gill microenvironment of rainbow trout: experimental observations and theory. J. Comp. Physiol. 159B, 527-537.
- Randall, D. J., Wilson, J. M., Peng, K. W., Kwok, T. W. K., Kuah, S. S. L., Chew, S.
 F., Lam, T. J. and Ip, Y. K. (1999). The mudskipper, *Periophthalmodon* schlosseri, actively transports NH₄⁺ against a concentration gradient. Am. J. Physiol. 277, R1562-1567.
- Randall, D. J. and Wright, P. A. (1987). The interaction between carbon dioxide and ammonia excretion and water pH in fish. *Can. J. Zool.* 67, 2936-2942.
- Rice, P., Longden, I. and Bleasby, A. (2000). EMBOSS: The European Molecular Biology Open Software Suite. *Trends Genet.* 16, 276-277.
- **Ripoche, P., Bertrand, O., Gane, P., Birkenmeier, C., Colin, Y. Cartron, J. -P.** (2006). Human role of RhAG and AQP1 in NH₃ and CO₂ gas transport in red cell ghosts: a stopped-flow analysis. *Transfus. Clin. Biol.* 13, 117-122.
- Salama, A., Morgan, I. J., Wood, C. M. (1999). The linkage between Na⁺ uptake and ammonia excretion in rainbow trout: kinetic analysis, the effects of (NH₄)₂SO₄ and NH₄HCO₃ infusion and the influence of gill boundary layer pH. J. Exp. Biol. 202, 697-709.
- Seshadri, R. M., Klein, J. D., Smith, T., Sands, J. M., Handlogten, M. E., Verlander, J. W. and Weiner, I. D. (2006). Changes in subcellular distribution of the ammonia transporter, Rhcg, in response to chronic metabolic acidosis. Am. J. Physiol. 290, F1443-1452.
- Soupene, E., Inwood, W. and Kustu, S. (2004). Lack of the Rhesus protein Rh1 impairs growth of the green alga *Chlamydomonas reinhardtii* at high CO₂. *Proc. Natl. Acad. Sci. USA* 101, 7787-7792.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving

the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* 22, 4673-4680.

- Verdouw, H., van Echteld, C. J. A. and Dekkers, E. M. J. (1978). Ammonia determinations based on indophenol formation with sodium salicylate. *Water Res.* 12, 399-402.
- Weiner, I. D. and Hamm, L. L. (2006). Molecular mechanisms of renal ammonia transport. Ann. Rev. Physiol. 69, 317-340.
- Westhoff, C. M. (2007). The structure and function of the Rh antigen complex. Semin. Hematol. 44, 42-50.
- Westhoff, C. M. and Wylie, D. E. (2006). Transport characteristics of mammalian Rh and Rh glycoproteins expressed in heterologous systems. *Transfus. Clin. Biol.* 13, 132-138.
- Wilkie, M. P. (2002). Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. J. Exp. Zool. 293, 284-301.
- Wilson, R. W. and Taylor, E. W. (1992). Transbranchial ammonia gradients and acidbase responses to high external ammonia concentration in rainbow trout (*Oncorhynchus mykiss*) acclimated to different salinities. J. Exp. Biol. 166, 95-112.
- Wilson, R. W., Wright, P. M., Munger, S. and Wood, C. M. (1994). Ammonia excretion in fresh water rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: lack of evidence for Na⁺/NH₄⁺ exchange. J. *Exp. Biol.* 191, 37-58.
- Wittbrodt, J., Meyer, A. and Schartl, M. (1998). More genes in fish? *BioEssays* 20, 511-515.
- Wolf, K. (1963). Physiological salines for fresh water teleosts. *Progve. Fish. Cult.* 25, 135-140.
- Wood, C. M. (1993). Ammonia and urea metabolism and excretion. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 379-425. Boca Raton: CRC Press.
- Wright, P. A., Heming, T. and Randall, D. J. (1986). Downstream pH changes in water flowing over the gills of rainbow trout. J. Exp. Biol. 126, 499-512.

Wright, P. A., Randall, D. J. and Perry, S. F. (1989). Fish gill boundary layer: a site of

linkage between carbon dioxide and ammonia excretion. J. Comp. Physiol. 158, 627-635.

- Wright, P. A. and Wood, C. M. (1985). An analysis of branchial ammonia excretion in the freshwater rainbow trout: effects of environmental pH change and sodium uptake blockage. J. Exp. Biol. 114, 329-353.
- Zheng, L., Kostrewa, D., Berneche, S., Winkler, F. K. and Li, X. -D. (2004). The mechanism of ammonia transport based on the crystal structure of AmtB of *Escherichia coli*. *Proc. Natl. Acad. Sci.* 101, 17090-17095.
- Zidi-Yahiaoui, N., Ripoche, P., Van Kim, C. L., Gane, P., D'Ambrosio, A. –M., Cartron, J. –P., Colin, Y. and Mouro-Chanteloup, I. (2006). Ammonium transport properties of HEK293 cells expressing RhCG mutants: preliminary analysis of structure/function by site-directed mutagenensis. *Transfus. Clin. Biol.* 13, 128-131.

CHAPTER 3

THE EFFECTS OF CO₂ AND EXTERNAL BUFFERING ON AMMONIA EXCRETION AND RHESUS GLYCOPROTEIN mRNA EXPRESSION IN RAINBOW TROUT

3.1 ABSTRACT

Rhesus (Rh) proteins were recently characterized as ammonia gas (NH₃) channels. Studies indicate however, that Rh proteins also facilitate CO₂ transport in a green alga and in human erythrocytes. Previously, we reported that Rh mRNA expression in various rainbow trout tissues responded to high environmental ammonia. To determine whether or not Rh proteins may also be involved in CO₂ transport in rainbow trout, we examined the effects of 12-hour exposure to external hypercapnia (1% CO₂ in air) on the Rh mRNA expression in the gill, skin, and erythrocytes. External hypercapnic conditions lowered the water pH and facilitated ammonia excretion, therefore we also studied the effects of hypercapnia and normocapnia in the presence of 10 mM Hepes-buffered water. Hepes treatment prevented water acidification, but resulted in elevated plasma ammonia levels and reduced ammonia excretion rates. Hypercapnia exposure without buffering did not elicit changes in Rh mRNA expression in the gill or skin. However, Rhcg2 was downregulated in the gills and upregulated in the skin of both normocapnia- and hypercapnia-exposed fish in Hepes-buffered water. mRNA expression of a newly cloned Rhbg2 cDNA was downregulated in the skin of fish exposed to buffered water and Rhag mRNA expression in erythrocytes was decreased with exposure to normocapnia in buffered water, but not with hypercapnia exposure in either buffered or unbuffered water. With the aid of Hepes buffering, we were able to observe the effects of both CO₂ and ammonia on Rh mRNA expression, the resulting pattern of which mirrored that in trout exposed to high environmental ammonia. This suggests that the Rh mRNA changes were in response to high plasma ammonia rather

than CO_2 , and therefore a dual function for Rh proteins is not evident from these results. Rhag however, responded differentially to high CO_2 and high ammonia, suggesting a possible dual role in the erythrocytes.

3.2 INTRODUCTION

Rh proteins are most commonly known for their role in erythrocyte antigenicity (Avent *et al.*, 2006). Several non-erythrocytic Rh proteins have now been identified in a variety of species ranging from bacteria to mammals (e.g. Chain *et al.*, 2003; Huang and Peng, 2005; Weihrauch, 2006). Structural and functional studies have revealed that Rh proteins are transporters of ammonia, a property shared with AmtB, their *Escherichia coli* homologue (see reviews by Planelles, 2007; Van Kim *et al.*, 2006; Weiner and Hamm, 2007). There is some conflict as to whether the primary substrate is NH₃ or NH₄⁺, but most evidence points to NH₃ (Javelle *et al.*, 2007).

A number of different Rh orthologues have been characterized in fish (Huang and Peng, 2005; Hung *et al.*, 2007; Nakada *et al.*, 2007 a, b; Nawata *et al.*, 2007). While Rhag appears to be limited to erythrocytes in mammals (Huang and Peng, 2005), it occurs in both erythrocytes and pillar cells in the pufferfish, *Takifugu rubripes* (Nakada *et al.*, 2007b). Additionally, Nakada *et al.* (2007b) showed in pufferfish that while Rhbg and Rhcg2 are situated basolaterally and apically in the pavement cells respectively, Rhcg1 is located apically in mitochondria-rich cells. Exactly how these Rh proteins function in ammonia transport in fish is not yet clear however, Rh mRNA expression

69

levels do change in response to high external ammonia in some fish species (Hung *et al.*, 2007; Nawata *et al.*, 2007), but not all (Nakada *et al.*, 2007a).

The bulk of studies implicate Rh proteins in NH₃ transport, although there remains the possibility that they also facilitate transfer of CO₂, another readily hydrated gas of similar size (Endeward *et al.*, 2006; Kustu and Inwood, 2006). Soupene *et al.* (2002, 2004) reported that the Rh1 protein of the green alga, *Chlamydomonas reinhardtii*, was upregulated by high CO₂ and that the absence of Rh1 impaired the growth of this alga under high CO₂, a condition that normally promotes rapid growth. Recently it was demonstrated, using ¹⁸O and mass spectrometry, that both NH₃ and CO₂ pass through the RhAG protein of human erythrocytes (Endeward *et al.*, 2007). These two substrates compete for entrance and passage through the RhAG channel, although the affinity appears to be greater for NH₃ than CO₂. Furthermore, structural studies performed on the Rh1 protein of the ammonia-oxidizing bacterium, *Nitrosomonas europaea*, revealed that it may have a much lower ammonium affinity than the Amt proteins (Lupo *et al.*, 2007) and a possible CO₂ binding site at the COOH-terminal end has been described (Li *et al.*, 2007).

A few studies suggest however, that CO_2 is not transported through the Rh channel. Ripoche *et al.* (2006) for example, found no difference in the permeability to CO_2 in human erythrocyte membranes lacking RhAG, using a stopped-flow method. More recently it was shown that both wild type (Weidinger *et al.*, 2007) and Rh1 knockout mutants (Cherif-Zahar *et al.*, 2007) of *N. europaea*, were unresponsive to CO_2 .

70

Like ammonia, CO_2 in fish is believed to pass through membranes mainly in its gaseous form (Henry and Heming, 1998), while the transport of CO_2 occurs in the plasma in its hydrated form, HCO_3^- (Perry and Gilmour, 2002). A small amount of CO_2 exits the apical gill epithelium as HCO_3^- in exchange for Cl⁻ after being hydrated in the gill by carbonic anhydrase and thus serves a role in acid-base regulation (Claiborne *et al.*, 2002; Perry, 1986; Perry and Gilmour, 2006).

In the present study, our goal was to determine if exposure to high external CO_2 levels could elicit changes in Rh mRNA expression in rainbow trout gill, skin, and erythrocytes, which would indicate a possible dual role for the Rh glycoproteins as NH₃ and CO₂ channels. However, studying the effects of high CO₂ on Rh mRNA expression in fish is problematic in that hypercapnia causes an increase in endogenous ammonia production (Claiborne and Heisler, 1986). Increased ammonia excretion rates have also been associated with exposure to external hypercapnia (Claiborne and Heisler, 1986) and this may be due to the favourable plasma to water ammonia gradient created by the lowered water pH as a result of the hydration of CO₂ in the water (Claiborne and Heisler, 1986; Larsen and Jensen, 1997; Lloyd and Herbert, 1960; Wright *et al.*, 1989). Buffering minimizes acidification of the water during hypercapnia, however this introduces the confounding effect of abolishing the acidified boundary layer at the gill which normally facilitates ammonia excretion (Salama *et al.*, 1999; Wilson *et al.*, 1994; Wright *et al.*, 1986, 1989).

We therefore examined three experimental conditions on the mRNA expression of Rh glycoproteins in trout: the effects of hypercapnia alone, hypercapnia with Hepes buffering, and effects of normocapnia with Hepes buffering. The hypercapnia treatment chosen (1% CO₂ for 12 hours) directly duplicated a previous study in our lab in identical water quality where internal acid-base and ion status (both extracellular and intracellular) was monitored (Wood and LeMoigne, 1991). Additionally we analyzed the ammonia excretion rates, plasma cortisol levels, and the mRNA expression of other gill transporters possibly involved in ammonia excretion in each experimental condition. Finally, we isolated three full-length Rhbg2 cDNA variants and assessed the mRNA expression of these in the gill and skin.

3.3 MATERIALS AND METHODS

3.3.1 Animals

Rainbow trout (*Oncorhynchus mykiss*, Walbaum), weighing 170–240g, were obtained from Humber Springs Trout Hatchery, Ontario, Canada and held in dechlorinated Hamilton tapwater (moderately hard: $[Na^+]=0.6$ mequiv L⁻¹, $[CI^-]=0.8$ mequiv L⁻¹, $[Ca^{++}]=0.8$ mequiv L⁻¹, $[Mg^{++}]=0.3$ mequiv L⁻¹, $[K^+]=0.05$ mequiv L⁻¹; titration alkalinity 2.1 mequiv L⁻¹; pH ~8.0; hardness ~140 mg L⁻¹ as CaCO₃ equivalents; temperature 12-16° C) and fed *ad libitum* until one week before experimentation, during which time food was withheld. Fish were transferred to individual, opaque boxes supplied with aerated, flowing dechlorinated tap water (15±0.5°C) and allowed to recover overnight. All procedures used were approved by the McMaster University Animal Research Ethics Board and are in accordance with the Guidelines of the Canadian Council on Animal Care.

3.3.2 Experimental conditions

3.3.2.1 Effect of external hypercapnia

Water flow was closed off to each box and the box water volume was set to 4 L. Fish were exposed for 12 hours to a 1% CO_2 in air mixture (hypercapnia), provided by a Wösthoff gas-mixing pump (Calibrated Instruments Inc., Ardsley, NY, USA), bubbled into the water. Control fish were treated identically with a 12-hour exposure to 100% air (normocapnia).

3.3.2.2 Effect of Hepes buffering

Bubbling of 1% CO₂ in air into the external water reduced the pH by approximately 1.22 pH units, a similar reduction to that reported earlier by other investigators (Larsen and Jensen, 1997; Wright *et al.*, 1988a). In order to minimize acidification of the water while fish were exposed to hypercapnia, Hepes (Sigma; St. Louis, MO, USA) was added to the external water to a nominal concentration of 10 mM using a 1M stock solution adjusted to pH 8.0 with KOH. This maintained the pH within the range of 7.76 and 7.39 during the course of 12 hours. To examine the effects of Hepes buffering alone on ammonia excretion and mRNA expression, another set of fish were exposed to normocapnia for 12 hours in the presence of 10 mM Hepes, as above.

3.3.2.3 Effect of high environmental ammonia (HEA)

In order to check some particular parameters highlighted in the results of the hypercapnia and Hepes buffering exposures, a high environmental ammonia (HEA) exposure protocol identical to that used by Nawata *et al.* (2007) was repeated using an ammonia concentration similar to that used by previous investigators (Cameron, 1986; Cameron and Heisler, 1983; Claiborne and Evans, 1988; Wilson and Taylor, 1992; Wilson *et al.*, 1994). Trout were exposed to a nominal total waterborne ammonia (T_{Amm}) concentration of 1.5 mmol L⁻¹ NH₄HCO₃ (pH 7.95 ± 0.05) for 12 hours. During this period the water flow was stopped and the box volume was set to 4 L. Water samples were removed every 3 hours and total water ammonia concentration remained at 1.41 ± 0.03 mmol L⁻¹ over the 12-hour period.

3.3.3 Analyses

Water samples (10 ml) were removed at 3-hour intervals, stored at -20° C and later analyzed in triplicate for total ammonia (T_{Amm}) using a modified salicylatehypochlorite method (Verdouw *et al.*, 1978). Because Hepes altered colour generation in this assay, additional ammonia standards containing 10 mM Hepes were prepared and analyzed in conjunction with the samples from the Hepes experiments. At the end of each experiment, fish were anaesthetized with 0.1 g L⁻¹ MS222 (Sigma) and caudal blood samples were collected into a heparinized syringe. Blood cells were separated from plasma by centrifugation and both blood fractions were snap frozen in liquid nitrogen and stored at -70° C.

Net flux rates of total ammonia $(J_{Amm}; \mu mol \text{ kg}^{-1}\text{h}^{-1})$ into the water were calculated as: $J_{Amm} = (T_{Ammi} - T_{Ammj}) \times V/(t \times M)$ where *i* and *f* refer to initial and final concentration ($\mu molL^{-1}$), *V* is the box water volume (L), *t* is the time elapsed (h) and *M* is

the fish mass (kg). A negative J_{Amm} indicates net excretion into the water. Plasma total ammonia was measured enzymatically (Raichem, Hemagen Diagnostics, Inc., San Diego, CA, USA) and reported as μ mol L⁻¹.

External water pH was monitored with a Radiometer GK2401C low ionic strength combination electrode thermostatted to the experimental water temperature.

3.3.3.1 Tissue sampling

Prior to tissue extraction, fish were perfused free of blood using Cortland saline (Wolf, 1963) as previously described (Nawata *et al.*, 2007). Samples of gill, skin, and blood were removed, snap frozen in liquid nitrogen and stored at -70° C until later processing. Gill samples from control fish were used for cloning of Rhbg2 and additional samples of brain, gill, intestine, kidney, liver, muscle and skin were removed for screening of Rhbg2 mRNA expression.

3.3.3.2 Total RNA isolation and reverse-transcriptase PCR amplification

Total RNA was extracted from blood and tissues using Trizol (Invitrogen, Burlington, ON, Canada) and concentrations were quantified and checked for quality with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and electrophoresed on 1% agarose gels stained with ethidium bromide, to verify integrity. First strand cDNA synthesis was performed using 1 μ g of DNaseItreated (Invitrogen) total RNA with oligo(dT₁₇) primer and Superscript II reverse transcriptase (Invitrogen).

75

McMaster - Biology

Full-length cDNA of Rhbg2 was obtained by 5'- and 3'-rapid amplification of cDNA ends (Smart RACE cDNA amplification kit, BD Bioscience Clontech, Mississauga, ON, Canada) using the RACE primers listed in Table 3.1 and the protocol described previously (Nawata *et al.*, 2007). Sequence analyses were performed with BioEdit (Hall, 1999) and CLUSTAL W (Thompson *et al.*, 1994). Hydropathy profile and N-glycosylation site predictions were made using SPLIT 4.0 (Juretic *et al.*, 2002), and ScanProsite (de Castro *et al.*, 2006), respectively.

Tissue distribution of Rhbg2 was determined by performing reverse transcriptase PCR (RT-PCR) on the above-mentioned cDNA with the primer set listed in Table 3.1 at 35 cycles for Rhbg2 and 25 cycles for elongation factor (EF-1 α). Products were electrophoresed on 1.5% agarose gels stained with ethidium bromide and sequenced to confirm identity.

3.3.3.3 mRNA expression

Quantitative real-time PCR (qPCR) was performed on the cDNA described above using the primers listed in Table 3.1. Rh mRNA expression was assessed in the gill, skin, and erythrocytes and the expressions of carbonic anhydrase (CA2; cytoplasmic), H⁺-ATPase (v-type, B-subunit), NHE2, and Na⁺/K⁺-ATPase (NKA; α 1a-subunit) were analyzed in the gill. Each 20 µl reaction contained 4 µl of cDNA, 8 pmoles of each primer and 10 µl of RT² Real-Time SYBR Green/ROX PCR Master Mix (SuperArray, Bioscience Corp, Frederick, MD). Analyses were performed at 95°C for 2 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. Melt-curve analysis and gel electrophoresis verified presence of a single product. No-template controls and nonreversed-transcribed controls were run in parallel. Data were extrapolated from standard curves generated by serial dilution of one randomly selected control sample. Three housekeeping genes, beta-actin, EF-1 α , and 18S proved to be unstable across treatments and data were therefore normalized to ng total RNA, another acceptable method of normalization (Bustin, 2000, 2002).

3.3.3.4 Plasma cortisol

Plasma cortisol levels were measured in duplicate on 25 μ l samples by radioimmunoassay (Cortisol ¹²⁵I RIA Kit, MP Biomedicals, Orangeburg, NY, USA) and values are reported in ng ml⁻¹.

3.3.3.5 Statistical analysis

Data are presented as means \pm s.e.m. (n, number of fish). One-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference *post-hoc* test was used to analyze both the effects of the experimental treatments, and the relative abundance of Rhbg2 mRNA in the skin. One-way repeated measures ANOVA was used to analyze ammonia excretion rates. Significance was set at α =0.05.

77

3.4 RESULTS

3.4.1 Identification of Rhbg2

Three Rhbg2 cDNA sequences were identified in this study (GenBank; EU660221, EU660222, and EU660223). Two of these, designated Rhbg2a and Rhbg2b, have identical open reading frames (ORFs) encoding for a protein 461 amino acid residues long. Analysis showed a 98% amino acid sequence identity with the ORF of Rhbg1 (Fig. 3.1A). These Rhbg2 variants are identical, but Rhbg2b has a 414 base-pair deletion in the 3'-untranslated region (UTR). The ORF of the third cDNA, designated Rhbg2c, is a truncated variant of the full-length ORF, with 166 amino acids deleted from the COOH-terminal end. The last 18 amino acids form an alternative end (Fig. 3.1A). Rhbg2b and Rhbg2c additionally have four different 5'-UTR variants (herein termed variants 1, 2, 3, and 4, in order of decreasing length) with an upstream ORF (uORF) that would encode for a polypeptide of 6 amino acids. The longest 5'-UTR variant (variant 1) has an additional uORF upstream to the common uORF that would encode for a polypeptide of 26 amino acids (Fig. 3.1B). Rhbg2a has three of the four 5'-UTR variants described; the longest one was not detected by RT-PCR analysis (Fig. 3.1C). Hydrophobicity analysis revealed 12 predicted transmembrane domains and two putative N-glycosylation sites for Rhbg2a and Rhbg2b (Asn48 and Asn212). The truncated variant, Rhbg2c retains the same predicted N-glycosylation sites as the full-length variants, but has only 8 predicted transmembrane domains (Fig. 3.1*A*).

3.4.2 Tissue distribution and expression of Rhbg2

PCR analysis of the various trout tissues from control fish revealed that Rhbg2 is expressed in the brain, gill, intestine, skin, and possibly in the liver and muscle (Fig. 3.2). The truncated form, Rhbg2c was only detected in the gill (data not shown). qPCR analysis performed on control samples of gill and skin showed that the 5'-UTR variants are present in equal abundance in the gill. In the skin however, variants 1 and 3 were significantly more abundant than the other two. To examine whether or not these variants respond differentially, we analyzed the expression in the skin of fish exposed to hypercapnia in Hepes-buffered water. Expression of all four variants decreased such that they were no longer differentially expressed (Fig. 3.3).

3.4.3 External hypercapnia

Exposure to external hypercapnia resulted in ammonia excretion rates that were elevated significantly over both the 3 and 12-hour control rates during the course of the exposure. The rates at 3 and 12 hours were 69% and 54% higher than the control rates, respectively (Fig. 3.4). Plasma ammonia levels in these fish at 12 hours were not significantly different from the control values (Fig. 3.5). There were no significant changes in Rh glycoprotein mRNA expression in the gill or skin, although Rhcg2 was decreased by almost 50% (Figs. 3.6 and 3.7). Similarly, no significant differences in Rhag and Rh30-like2 mRNA expression were noted in the erythrocytes, however Rhag was increased by about 1.7-fold (Fig. 3.8). Expressions of CA2, H⁺-ATPase, NHE2, and

NKA mRNA in the gill were unchanged (Fig. 3.9). The plasma cortisol concentration was not significantly different from that of the control fish (Fig. 3.10).

3.4.4 External hypercapnia with Hepes buffering

Throughout the 12-hour exposure to hypercapnia in Hepes-buffered water, trout exhibited ammonia excretion rates that were significantly lower than those in fish exposed to hypercapnia alone, with a 57% reduction at 3 hours and a 42% reduction at 12 hours. At 9 hours however, the rates were significantly increased above the 3-hour control rates and by 12 hours they were not different from the 12-control rates (Fig. 3.4). Plasma ammonia levels were significantly increased 2.3-fold above the control value after 12 hours of exposure (Fig. 3.5). In the gill, CA2 and NHE mRNA levels were significantly increased (Fig. 3.9) and Rhcg2 was a significantly decreased by 60% (Fig. 3.6). No significant changes were noted in the mRNA expression of Rhag or Rh30-like2 in the erythrocytes, although expression of Rhag was 2-fold higher than the control (Fig. 3.8). In the skin, there was a 67% reduction in Rhbg2 but a 30-fold increase in Rhcg2. Rhcg1 was decreased, but not significantly (Fig. 3.7). Plasma cortisol was not significantly different from the control value at 12 hours (Fig. 3.10).

3.4.5 External normocapnia with Hepes buffering

The ammonia excretion rate in fish exposed to normocapnia in Hepes-buffered water was not significantly different from the 3-hour control rate, throughout the 12-hour exposure. However, there was a trend towards decreased excretion and by 12 hours, the

rate was reduced to 71% of the 12-hour control value (Fig. 3.4). Plasma ammonia was significantly elevated over the control value at 12 hours (Fig. 3.5). Rhcg2 mRNA levels were significantly reduced in the gill to about half of the control level (Fig. 3.6), but no changes were noted in the other gill mRNAs (CA2, H⁺-ATPase, NHE2, NKA; Fig. 3.9). In the skin, while there was a 2-fold reduction in Rhbg1 and a 4-fold reduction in Rhbg2, Rhcg1 and Rhcg2 expressions were increased by 4- and 58-fold, respectively (Fig. 3.7). In the erythrocytes, there were 3-fold reductions in both Rhag and Rh30-like2 mRNA expression (Fig. 3.8) and plasma cortisol levels were low, but not significantly different from control values (Fig. 3.10).

3.4.6 High environmental ammonia

Because of the high nucleotide sequence identity between the Rhbg1 and the Rhbg2 cDNAs (the highest being 91.5%, between the Rhbg1a and Rhbg2a variants), we designed a primer set more specific for Rhbg1 than that used previously by Nawata *et al.*, (2007), and assessed the expression of Rhbg1 and Rhbg2 in the gill and skin of HEA-exposed fish. Both Rhbg1 and Rhbg2 mRNA expression levels remained unchanged in the gill during HEA (Fig. 3.6). In the skin, Rhbg1 mRNA levels were not significantly different from that of the control, but Rhbg2 mRNA expression was significantly down-regulated by about 4-fold (Fig. 3.7). Expressions of Rhcg1 and Rhcg2 mRNA in the gill and skin, Rhag and Rh30-like2 mRNA in the erythrocytes, and CA2, H⁺-ATPase, NHE2, and NKA mRNA in the gill were also measured and confirmed the results reported earlier by Nawata *et al.* (2007) and are therefore not reported here. Plasma ammonia and

cortisol levels were both significantly elevated above the control values by 10-fold and 4-fold, respectively (Figs 3.5 and 3.10).

3.5 DISCUSSION

Recently, we established a link between Rh proteins and ammonia by showing that rainbow trout exposed to high environmental ammonia (HEA) responded with upregulated Rhcg2 mRNA expression in the gill (Nawata et al., 2007). This response coincided with the re-establishment and enhancement of ammonia excretion that was initially inhibited by the reversed plasma to water ammonia gradient. In the present study, we used hypercapnia as a tool to assess the response of Rh mRNA transcription levels to high CO₂ in the rainbow trout. This proved problematic in that, like the study by Claiborne and Heisler (1986), hypercapnia caused an increase in internal ammonia production as evidenced by the elevated rate of ammonia excretion (Fig. 3.4) without alteration of plasma total ammonia concentration (Fig. 3.5). Bubbling of CO₂ into the water lowered the pH, creating a sink for NH₃, thus enhancing the rate of excretion. This increased excretion rate was sufficient to maintain plasma ammonia at control levels (Fig. 3.5) and may explain why changes in Rh mRNA levels were not observed. Any effects that elevated CO₂ may have had were not reflected as changes in mRNA transcription levels in the gill or skin.

Buffering of the external water effectively reduced the acidification caused by the CO_2 , but it also likely abolished the acidified gill boundary layer. It has been well documented that the boundary layer, whether acidified by hydration of CO_2 by carbonic

McMaster - Biology

anhydrase in the mucus (Wright *et al.*, 1986, 1989), or by release of protons from an apical H^+ -ATPase (Lin *et al.*, 1994; Lin and Randall, 1990), normally facilitates ammonia excretion. Indeed, the present study confirms previous reports (Salama *et al.*, 1999, Wright *et al.*, 1989) showing that there was a decline in the ammonia excretion rate when fish were exposed to normocapnia in Hepes-buffered water, with the rate reduced to a third of the control rate by 12 hours (Fig. 3.4). As a consequence of the reduced excretion, plasma ammonia levels in these fish were elevated after 12 hours of treatment (Fig. 3.5). Despite this elevation, and unlike HEA-exposed fish that also have high plasma ammonia (Nawata *et al.*, 2007), the Rhcg2 mRNA transcription level was decreased in the gill (Fig. 3.6). Upregulated or enhanced ammonia transport capacity at the gill would likely prove futile in the presence of Hepes since any protons formed or released at the apical gill surface would be rapidly removed by the buffer, effectively eliminating a favourable ammonia gradient.

However, in the skin of fish exposed to Hepes in the presence of normocapnia both Rhcg1 and Rhcg2 mRNA levels were highly elevated (Fig. 3.7). Rhcg2 exhibited the greatest fold increase in mRNA expression suggesting that this protein may have a dominant role in the skin, but this requires further investigation. Upregulation of Rhcg2 in the skin also occurred when excretion was blocked at the gill after 12 hours of exposure to HEA (Nawata *et al.*, 2007). Fish skin is generally thought to have low permeability (Fromm, 1968) and therefore its role in gas and ion exchange has been largely ignored. A few studies have measured ammonia excretion from fish skin (see Wood, 1993 for a review), but it appears that the skin has a secondary role to the gill if any, in freshwater fish. This may be true especially under normal circumstances, but under conditions when excretion is blocked at the gill, the skin may become an important alternate site of ammonia release and this possibility needs to be explored further in freshwater species. Indeed the skin becomes a site of ammonia excretion via volatilization during aerial exposure in the mangrove killifish, *Kryptolebias marmoratus*, and skin Rh mRNA levels are also upregulated during this time (Hung *et al.*, 2007).

The acid-base disturbance in response to hypercapnia has been well described in catheterized fish (see Claiborne 1998; Heisler, 1993, for reviews). Catheterization was avoided in the present study, as our initial trials indicated that catheterization alone may alter Rh expression (Nawata and Wood, unpubl.). However, we used an exposure regime in which the acid-base effects had been documented previously. In an identical 1% CO₂ exposure protocol in Hamilton tapwater, chronically catheterized rainbow trout exhibited a 0.3 unit depression in arterial pH at 3 hours with partial recovery by 12 hours (see Fig. 3.7 of LeMoigne and Wood, 1991). Although gill cell intracellular ion levels (Na⁺ and Cl⁻) declined, gill intracellular pH did not change.

The associated increase in acid excretion which helps correct internal pH by building up internal HCO_3^- levels during hypercapnia involves the hydration of CO_2 by carbonic anhydrase within the gill (Henry and Heming, 1988; Perry, 1986; Perry and Gilmour, 2006). Apically located H⁺-ATPase and/or NHE may then be involved in the release of protons into the water (Edwards *et al.*, 2005). Although buffering during hypercapnia would cause an internal elevation of both CO_2 and ammonia, results from our study suggested that fish in this treatment group were responding to an acidosis

McMaster - Biology

induced by the high CO₂. The upregulation of NHE2 mRNA levels in the gill corresponds well with the findings of Edwards *et al.* (2005), who reported an increase in NHE2-like protein expression in *Fundulus heteroclitus* exposed to external hypercapnia. This suggests that although H⁺-ATPase is believed to play the major role in acid secretion in freshwater teleosts (Edwards *et al.*, 2005), NHE2 may also have a role during acidosis in rainbow trout. H⁺-ATPase mRNA expression levels were not upregulated at 12 hours (Fig. 3.9), but this should not be interpreted to mean that the corresponding protein is not functional during acidosis. The expression levels of H⁺-ATPase mRNA may in fact peak as early as 2 hours after hypercapnia exposure (Perry *et al.*, 2000) that could then result in a sufficient quantity of protein by 12 hours. The increased CA2 mRNA levels that occurred are also in agreement with previous studies that reported increased carbonic anhydrase activity (Dimberg and Hoglund, 1987) and increased CA2 mRNA and protein expression (Georgalis *et al.*, 2006) in the gills of hypercapnia-exposed trout.

Fish exposed to hypercapnia in Hepes-buffered water also experienced inhibition of ammonia excretion at the gill as evidenced by the elevated plasma ammonia (Fig. 3.5) as well as the excretion rates that were significantly lower than those of the hypercapniaexposed fish in unbuffered water (Fig. 3.4). Although the excretion rates started to rise at 9 hours (Fig. 3.4), the downregulation of Rhcg2 and H⁺-ATPase mRNA levels in the gill indicate that these fish were probably not using the same mechanism to enhance ammonia excretion as that used during HEA. Both treatments resulted in blockage of ammonia excretion from the gill, but it appears that in hypercapnic fish, the primary mRNA response in the gill was to CO_2 rather than high ammonia. Similar to the fish exposed to normocapnia in Hepes-buffered water, there was an upregulation of Rhcg2 mRNA in the skin (Fig. 3.7), again reinforcing the idea that this may be an alternate route for ammonia excretion.

Previously we reported that Rhag as well as the Rh30-like mRNAs were downregulated during HEA, when plasma ammonia levels were high (Nawata *et al.*, 2007). In the present study, both groups of fish exposed to Hepes-buffered water had elevated plasma ammonia levels, albeit lower than HEA-exposed fish (Fig. 3.5), so we would have expected a similar down-regulation of Rhag in these two groups. Fish exposed to normocapnia did indeed exhibit lowered Rhag, however the hypercapnia-exposed fish did not. Instead, the Rhag mRNA expression in the hypercapnia-exposed fish was increased 2-fold over that of the control fish, a response similar to that of the hypercapnia-exposed fish in unbuffered water (Fig. 3.8). This suggests that Rhag was responding to both high CO_2 and high ammonia, but in a differential fashion.

Ammonia accumulates in trout erythrocytes during hypercapnia (Wright *et al.*, 1988b). If Rhag allows passage of NH₃, then downregulation when plasma ammonia levels are elevated may be a protective response. Transport of NH₃ with CO₂ and H₂O into the erythrocyte would produce NH₄⁺ and HCO₃⁻ and once HCO₃⁻ is exchanged for CI⁻, the formation of NH₄Cl would lead to H₂O uptake and result in swelling (Bruce, 2008). Also, the buffering effect of NH₃ could interfere with the Bohr and Haldane effects, and therefore with O₂ and CO₂ transport. In human erythrocyte membranes, RhAG forms a macrocomplex with a number of proteins including the band-3 anion exchanger (AE1) and carbonic anhydrase, and this complex is thought to function in
CO_2/O_2 gas exchange (Bruce *et al.*, 2003). Trout erythrocytes also have AE1 present (Michel and Rudloff, 1989), but whether or not it forms a similar complex with Rhag is unknown. However, if Rhag functions as a CO_2 channel, then upregulation along with AE1 could be beneficial during hypercapnia when plasma HCO_3^- is high.

It has also been speculated that the Rh30 proteins could allow CO_2 passage (Callebaut *et al.*, 2006), although Endeward *et al.* (2007) found no difference in CO_2 uptake in erythrocytes lacking the RhD (Rh30) protein. Data from our study also suggests that the Rh30-like proteins are not involved in CO_2 transport as there were no notable changes in mRNA expression in erythrocytes from either of the two groups exposed to hypercapnia (Fig. 3.8). Rh30-like2 mRNA expression however, was downregulated in the fish exposed to normocapnia and Hepes, like HEA-exposed fish (Nawata *et al.*, 2007), suggesting a response to high ammonia only.

So far, studies have indicated that fish have multiple Rh genes (Huang and Peng, 2005; Hung *et al.*, 2007; Nakada *et al.*, 2007a,b; Nawata *et al.*, 2007) but the significance of this seeming redundancy is unclear. Nakada *et al.* (2007b) proposed that Rhag in the pillar cells of pufferfish may work in conjunction with the basolaterally located Rhbg and apically situated Rhcg2 in the lamellae to keep plasma ammonia levels low, with additional excretion aided by Rhcg1 in the mitochondria-rich cells. Others have shown that some Rh proteins may be dispensable like Rhbg in the mouse (Chambrey *et al.*, 2005) and Rh1 in the slime mold (Benghezal, 2001). Likewise, the physiological significance of the multiple variants of Rhbg2 (Fig. 3.1) is puzzling, but the presence of uORFs and splicing in the 5'- and 3'-UTRs of the mRNAs suggests complex regulation

of this protein. uORFs have the potential to impact gene expression and some may serve as *cis*-acting regulatory elements modulating translation of the main ORF (Meijer and Thomas, 2002; Morris and Geballe, 2000). Similarly, the UTRs are involved in many post-transcriptional pathways that control the localization, stability and translation efficiency of mRNAs (Pesole *et al.*, 2001). The existence of a truncated version of the Rhbg2 protein suggests yet another level of complexity. Truncation alters function and/or intracellular location of some transporters while some alternative-splice products negatively regulate the wild-type protein (Kitayama *et al.*, 1999; Mangravite *et al.*, 2003). Whether the truncated version of Rhbg2 functionally interacts with the full form or whether it has a completely different function has yet to be analyzed.

Interestingly, the same 5'-UTR Rhbg2 variants are present in both the skin and gill, although in the gill they are expressed equally while in the skin they are expressed differentially (Fig. 3.3). The downregulation of Rhbg2 that occurred in the skin with Hepes treatment during both hypercapnia and normocapnia appears to be in response to high plasma ammonia rather than high CO₂ as suggested by the unchanged expression in the hypercapnia-treated fish in unbuffered water. To support this idea, we found that HEA-exposed fish exhibited a similar down-regulation (Fig. 3.7). Rhbg was reported to be nonessential during acidosis in knockout mice (Chambrey *et al.*, 2005). Abundance of Rhbg2 in fish skin may similarly be unnecessary during hypercapnia and HEA, and instead Rhbg2 could have a different role other than ammonia transport.

Glucocorticoids are known for their role in regulating gene expression in mammals and the same is likely true for fish (Mommsen *et al.*, 1999). Data from this

88

study however, showed no correlation between Rh mRNA abundance and cortisol levels. Plasma cortisol and gill Rhcg2 mRNA levels were both elevated during HEA but in the two Hepes-exposed groups, Rhcg2 mRNA was elevated in the skin while the cortisol remained at control levels (Fig. 3.10). A lack of correlation however, does not exclude the possibility that cortisol could be involved either directly or indirectly in the control of Rh protein expression. Indeed, cortisol levels need not be elevated in order to have an influence on physiological processes (e.g. Wood *et al.*, 2001) and other factors such as the clearance rate and non-genomic actions of cortisol need to be taken into consideration (Mommsen *et al.*, 1999).

We have evaluated the expression of Rh protein mRNAs in rainbow trout exposed to hypercapnic and normocapnic conditions in the presence and absence of Hepes buffer. Previously, we showed that at the mRNA transcript level, Rh proteins clearly respond to high ammonia conditions in rainbow trout tissues (Nawata *et al.*, 2007). A similar response to high CO₂ would suggest that Rh proteins may have a dual role as CO₂ and NH₃ gas channels in trout tissues. Data from our present study revealed however, that high CO₂ did not directly elicit changes in Rh mRNA transcription levels in the gill and skin. Instead, the changes that did occur likely reflected responses to high plasma ammonia, and thus reinforce the connection between the Rh proteins and ammonia. As such, a dual role for Rh proteins in the rainbow trout is not apparent from this study. There does remain however, the possibility that Rhag interacts with both CO₂ and ammonia in the erythrocyte and further work is needed to clarify this.

3.6 TABLES AND FIGURES

Name	Forward / reverse sequence (5'- 3')	Application	Accession #
Rhbg2	cctggctgtgaccctcggcattg / ctccctggctgtgaccctcggcattg	RACE	EU660221
Rhbg1	gaagatacaggacacgtgtg / gatgcccagggttacggc	qPCR	EF051113
Rhbg2	ctgtgaccctcggcattgc / ggatctcaagtgtgtgtgtg	qPCR, RT-PCR	EU660221
Rhcg1	catectcageetcatacatge / tgaatgacagaeggagecaate	qPCR	DQ431244
Rhcg2	cctcttcggagtcttcatc / ctatgtcgctggtgatgttg	qPCR	AY619986
Rh30-like2	gctgctgcactgatatcaac / cgcccgttgtgagatcatc	qPCR	EF062577
Rhag	ctggcggccaatgatgttg / atggcgaagaggtcagagtg	qPCR	EF667352
CA	gccagtctcccattgacatc / cctgtacgtccctgaaatgg	qPCR	AY514870
HAT	tcagccttggttgtgagatg / caacattggtgggaaacagg	qPCR	AF140002
NHE	tatggccattgtgacctgtg / caggcctctccacactaagg	qPCR	EF446605
NKA	ttgacctggatgaccacaag / ggatctccttagcccgaac	qPCR	AY319391
EF1a	ggaaagtcaaccaccacag / gataccacgctccctctcag	RT-PCR	AF498320
V1	ggcagaccaactcatgtgtc / gttgccgaattcgtcatgg	qPCR	EU660222
V2	ggactccctgcactccca / gttcaactcagctccagagc	qPCR	EU660222
V3	ggactcccactctcccatc / gttgccgaattcgtcatgg	qPCR	EU660222
V4	ggactctcccatcgcatc / gttgccgaattcgtcatgg	qPCR	EU660222

Table 3.1 Primer list

MTNSATNLRLKLFMACTILEVINITILFGELVQYDDETDAKLNHKKTANGSSNYDNDFYYR 60 A Rhbg1 MTNSATNLRLKLPMACIILEVITIILFGALVQYDDETDAKLWHKEIANGSSNYDNDFYYR 60 Rhbg2a Rhbg2c MTNSATNLRLKLPMACIILEVIIIILFGALVQYDDETDAKLNHKEIANGSSNYDNDFYYR 60 YPSFQDVHVMIFIGFGFLMTFLQRYGFSSVGFNFLIAAFALQWATLMQGFVHGMHGGKIH 120 Rhbgl Rhbg2a YPSFQDVHVMIFIGFGFLMTFLQRYGFSSVGFNFLIAAFALQWATLMQGFVHGMHGGKIH 120 Rhbg2c YP3FQDVEVMIFIGFGFLMTFLQRYGF3SVGFNFLIAAFALQWATLMQGFVHGMHGGKIE 120 Rhbg1 VGIESNINADFCTGSVIISFGAVEGKTSPUQLLUMSVIEVTLFAVNEPIVLSVLGAKDAG 180 Rhbg2: VGIESMINADFCTGSVLISFGAVLGKTSFVQLLVMSVIEVTLFAVNEFIVLTVLGAKDAG 180 Rhbg2c VGIESMINADFCTGSVLISFGAVLGKTSFVQLLVMSVIEVTLFAVNEFIVLTVLGAKDAG 180 GSMTIHTFGAYFGLMVARVLYRPNLDKSKHKNCSVYHSDIFAMIGTIYLWMFWPSFNSAV 240 Rhbg1 Rhbg2: GSMTIHTFGAYFGLMVARVLYRPMLDKSKHKNCSVYHSDLFAMIGTIYLWMFWPSFNSAV 240 Rhbg2c GSMTIHTFGAYFGLMVARVLYRPNLDKSKHKNCSVYHSDLPAMIGTIYLWMFWPSFNSAV 240 Rhbg1 TAHGDDQHRTAMNTYYSLAACTLATYGLSAVVAHDGKLDMVHIQNAALAGGVAVGTAGEM 300 Rhbg2: TAHGDDQHRTAMNTYYSLAACTLATYGLSAVVAHDGKLDMVHIQNAALAGGVAVGTAGEM 300 Rhbg2c TAHGDDQHRTAMNTYYSLAACTLATYGLSAVVAHDGKLDMURELVQCGDLRKSRSLKUV 299 Rhog1 MLTPPCSMAVG24AG4M4KG4M4KG271LLQKMKLQDTCGVENILHGM2GM4GA4VGAV9A 260 Rhbg2: MLTPFGSMIVGFLAGTESVLGFKFLSPILEQKLKIQDTCGVHNLKGMPGVLGAIVGAVTA 360 Rhbg1 ALATKDVYGDGMADVFPDIHSGDVEASPQGVRQAISLAVTLGIALLGGLITGFILKLPIY 420 Rhbg2: ALATKDVYGDGMADVF9DIQ5GDVEASFQGIRQAISLAVTLGIALLGGLITGFILKLPIY 420 Rhbg1 GAPPDTICPEDGIYWZ1PGEGGSGEELTTVRTPDEAEXLNA 461 Rhbg2= GAPPDTICPEDGIYWZ1PGEEGSHEELTTVRTPDEAEXLNA 461 R GCAGACCAACTCATGTGTCAGCCTGAGTCCTCACTCCCTGCACTCCCACTCTCCCATCGC <u>XCOPESSLPALPLS</u> H X ATCACTGCAGCCACCAGTGCTTGTGAGTGTTAGTGTATGTTGCTGTGTTCCCCGCTCTGG ITAATSACEC 'CHILCSPLE AGCTGAGTTGAACTTTTAACCATTCACCCCTAAGGCCATAGTGCAGAGCCAGGAGAACAC S * V E L L T I H P * G H S A E P G E H CCAGGTAACCCCACAGCC **FGNFTA** v2 C Rhbo2a Leeves. ¥3 12222 24 Rhbg2b V2 v3 Rhbg2c v2 v3

Figure 3.1 (A) Sequence alignment of Rhbg1, Rhbg2a, and Rhbg2c. Lines above the sequences represent putative transmembrane domains and asterisks indicate predicted N-glycosylation sites. The alternative COOH-terminal end of Rhbg2c is underscored. Shading highlights amino acid differences between Rhbg1 and Rhbg2. (B) 5'-untranslated region of the longest Rhbg2 variant. Two upstream open reading frames are underscored. (C) Schematic representation of the mRNA variants identified in Rhbg2. Shading and stippling identifies regions of identity between variants. White background represents the 5'-untranslated region, black background represents the open reading frame, and gray background represents the 3'-untranslated region. Four variants were detected in the 5'-untranslated regions, three of which were detected in Rhbg2a.

vid



Figure 3.2 Tissue distribution of Rhbg2 in rainbow trout exposed to control conditions (100% air for 12 hours) as determined by reverse transcriptase PCR. Elongation factor (EF-1 α) was used as an internal control. (NTC; no template control).



Figure 3.3 Relative abundance of 5'-untranslated region variants (v1,2,3,4) in the gill under control conditions (12-hours exposure to 100% air) and skin during control conditions and after 12 hours of exposure to hypercapnia (1% CO₂ in air) in 10 mM Hepes-buffered water. Expression data were normalized to ng total RNA concentration. Different letters indicates significant differences in mRNA abundance between variants in the skin during control conditions. Data are means \pm s.e.m. (*n*=6).



Figure 3.4 The effect of 12-hour exposures of rainbow trout to: hypercapnia (1% CO₂ in air) in unbuffered water, hypercapnia in 10 mM Hepes-buffered water, and normocapnia (100% air) in Hepes-buffered water on the net ammonia flux (J_{Amm}). Control fish were exposed to normocapnia for 12 hours in unbuffered water. The negative values indicate excretion into the water. Crosses represent values significantly different from the 3-hour control value. Asterisks represent values significantly different from the 12-hour control value. Control values are significantly different from each other (P<0.05). Data are means ± s.e.m. (n=6-10).



Figure 3.5 Plasma total ammonia after 12 hours of exposure of rainbow trout to 1% CO₂ in air (CO₂), 1% CO₂ in air with 10 mM Hepes buffered-water (CO₂ + Hepes), 100% air with 10 mM Hepes-buffered water (Air + Hepes), and high environmental ammonia (HEA; 1.5 mmoL NH₄HCO₃). Control fish were exposed to 100% air for 12 hours in unbuffered water. Asterisks represent plasma values significantly different from the control value (*P*<0.05). Data are means ± s.e.m. (*n*=6-10).

Ph.D. Thesis - C. M. Nawata



Figure 3.6 Gill Rh mRNA expression in rainbow trout after 12 hours of exposure to 1% CO₂ in air (CO₂), 1% CO₂ in air with 10 mM Hepes buffered-water (CO₂ + Hepes), 100% air with 10 mM Hepes-buffered water (Air + Hepes), and high environmental ammonia (HEA; 1.5 mmoL NH₄HCO₃). Control fish were exposed to 100% air for 12 hours in unbuffered water. Expression data were normalized to ng total RNA concentration with the control value set to one. Asterisks indicate significant difference from the control (P<0.05). Data are means ± s.e.m. (n=6).



Figure 3.7 Skin Rh mRNA expression in rainbow trout after 12 hours of exposure to 1% CO_2 in air (CO_2), 1% CO_2 in air with 10 mM Hepes buffered-water (CO_2 + Hepes), 100% air with 10 mM Hepes-buffered water (Air + Hepes), and high environmental ammonia (HEA; 1.5 mmoL NH₄HCO₃). Control fish were exposed to 100% air for 12 hours in unbuffered water. Expression data were normalized to ng total RNA concentration with the control value set to one. Asterisks indicate significant difference from the control (P<0.05). Data are means ± s.e.m. (n=6).



Figure 3.8 Erythrocyte Rh mRNA expression in the rainbow trout exposed to 12 hours of to 1% CO₂ in air (CO₂), 1% CO₂ in air with 10 mM Hepes buffered-water (CO₂ + Hepes), and 100% air with 10 mM Hepes-buffered water (Air + Hepes). Control fish were exposed to 100% air for 12 hours in unbuffered water. Expression data were normalized to ng total RNA concentration with the control value set to one. Asterisk indicates significant difference from the control (P<0.05). Data are means ± s.e.m. (n=6).



Figure 3.9 The effect of 12-hour exposure to 1% CO₂ in air (CO₂), 1% CO₂ in air with 10 mM Hepes buffered-water (CO₂ + Hepes), and 100% air with 10 mM Hepes-buffered water (Air + Hepes) on the mRNA expression of carbonic anhydrase (CA2), H⁺-ATPase, NHE2, and Na⁺/K⁺-ATPase α -1a (NKA) in the gills of rainbow trout. Control fish were exposed to 100% air for 12 hours in unbuffered water. Expression data were normalized to ng total RNA concentration with the control value set to one. Significant differences from the control are indicated by an asterisk (*P*<0.05). Data are means ± s.e.m. (*n*=6).



Figure 3.10 Plasma cortisol levels after 12 hours of exposure to 1% CO₂ in air (CO₂), 1% CO₂ in air with 10 mM Hepes buffered-water (CO₂ + Hepes), 100% air with 10 mM Hepes-buffered water (Air + Hepes), and high environmental ammonia (HEA; 1.5 mmoL NH₄HCO₃). Control fish were exposed to 100% air for 12 hours in unbuffered water. Asterisk indicates significant difference from the control value (P<0.05). Data are means ± s.e.m. (n=6-10).

3.7 REFERENCES

- Avent, N. D., Madgett, T. E., Lee, Z. E., Head, D. J., Maddocks, D. G. and Skinner, L. H. (2006). Molecular biology of Rh proteins and relevance to molecular medicine. *Expert Rev. Mol. Med.* 8, 1-20.
- Benghezal, M., Gotthardt, D., Cornillon, S. and Cosson, P. (2001). Localization of the Rh50-like protein to the contractile vacuole in *Dictylostelium*. *Immunogenetics* 52, 284–288.
- Bruce, L. J. (2008). Red cell membrane transport abnormalities. *Curr. Opin. Hematol.* 15, 184-190.
- Bruce, L. J., Beckmann, R., Ribeiro, M. L., Peters, L. L., Chasis, J. A., Delaunay, J., Mohandas, N., Anstee, D. J. and Tanner, M. J. A. (2003). A band 3-based macrocomplex of integral and peripheral proteins in the RBC membrane. *Blood* 101, 4180-4187.
- **Bustin, S. A.** (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrin.* 25, 169–193.
- Callebaut, I., Dulin, F., Bertrand, O., Ripoche, P., Mouro, I., Colin, Y., J,-P. Mornon, and J. P. Cartron. (2006). Hydrophobic cluster analysis and modeling of the human Rh protein three-dimensional structures. *Transfus. Clin. Biol.* 13, 70-84.
- **Cameron, J. N.** (1986). Responses to reversed NH₃ and NH₄⁺ gradients in a teleost (*Ictalurus punctatus*), an elasmobranch (*Raja erinacea*), and a crustacean (*Callinectes sapidus*): Evidence for NH₄⁺/H⁺ exchange in the teleost and the elasmobranch. J. Exp. Zool. 239, 183-195.
- **Cameron, J. N. and Heisler, N.** (1983). Studies of ammonia in the trout: physicochemical parameters, acid-base behaviour and respiratory clearance. *J. Exp. Biol.* 105, 107-125.
- Chain, P., Lamerdin, J., Larimer, F., Regala, W., Lao, V., Land, M., Hauser, L., Hooper, A., Klotz, M., Norton, J., Sayavedra-Soto, L., Arciero, D., Hommes, N., Whittaker, M. and Arp, D. (2003). Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea. J. Bacteriol.* 185, 2759-2773.

Chambrey, R., Goossens, D., Bourgeois, S., Picard, N., Bloch-Faure, M., Leviel, F.,

Geoffroy, V., Cambillau, M., Colin, Y., Paillard, M., Houillier, P., Cartron, J. P. and Eladari, D. (2005). Genetic ablation of Rhbg in the mouse does not impair renal ammonium excretion. *Am. J Physiol. Renal Physiol.* 289, F1281–1290.

- Cherif-Zahar, B., Durand, A., Schmidt, I., Hamdaoui, N. Matic, I., Merrick, M. and Matassi, G. (2007). Evolution and functional characterization of the RH50 gene from the ammonia-oxidizing bacterium *Nitrosomonas europaea*. J. Bacteriol. 189, 9090-9100.
- Claiborne, J. B. (1998). Acid-base regulation. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 179-200. Boca Raton: CRC Press.
- Claiborne, J. B., Edwards, S. L., and Morrison-Shetlar, A. I. (2002). Acid-base regulation in fishes: cellular and molecular mechanisms. *J. Exp. Zool.* 293, 302-319.
- Claiborne, J. B. and Evans, D. H. (1988). Ammonia and acid-base balance during high ammonia exposure in a marine teleost (*Myoxocephalus octodecimspinosus*). J. *Exp. Biol.* 140, 889-105.
- Claiborne, J. B. and Heisler, N. (1986). Acid-base regulation and ion transfers in the carp (*Cyprinus carpio*): pH compensation during graded long- and short-term environmental hypercapnia, and the effect of bicarbonate infusion. J. Exp. Biol. 126, 41-61.
- de Castro, E., Sigrist, C. J. A., Gattiker, A., Bulliard, V., Petra, S., Langendijk-Genevaux, P. S., Gasteiger, E., Bairoch, A. and Hulo, N. (2006). ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. *Nucleic Acids Res.* 34, W362-W365.
- **Dimberg, K. and Hoglund, L. B.** (1987). Carbonic anhydrase activity in the blood and the gills of rainbow trout during long-term hypercapnia in hard, bicarbonate-rich freshwater. *J. Comp. Physiol.* 157B, 405-412.
- **Endeward, V., Cartron, J. P., Ripoche, G. and Gros, G.** (2006). Red cell membrane CO₂ permeability in normal human blood and in blood deficient in various blood groups, and effect of DIDS. *Transfus. Clin. Biol.* 13, 123-127.
- Endeward, V., Cartron, J. -P., Ripoche, P., and Gros, G. (2007). RhAG protein of the complex is a CO₂ channel in the human red cell membrane. *FASEB J.* 22, 64-73.
- Edwards, S. L., Wall, B. P., Morrison-Sheltar, A., Sligh, S., Weakley, J. C. and Claiborne, J. B. (2005). The effect of environmental hypercapnia and salinity on

the expression of NHE-like isoforms in the gills of a euryhaline fish (*Fundulus heteroclitus*). J. Exp. Zool. 303A, 464-475, 2005.

- Georgalis, T., Perry, S. F. and Gilmour, K. M. (2006). The role of branchial carbonic anhydrase in acid-base regulation in rainbow trout (*Oncorhynchus mykiss*). J. *Exp. Biol.* 209, 518-530.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser.* 41, 95-98.
- Heisler, N. (1993). Acid-base regulation. In *The Physiology of Fishes* (ed. D. H. Evans) pp.343-378. Boca Raton: CRC Press.
- Fromm, P. O. (1968). Some quantitative aspects of ion regulation in teleosts. *Comp. Biochem.* 27, 865-869.
- Henry, R. P. and Heming, T. A. (1998). Carbonic anhydrase and respiratory gas exchange. In *Fish Respiration* (ed. S. F. Perry and B. L. Tufts), pp. 75-111. San Diego: Academic Press.
- Huang, C. H. and Peng, J. (2005). Evolutionary conservation and diversification of Rh family genes and proteins. *Proc. Natl. Acad. Sci. USA* 102, 15512-15517.
- Hung, C. Y. C., Tsui, K. N. T., Wilson, J. M., Nawata, C. M., Wood, C. M. and Wright, P. A. (2007). Rhesus glycoprotein gene expression in the mangrove killifish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air. J. Exp. Biol. 210, 2419-2429.
- Javelle, A., Lupo, D., Li, X. D., Merrick, M., Chami, M., Ripoche, P. and Winkler, F. K. (2007). Structural and mechanistic aspects of Amt/Rh proteins. J. Struct. Biol. 158, 472-481.
- Juretic, D., Zoranic, L. and Zucic, D. (2002). Basic charge clusters and prediction of membrane protein topology. J. Chem. Inf. Comput. Sci. 42, 620-632.
- Kitayama, S., Ikeda, T., Mitsuhata, C., Sato, T., Morita, K. and Dohi, T. (1999). Dominant negative isoform of rat norepinephrine transporter produced by alternative RNA splicing. J. Biol. Chem. 274, 10731-10736.
- Kustu, S. and Inwood, W. (2006). Biological gas channels for NH₃ and CO₂: evidence that Rh (Rhesus) proteins are CO₂ channels. *Transfus. Clin. Biol.* 13, 103-110.

Larsen, B. K. and Jensen, F. B. (1997). Influence of ionic composition on acid-base

regulation in rainbow trout (*Oncorhynchus mykiss*) exposed to environmental hypercapnia. *Fish Physiol. Biochem.* 16, 157-170.

- Li, X., Jayachandran, S., Nguyen, H. H. T., and Chan, M. (2007). Structure of the Nitrosomonas europaea Rh protein. Proc. Natl. Acad. Sci. USA 104, 19279-19284.
- Lin, H., Pfeiffer, D. C., Wayne, V. A., Pan, J., and Randall, D. J. (1994). Immunolocalization of H⁺-ATPase in the gill epithelia of rainbow trout. J. Exp. Biol. 195, 169-183.
- Lin, H. and Randall, D. J. (1990). The effect of varying water pH on the acidification of expired water in rainbow trout. *J. Exp. Biol.* 141, 14-160.
- Lupo, D., Li, X. D., Curand, A., Tomikazi, T., Cherif-Zahar, B., Matassi, G., Merrick, M. and Winkler, F. K. (2007). The 1.3Å resolution structure of *Nitrosomonas europaea* Rh50 and mechanistic implications for NH₃ transport by Rhesus family proteins. *Proc. Natl. Acad. Sci. USA* 104, 19303-19308.
- Lloyd, R. and Herbert, D. W. M. (1960). The influence of carbon dioxide on the toxicity of un-ionized ammonia to rainbow trout (*Salmo gairdnerii* Richardson). *Ann. Appl. Biol.* 48, 399-404.
- Mangravite, L. M., Xiao, G., and Giacomini, K. M. (2003). Localization of human equilibrative nucleoside transporters, hENT1 and hENT2 in renal epithelial cells. *Am. J. Physiol.* 284, F902-910.
- Meijer, H. A. and Thomas, A. A. M. (2002). Control of eukaryotic protein synthesis by upstream open reading frames in the 5'-untranslated region of an mRNA. *Biochem. J.* 367, 1-11.
- Michel, F. and Rudloff, V. (1989). Isolation and characterization of the rainbow trout erythrocyte band-3 protein. *Eur. J. Biochem.* 181, 182-187.
- Mommsen, T. P., Vijayan, M. M. and Moon, T. W. (1999). Cortisol in teleosts: dyanamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol. Fish.* 9, 211-268.
- Morris, D. R. and Geballe, A. P. (2000). Upstream open reading frames as regulators of mRNA translation. *Mol. Cell. Biol.* 20, 8635-8642.

Nakada, T., Hoshijima, K., Esaki, M., Nagayoshi, S., Kawakami, K. and Hirose, S.

(2007a). Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. *Am. J. Physiol.* 293, R1743-1753.

- Nakada, T., Westhoff, C. M., Kato, A. and Hirose, S. (2007b). Ammonia secretion from fish gill depends on a set of Rh proteins. *FASEB J.* 21, 1067-1074.
- Nawata, C. M., Hung, C. C. Y., Tsui, T. K. N., Wilson, J. M., Wright, P. A. and Wood, C. M. (2007). Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H⁺-ATPase involvement. *Physiol. Genomics* 31, 463-474.
- Perry, S. F. (1986). Carbon dioxide excretion in fishes. Can. J. Zool. 64, 565-572.
- Perry, S. F. and Gilmour, K. M. (2002). Sensing and transfer of respiratory gases at the fish gill. J. Exp. Zool. 293, 249-263.
- **Perry, S. F. and Gilmour, K. M.** (2006). Acid-base balance and CO₂ excretion in fish: unanswered questions and emerging models. *Resp. Physiol. Neuro.* 154, 199-215.
- **Perry, S. F., Beyers, M. L., and Johnson, D. A.** (2000). Cloning and molecular characterization of the trout (*Oncorhynchus mykiss*) vacuolar H⁺-ATPase B subunit. *J. Exp. Biol.* 203, 459-470.
- Pesole, G., Mignone, F., Gissi, C., Grillo, G., Licciulli, F. and Liuni, S. (2001). Structural and functional features of eukaryotic mRNA untranslated regions. *Gene* 276, 73-81.
- Planelles, G. (2007). Ammonium homeostasis and human Rhesus glycoproteins. *Nephron Physiol.* 105, 11-17.
- Ripoche, P., Goossens, D., Devuyst, O., Gane, P., Colin, Y., Verkman, A. S. and Cartron, J. P. (2006). Role of RhAG and AQP1 in NH₃ and CO₂ gas transport in red cell ghosts: a stopped-flow analysis. *Transfus. Clin. Biol.* 13, 117-122.
- Salama, A., Morgan, I. J. and Wood, C. M. (1999). The linkage between sodium uptake and ammonia excretion in rainbow trout - kinetic analysis, the effects of (NH₄)₂ SO₄ and NH₄ HCO₃ infusion, and the influence of gill boundary layer pH. J. Exp. Biol. 202, 697-709.
- Soupene, E., Inwood, W. and Kustu, S. (2004). Lack of the Rhesus protein Rh1 impairs growth of the green alga *Chlamydomonas reinhardtii* at high CO₂. Proc. Natl. Acad. Sci. USA 101, 7787-7792.

- Soupene, E., King, N., Field, E. Liu, P., Niyogi, K. K., Huang, C. H. and Kustu, S. (2002). Rhesus expression in a green alga is regulated by CO₂. *Proc. Natl. Acad. Sci. USA* 99, 7769-7773.
- **Thompson, J. D., Higgins, D. G. and Gibson, T. J.** (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* 22, 4673-4680.
- Tipsmark, C. K., Baltzegar, D. A., Ozden, O., Grubb, B. J. and Borski, R. J. (2008). Salinity regulates mRNA and protein expression in the teleost gill. *Am. J. Physiol.* 294, R1004-1014.
- Van Kim, C. L., Colin, Y. and Cartron, J. P. (2006). Rh proteins: key structural and functional components of the red cell membrane. *Blood Rev.* 20, 93-110.
- Verdouw, H., van Echteld, C. J. A. and Dekkers, E. M. J. (1978). Ammonia determinations based on indophenol formation with sodium salicylate. *Water Res.* 12, 399-402.
- Weidinger, K., Neuhauser, B., Gilch, S., Ludewig, U., Meyer, O. and Schmidt, I. (2007). Functional and physiological evidence for a Rhesus-type ammonia transporter in *Nitrosomonas europaea*. *FEMS Microbiol. Lett.* 273, 260-267.
- Weihrauch, D. (2006). Active ammonia absorption in the midgut of the Tobacco hornworm *Manduca sexta* L: transport studies and the mRNA expression analysis of a Rhesus-like ammonia transporter. *Insect Bio. Mol. Biol.* 36, 808-821.
- Weiner, I. D. and Hamm, L. L. (2007). Molecular mechanisms of renal ammonia transport. *Annu. Rev. Physiol.* 69, 317-340.
- Wilson, R. W. and Taylor, E. W. (1992). Transbranchial ammonia gradients and acidbase responses to high external ammonia concentration in rainbow trout (*Oncorhynchus mykiss*) acclimated to different salinities. J. Exp. Biol. 166, 95-112.
- Wilson, R. W., Wright, P. M., Munger, S. and Wood, C. M. (1994). Ammonia excretion in fresh water rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: lack of evidence for Na⁺/NH₄⁺ exchange. J. *Exp. Biol.* 191, 37-58.
- Wolf, K. (1963). Physiological salines for freshwater teleosts. *Prog. Fish Cult.* 25, 135-140.

- Wood, C. M. (1993). Ammonia and urea metabolism and excretion. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 379-425. Boca Raton: CRC Press.
- Wood, C. M. and LeMoigne, J. (1991). Intracellular acid-base responses to environmental hyperoxia and normoxic recovery in rainbow trout. *Resp. Physiol.* 86, 91-113.
- Wood, C. M., Warne, J. M., Wang, Y., McDonald, M. D., Balment, R. J., Laurent,
 P. and Walsh, P. (2001). Do circulating plasma AVT and/or cortisol levels control pulsatile urea excretion in the gulf toadfish (*Opsanus beta*)? Comp. Biochem. Physiol. 129A. 859-872.
- Wright, P. A., Heming, T. and Randall, D. J. (1986). Downstream pH changes in water flowing over the gills of rainbow trout. *J. Exp. Biol.* 126, 499-512.
- Wright, P. A., Randall, D. J. and Perry, S. F. (1989). Fish gill boundary layer: a site of linkage between carbon dioxide and ammonia excretion. J. Comp. Physiol. 158, 627-635.
- Wright, P. A., Randall, D. J. and Wood, C. M. (1988a). The distribution of ammonia and H⁺ between tissue compartment in lemon sole (*Parophrys vetulus*) at rest, during hypercapnia and following exercise. J. Exp. Biol. 136, 149-175.
- Wright, P. A., Wood, C. M. and Randall, D. J. (1988b). An *in vitro* and *in vivo* study of the distribution of ammonia between plasma and red cells of rainbow trout (*Salmo gairdneri*). J. Exp. Biol. 134, 423-428.

CHAPTER 4

mRNA ANALYSIS OF THE PHYSIOLOGICAL RESPONSES TO AMMONIA INFUSION IN RAINBOW TROUT

108

4.1 ABSTRACT

We recently reported that tissue levels of Rhesus (Rh) mRNA in rainbow trout changed in response to high external ammonia (HEA). To investigate whether or not these changes could be due to elevated plasma ammonia levels, we infused rainbow trout for 12 hours with 140 mmol L⁻¹ NH₄HCO₃, or with 140 mmol L⁻¹ NaCl as a control for the effects of infusion. We also analyzed the effects of dorsal aortic catheterization alone, without infusion. Catheterization alone resulted in an elevated ammonia excretion rate, a downregulation of Rhbg mRNA in the brain, and mRNA upregulations of Rhbg, Rhcg1, and Rhcg2 in the gill, Rhbg and Rhcg1 in the skin, and Rhag in the erythrocytes. In NH₄HCO₃-infused fish, plasma cortisol peaked at 6 hours, erythrocyte Rhag mRNA was downregulated, gill Rhbg, Rhcg1, and Rhcg2 mRNA were upregulated, and skin Rhbg mRNA was also upregulated. NaCl infusion resulted in elevated plasma ammonia and ammonia excretion rates as well as gill mRNA upregulations of Rhbg, carbonic anhydrase, NHE2, H⁺-ATPase, Na⁺/K⁺-ATPase. Taken together, the results indicated that infusion of NH₄HCO₃ induced a similar pattern of Rh transcript changes as that seen when fish were exposed to HEA. Secondly, catheterization alone, as well as isotonic NaCl infusion, significantly altered mRNA levels, highlighting the necessity for careful data interpretation and inclusion of appropriate controls for gene expression studies in fish that have undergone anaesthesia/surgery and infusion procedures. Lastly, elevated plasma ammonia and cortisol may both be involved in the signaling mechanism for Rh gene regulation.

4.2 INTRODUCTION

Like most freshwater teleost fish, rainbow trout are ammoniotelic, excreting the majority of their nitrogenous waste in the form of ammonia across the gills (Wood, 1993). Elevated levels of environmental ammonia due to pollution in natural waters and those associated with aquacultural systems can lead to elevated internal levels of ammonia which can have detrimental effects on the health of fish (Eddy, 2005; Mommsen and Walsh, 1992; Randall and Tsui, 2002). Therefore the understanding of ammonia handling at the gill has been the subject of much research over the years and the general consensus is that ammonia gas (NH₃) is excreted across the gills by simple diffusion aided by an acidified gill-water boundary layer that traps NH₃ to NH₄⁺, in freshwater fish (Wilkie, 2002). This model of ammonia excretion however, is now being modified by the identification of the newest members of the ammonia transporter superfamily, the Rhesus (Rh) proteins. In mammals, Rhag is erythroid-specific whereas Rhbg and Rhcg are non-erythroid proteins present in renal and extra-renal tissues (Huang and Liu, 2001). These Rh proteins appear to function as ammonia channels (likely for NH_3 , but possibly for NH_4^+) with the additional, albeit controversial function of Rhag as a CO₂ channel (see Cartron, 2008).

Rh proteins have also been identified in several fish species (Huang and Peng, 2005; Hung *et al.*, 2007; Nakada *et al.*, 2007a, b; Nawata *et al.*, 2007). In two species, the mangrove killifish and the rainbow trout, exposures to high external ammonia (HEA) concentrations have been associated with changes in tissue levels of Rh mRNA, notably the upregulation of Rhcg2 mRNA in the gill (Hung *et al.*, 2007; Nawata *et al.*, 2007). In

the rainbow trout, this upregulation of Rhcg2 coincides with the resumption and enhancement of ammonia excretion that was initially blocked by the reversed plasma to water ammonia gradient created by the HEA treatment (Nawata *et al.*, 2007).

In mammals, an increase in ammonia metabolism induced by chronic metabolic acidosis or reduced renal mass is associated with an increase in Rhcg protein expression (Kim et al., 2007; Seshadri et al., 2006) and Rhcg knockdown mice have impaired ammonia excretion (Biver et al., 2008). Therefore a clear link has been established between ammonia and Rh proteins in both fish and mammals. Presently however, little is Studies with known about the signaling pathway regulating Rh gene expression. rainbow trout suggest that the observed changes in tissue Rh mRNA levels are directly linked to elevated plasma ammonia levels after exposure to HEA, or when ammonia excretion is blocked at the gill after exposure to Hepes buffer (Nawata et al., 2007; Nawata and Wood, 2008). We wanted to further examine this relationship between plasma ammonia levels and Rh mRNA expression, this time by elevating the plasma ammonia concentration in rainbow trout via infusion of 140 mmol L⁻¹ NH₄HCO₃ (approximately isotonic to the blood plasma). NH₄HCO₃ infusion avoids the well-known acidosis-inducing effects of ammonium salts containing strong anions (e.g. NH₄Cl or (NH₄)₂SO₄ - Cameron and Heisler, 1983; McDonald and Prior, 1988; Salama et al., 1999; Wilson et al., 1994). Comparable infusion with 140 mmol L⁻¹ NaCl served as a control for the effects of isotonic volume loading. These procedures however require prior anaesthesia and the surgical placement of a dorsal aortic catheter. Currently nothing is

known about the potential impact that catheterization or infusion may have on gene expression.

The use of chronic indwelling dorsal aortic catheters has greatly facilitated physiological studies in fish, allowing for repeated blood sampling and infusion of substances with minimal disturbance to the fish (e.g. Goss and Wood, 1990; McGeer and Eddy, 1998; Salama *et al.*, 1999; Wilson *et al.*, 1994; Yesaki and Iwama, 1992). Although it has been well recognized that anaesthesia and surgical procedures impose stress on fish (Houston, 1990; Soivio and Nynholm, 1975), the fact that this stress may bias the results in physiological studies has been largely overlooked. Indeed it is commonly assumed that once the plasma cortisol in surgically manipulated fish has reached basal levels after a period of recuperation, the fish are physiologically stable (Tashjian and Hung, 2005). At the molecular level however, the picture may be very different. In fact it has been shown in mammalian studies, that surgical manipulations can have an impact on gene expression (e.g. Gerloff *et al.*, 1999; Lin *et al.*, 2006; Zhao *et al.*, 2004) and therefore incorporation of appropriate controls is necessary in gene expression studies involving surgical procedures.

The objectives of this study were two-fold. The first was to determine whether or not elevated internal plasma ammonia concentrations achieved by ammonia infusion could elicit a similar pattern of mRNA expression in trout tissues as that when trout were exposed to HEA as previously reported (Nawata *et al.*, 2007). The second goal was to determine whether or not surgical procedures or infusion alone could influence mRNA expression levels. An uncatheterized (no-surgery) control and a catheterized control were

112

included to determine the effects of the surgical procedures, and a NaCl-infusion control was employed to examine the effects of infusion itself. The tissue mRNA expression levels of Rhag, Rhbg, Rhcg1, and Rhcg2, as well as carbonic anhydrase (CA2), H⁺-ATPase, Na⁺/H⁺ exchanger 2 (NHE2), and Na⁺/K⁺-ATPase (NKA) were analyzed. Ammonia excretion rates, and levels of plasma ammonia and cortisol were also evaluated.

4.3 MATERIALS AND METHODS

4.3.1 Experimental animals

Rainbow trout, *Oncorhynchus mykiss* (Walbaum), weighing 176-265g, were acquired from Humber Springs Trout Hatchery, Ontario, Canada and held in dechlorinated Hamilton tapwater ($[Na^+]=0.6$ mequivL⁻¹, $[Cl^-]=1.8$ mequiv L⁻¹, $[Ca^{++}]=0.8$ mequiv L⁻¹, $[Mg^{++}]=0.3$ mequiv L⁻¹, $[K^+]=0.05$ mequiv L⁻¹; titration alkalinity 2.1 mequiv L⁻¹; pH ~8.0; hardness ~140 mg L⁻¹ as CaCO₃ equivalents; temperature 12-16° C). Feeding was suspended one week prior to experimentation. Fish were fitted with dorsal aortic catheters under anaesthesia (0.07 g L⁻¹ neutralized MS222, Sigma; St. Louis, MO), transferred to individual darkened boxes with aerated, flowing tap water (15± 0.5°C) and allowed to recover for a minimum of 48 hours before experimentation.

4.3.2 Infusions and analyses

Fish were infused over a period of 12 hours with 140 mmol L^{-1} NH₄HCO₃ (pH 7.8) or as an infusion control, separate fish were infused with 140 mmol L^{-1} NaCl (pH

7.8). The infusion rate was set at the lowest allowable with a polystaltic pump (Buchler Instruments, Inc., Kansas, MO), resulting in an average rate of 6.18 ± 0.34 ml kg⁻¹ h⁻¹. A set of catheterized fish were not infused and served as a catheterized control and an additional set of fish that did not undergo surgery served as an additional (no-surgery) control.

Ammonia excretion rates were measured in both sets of control fish as well as in the infused fish after 3, 6, 9, and 12 hours of infusion. During this time, the boxes were closed off to water flow for one hour and the box water volume was set to 4 L. A 10-mL water sample was removed at the beginning and end of each flux period, frozen at -20° C and later assayed for total ammonia (T_{Amm}) in triplicate, using a modified salicylatehypochlorite method (Verdouw *et al.* 1978). Net flux rates of total ammonia (J_{Amm} , µmol kg⁻¹h⁻¹) were calculated as:

$$J_{\text{Amm}} = (T_{\text{Amm}i} - T_{\text{Amm}f}) \times V/(t \times M)$$

where *i* and *f* are respectively, initial and final concentration (μ mol L⁻¹), *V* is box water volume (L), *t* is the total flux time (h) and *M* is fish mass (kg). A negative J_{Amm} indicates a net excretion of ammonia to the water.

Blood samples (400 μ l) were collected via the dorsal aortic catheter at 3, 6, 9, and 12 hours. The infusion line was disconnected, cleared with Cortland saline (Wolf, 1963) and blood was drawn into the catheter three times prior to withdrawing the sample in order to minimize contamination with infusate. In the case of uncatheterized fish, blood samples were removed via caudal puncture after terminal, rapid anaesthetization with neutralized MS222 (0.1g L⁻¹). The durations of these caudal collections were always

under 30 s in order to minimize blood chemistry alterations due to anaesthetization and handling stress. Samples were immediately centrifuged to separate plasma from erythrocytes (2 min, 20,000 g). Plasma samples were frozen in liquid nitrogen and stored at -70° C and later analyzed in duplicate for T_{Amm} by enzyme assay (Raichem, Hemagen Diagnostics, Inc., San Diego, CA) and for cortisol by radioimmunoassay (Cortisol ¹²⁵I RIA Kit, MP Biomedicals, Orangeburg, NY).

4.3.3 Tissue sampling

Tissues were extracted from fish at the end of 3, 6, and 12 hours of NH₄HCO₃ infusion, and at the end of 3 and 12 hours of NaCl infusion. After blood samples were removed, fish were terminally anaesthetized with neutralized MS222. Although the contribution of erythroid Rhbg mRNA to the overall Rhbg mRNA expression in trout tissues is extremely low (Nawata *et al.*, 2007), the same protocol that was used in this previous study was maintained; tissues were perfused with ice-cold, heparinized Cortland saline via the *bulbus arteriosus* in order to minimize erythrocyte contamination. Brain, gill and skin were quickly excised and flash frozen together with the erythrocytes (separated from plasma as described above), in liquid nitrogen and stored at –70°C. The same procedure was carried out for both sets of control fish. For the two infused groups, gill samples were analyzed at all sample times while erythrocytes, brain, and skin samples were analyzed at the 12-hour time point. Similarly, only 12-hour samples were analyzed in both control groups.

4.3.4 RNA extraction and cDNA synthesis

Total RNA was extracted from erythrocytes and tissues with Trizol (Invitrogen, Burlington, ON), quantified with a Nanodrop spectrophotometer (ND-1000; Nanodrop Technologies, Wilmington, DE), and electrophoresed on 1% agarose gels stained with ethidium bromide to verify integrity. First strand cDNA was synthesized from 1 μ g total RNA (DNaseI-treated, Invitrogen) with an oligo(dT₁₇) primer and Superscript II reverse transcriptase (Invitrogen).

4.3.5 Real-time quantitative PCR (qPCR)

Rh mRNA expression was assessed in the brain, gill, skin, and erythrocytes and carbonic anhydrase (CA2; cytoplasmic), H⁺-ATPase (v-type, B-subunit), NHE2, and Na⁺/K⁺-ATPase (NKA; α 1a-subunit) mRNA expression was analyzed in the gill using the cDNA described above and primers previously published (Nawata *et al.*, 2007). Reactions (20 µl) containing 4 µl of cDNA, 8 pmoles of each primer, and 10 µl of RT² Real-Time SYBR Green/ROX PCR Master Mix (SuperArray, Bioscience Corp, Frederick, MD) were performed at 95°C for 2 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. Production of a single product was verified by melt-curve analysis and gel electrophoresis. No-template controls and non-reversed-transcribed controls were run in parallel. Values were extrapolated from standard curves generated by serial dilution of one randomly selected control sample. The expression of three reference genes, beta-actin, elongation factor (EF1- α) and 18S proved unstable across treatments,

therefore data were normalized against ng total RNA, an acceptable normalization method (Bustin, 2000, 2002) as described previously (Nawata and Wood, 2008).

4.3.6 Statistical analysis

Data are presented as means \pm s.e.m. (*n*, number of fish). The effects of NaCl and NH₄HCO₃ infusions were analyzed by one-way analysis of variance (ANOVA), followed by Fisher's Least Significant Difference post-hoc test. A paired t-test was used to compare the two control sets. Significance was set at α =0.05.

4.4 RESULTS

4.4.1 Ammonia and cortisol responses

Catheterization (which involved anaesthesia and surgery) caused a significant 2.5fold elevation in ammonia excretion relative to the no-surgery control (Fig. 4.1). Infusion of 140 mmoL L⁻¹ NH₄HCO₃ resulted in an average ammonia-loading rate of 918 μ mol kg⁻¹ h⁻¹ and the ammonia excretion rate of NH₄HCO₃-infused fish was elevated at all time points relative to both controls. By 12 hours the ammonia excretion rate exceeded the ammonia loading rate and was elevated 7-fold over the no-surgery control rate and 3-fold over that of the catheterized control (Fig. 4.1). The ammonia excretion rate in NaClinfused fish was elevated by an average of 3.5-fold above the no-surgery control level at 3, 6 and 12 hours, but the rates were not significantly different from those of the catheterized control (Fig. 4.1). Plasma ammonia levels were not significantly different in the two control groups of fish, but were significantly elevated in the NH₄HCO₃-infused fish at all time points, reaching a level 29-fold above that of the controls by 12 hours (Fig. 4.2). The NaClinfused fish also had increased plasma ammonia levels after 6 hours and were 1.7-fold higher than the controls by 12 hours (Fig. 4.2). Plasma cortisol levels were not significantly different between the two control groups but were elevated 6.8-fold over both control levels after 6 hours of NH₄HCO₃ infusion (Fig. 4.3). However, this effect disappeared by 12 hours of infusion. Plasma cortisol concentrations were not significantly altered by NaCl infusion.

4.4.2 Expression of Rh genes

In the gills, the mRNA expression levels of Rhbg, Rhcg1 and Rhcg2 were significantly increased in the catheterized control when compared to the no-surgery control by 4-, 2-, and 4.7-fold, respectively. Similarly, gill mRNA levels of Rhbg, Rhcg1, and Rhcg2 in the NH₄HCO₃-infused fish were all elevated at 6 hours and by 12 hours, Rhbg was still 7-fold higher while Rhcg2 was 12-fold higher than the levels in the no-surgery controls. At 3 hours, gill Rhbg mRNA in the NaCl-infused fish was 10.5-fold higher than that in the no-surgery controls (Fig. 4.4). There were no significant differences between the catheterized control fish and either set of infused fish.

Erythrocyte Rhag mRNA was significantly upregulated in the catheterized controls by 2-fold relative to the no-surgery control (Fig. 4.5). After 12 hours of NH₄HCO₃ infusion, Rhag was significantly down-regulated by 7-fold when compared to

levels in the catheterized control fish, but there was no significant difference when compared to the no-surgery control levels. Similarly, no significant change was seen in Rhag expression in the erythrocytes after 12 hours of NaCl infusion when compared to that in both controls (Fig. 4.5). Rhcg1 and Rhcg2 are not expressed in trout erythrocytes and Rhbg is very weakly expressed (Nawata *et al.*, 2007) therefore, these mRNAs were not analyzed.

Brain levels of Rhbg mRNA were about 50% lower in the catheterized control fish when compared to no-surgery controls, but there were no significant changes after 12 hours of either NaCl or NH₄HCO₃ infusion when compared to either of the control groups (Fig. 4.6*A*). In the skin, both Rhbg and Rhcg1 mRNA were significantly elevated in the catheterized control when compared to the no-surgery control by 3- and 2-fold respectively (Fig. 4.6*B*). Rhbg mRNA was elevated 5-fold in the skin of NH₄HCO₃-infused fish at 12 hours compared to the no-surgery controls. However, there were no significant differences between the catheterized control fish and either group of infused fish (Fig. 4.6*B*).

4.4.3 Expression of other transport genes in the gills

Catheterization alone resulted in a 10-fold elevation of gill CA2 mRNA and 3 hours of NaCl infusion elevated the level to almost 28-fold over the no-surgery control (Fig. 4.7). Catheterization also caused a 4-fold increase in gill NHE2 mRNA expression when compared to the no-surgery control (Fig. 4.7). At 3 hours however, NHE2 mRNA expression in the gills of the NaCl-infused fish was significantly higher than both

controls while in the NH₄HCO₃ -infused fish, the expression was elevated 5-fold over the no-surgery control at 6 and 12 hours. Gill H⁺-ATPase mRNA expression was similar in both controls but 3 hours of NaCl infusion resulted in a 3-fold increase in H⁺-ATPase mRNA over that in the controls (Fig. 4.7). NH₄HCO₃ infusion also resulted in 3.5-fold higher levels of H⁺-ATPase mRNA in the gills compared to that in the no-surgery controls at 3 and 12 hours. NKA mRNA levels in the gill were similar in the no-surgery and catheterized controls (Fig. 4.7). However, 3 hours of NaCl-infusion resulted in a 6-fold increase and 12 hours of NH₄HCO₃ infusion caused a 4.5-fold increase in NKA mRNA when compared to that in both control sets of fish.

4.5 DISCUSSION

4.5.1 Overview

The recent identification of the Rh glycoprotein ammonia transporters has fueled several studies related to ammonia transport in fish (Hung *et al.*, 2007, 2008; Nakada *et al.*, 2007a,b; Nawata *et al.*, 2007; Nawata and Wood, 2008; Shih *et al.*, 2008; Tsui *et al.*, 2009). In two of those studies (Nawata *et al.*, 2007; Nawata and Wood, 2008) an association was made between high plasma ammonia levels and changes in Rh mRNA expression in rainbow trout tissues. These high plasma ammonia levels were achieved after trout were exposed to high external ammonia (HEA) or after the acidified gill boundary layer was eliminated with Hepes treatment. In the present study, plasma ammonia levels were elevated in rainbow trout via infusion with NH₄HCO₃. This ammonium salt was chosen to avoid acidosis, although we have previously shown that

acidosis produced under hypercapnic conditions had no effect on Rh mRNA expression (Nawata and Wood, 2008). This allowed us to correlate changes in Rh mRNA levels to changes in plasma ammonia levels and at the same time, assess the relative impact of surgical procedures (includes anaesthesia) and infusion on the results. The results clearly show that elevated plasma ammonia levels as well as surgical procedures and infusion have marked effects on physiology and on the mRNA expression levels of Rh and other genes. However, in general, internal ammonia loading induced a similar pattern of Rh transcript changes as that seen when fish were exposed to HEA.

4.5.2 The influence of surgical procedures and infusion

Catheterization of the dorsal aorta under anaesthesia, followed by recovery, so as facilitate serial blood sampling and infusion of substances for physiological studies in fish has a long history (see Tashjian and Hung, 2005), but the possible influence of this surgical procedure on gene expression has not been explored. From the present study, it was clear that catheterization alone (followed by 48h recovery), without infusion, resulted in many changes in mRNA expression levels when compared to the no-surgery control. Dramatic differences were seen in Rh mRNA levels in the gill; the mRNA expressions of all three Rh genes were significantly elevated (Fig. 4.4). These catheterized control fish also had an elevated ammonia excretion rate (Fig. 4.1), suggesting that endogenous ammonia production was high. This increased ammonia excretion, possibly facilitated by an upregulation of Rh ammonia transporters, likely explains the unaltered control levels of plasma ammonia (Fig. 4.2).

121

McMaster - Biology

What is not clear however, is which component of the surgical procedure may have affected mRNA levels. Anaesthetics are known to alter the blood chemistry of fish (Iwama *et al.*, 1989), so it is plausible that they could also elicit changes in mRNA levels. Although it is possible to study the effects of anaesthesia alone on mRNA expression, it is not possible to isolate the influence of the surgery itself since anaesthesia is a requisite part of the surgical procedure. Therefore, the effects of the surgical procedures could have been due to the anaesthesia, the surgery, or a combination of both. If indeed anaesthesia has an effect on mRNA expression levels, then timing could make a difference. A 48-h recovery period was used in the present study. In future studies, it will be worthwhile to evaluate the effects of anaesthesia alone on mRNA expression levels and plasma parameters, and also to investigate whether or not there are any correlations between the time elapsed following anaesthesia/surgery and changes in these parameters.

Studies that utilize infusion to examine physiological parameters typically incorporate isotonic NaCl infusion as a control. This causes expansion of the extracellular fluid volume and diuresis, but generally has little effect on plasma Na and Cl levels or acid-base status (e.g. Curtis and Wood, 1992; Goss and Wood, 1990; Vermette and Perry, 1987a,b,c). The infusion rate employed in the present study was 1.5-2-fold higher than that used in these earlier studies and it was apparent from our data that this NaCl infusion induced changes in ammonia metabolism and gene expression. Both plasma T_{Amm} and the ammonia excretion rates were elevated at most time points (Figs 4.1, 4.2). Like the catheterized control, this suggests that endogenous ammonia production was high, but the elevated plasma ammonia levels during NaCl infusion additionally indicate that the rate
of ammonia excretion was insufficient to keep up with the production of endogenous ammonia. Furthermore, the elevation in plasma ammonia was apparently not of sufficient magnitude to elicit the same changes in Rh mRNA expression as seen in the NH₄HCO₃-infused fish (Fig. 4.4). The only change noted was the upregulation of Rhbg mRNA at 3 hours and this mRNA was also upregulated in the catheterized controls. This upregulation appears to be unrelated to plasma ammonia since a similar upregulation did not occur in the NH₄HCO₃-infused fish (Fig. 4.2). As with catheterization alone, NaCl infusion did not significantly elevate cortisol levels (Fig. 4.3).

Previous studies on trout which used lower rates of isotonic NaCl infusion, showed negligible disturbances of blood acid-base status or plasma NaCl levels (Curtis and Wood, 1992; Goss and Wood, 1990; Vermette and Perry, 1987a,b,). However, in humans at least, metabolic acidosis can result from high rates of NaCl infusion because the anion gap is reduced - i.e. HCO₃⁻ concentration is reduced (Constable, 2003; Scheingraber *et al.*, 1999). In fact when we examined other gill proteins, the upregulations at 3 hours of NKA and H⁺-ATPase (Fig. 4.7) were clearly related to the NaCl-infusion itself and not to the surgical procedure since there were no differences between the two control levels of these mRNAs. Indeed Perry *et al.* (2006) reported that NKA mRNA and protein expression were upregulated in trout that were subjected to high NaCl loading through the diet. Similarly, although NHE2 mRNA was upregulated in the catheterized control, it was further upregulated over this control at 3 hours after NaCl-infusion, suggesting that NaCl-infusion itself stimulated the upregulation of NHE2 mRNA (Fig.4.7). There was also an upregulation of CA2 at 3 hours, but this mRNA was

also upregulated in the catheterized control, suggesting that the change may be due in part to the surgical procedures (including anaesthesia). CA2, NHE2, and H⁺-ATPase have all been implicated in the branchial acid secretion mechanism of fish at the transcript level (Edwards *et al.*, 2005; Georgalis *et al.*, 2006; Perry *et al.*, 2000). Therefore, the upregulations of CA2, NHE2, and H⁺-ATPase mRNA in the NaCl-infused fish may be indicative of an acidosis resulting from the high rate of infusion and reduced anion gap, and/or a response to extracellular volume expansion. Overall, we conclude that despite the elevated plasma ammonia level and ammonia excretion rate, isotonic NaCl infusion resulted in changes in mRNA levels that reflected responses to the sideeffects of infusion itself, rather than specific responses to ammonia.

4.5.3 Responses to internal ammonia loading

In trout, natural elevations in plasma ammonia that occur post-prandially are approximately 3-fold higher than the control level (Bucking and Wood, 2008; Wicks and Randall, 2002a,b). Similar elevations occur after strenuous exercise (Mommsen and Hochachka, 1988; Wood, 1988). Our present study was designed to elevate plasma ammonia to levels 10-fold or greater above the control value, comparable to levels attained previously with HEA exposure (Nawata *et al.*, 2007; Nawata and Wood, 2008). This was done in order to determine whether or not the changes in Rh mRNA expression were in response to high plasma ammonia, rather than to externally elevated ammonia. Although these values were unnaturally elevated and approaching levels thought to be toxic (Wilkie, 2002), no mortalities occurred. Indeed it has been reported that trout

McMaster - Biology

survived a seven-day external exposure to 2000 μ mol L⁻¹ NH₄Cl with circulating plasma ammonia levels elevated 10-fold above that of the control (Tsui *et al.*, 2009).

NH₄HCO₃ infusion resulted in the upregulations of Rhbg and Rhcg2 mRNA in the gill (Fig. 4.4) similar to HEA-exposure which resulted in mRNA upregulations of gill Rhcg2 after 12 hours, and Rhbg in the gill pavement cells after 48 hours as demonstrated previously (Nawata *et al.*, 2007). As with the HEA-exposed fish, these upregulations appeared in conjunction with enhanced ammonia excretion (Fig. 4.1). Unlike the HEAexposed fish however, expression of Rhcg1 mRNA was also elevated in the gill (Fig. 4.4). It is possible that an early peak in Rhcg1 mRNA also occurred under HEA conditions but was missed during the time course of the study, or it could be a response that was specific to NH₄HCO₃ infusion. Since it only occurs after infusion of NH₄HCO₃ and not after infusion of NaCl, this suggests that it was a response related to high plasma ammonia and not the infusion itself.

In HEA-exposed trout, the mRNA expression and activity of CA2 were decreased while the NHE2 mRNA expression remained unchanged (Nawata *et al.*, 2007). In the current study, the mRNA expression levels of CA2 in NH₄HCO₃-infused fish remained unchanged and the NHE2 mRNA levels were significantly elevated at 6 and 12 hours, relative to the no-surgery control (Fig. 4.7). Both of these mRNAs were elevated in the catheterized controls and therefore true levels of CA2 mRNA could have been masked by the upregulation caused by the surgical procedures (including anaesthesia). Similarly, elevations of NHE2 may also be related to these surgical procedures and not to the infusion. It is possible however, that at very high concentrations of plasma ammonia,

NHE2 is recruited into the ammonia excretion mechanism. A recent in vitro study showed that NHE2 mRNA levels increased after cultured rainbow trout gill epithelia were pre-exposed to 2 mM NH₄Cl and 1000 ng ml⁻¹ cortisol (Tsui *et al.*, 2009). In the present study, the plasma ammonia was very close to 2 mM by 12 hours of NH₄HCO₃ infusion, but in the HEA study, plasma ammonia levels only reached about half of this value (Nawata *et al.*, 2007). Additionally, Ivanis *et al.* (2008) reported that NHE2 mRNA levels in rainbow trout increased in the presence of cortisol. Elevated cortisol levels were observed at 6 hours of NH₄HCO₃ infusion in the present study (Fig. 4.3) and therefore could have also contributed to the upregulation of NHE2 mRNA.

The early elevation of H^+ -ATPase mRNA at 3 hours, which was also seen in the NaCl-infused fish, is likely a consequence of the infusion and not the surgical procedures, since levels in the two control sets were not significantly different from each other (Fig. 4.7). The later upregulation of H^+ -ATPase mRNA at 12 hours however, does correspond with the elevation of H^+ -ATPase activity and mRNA level seen after 12 and 48 hours of HEA exposure (Nawata *et al.*, 2007), thus reinforcing the importance of this transporter in the acid-trapping model of ammonia excretion. Finally, the upregulation of NKA mRNA at 12 hours (Fig. 4.7) corresponds with the upregulation seen in the gill pavement cells of HEA-exposed trout after 48 hours of exposure (Nawata *et al.*, 2007).

Previously, it was shown that both Rhbg and Rhcg1 were downregulated in the brain after 48 hours of HEA (Nawata *et al.*, 2007). In the present study, the surgical procedures (including anaesthesia) resulted in a significant downregulation of Rhbg in the brain, but infusions of either NaCl or NH_4HCO_3 did not lead to significant changes

126

(Fig. 4.6*A*). The fact that a downregulation of Rhbg occurred in the catheterized controls suggests that this change may be related to other factors, rather than high ammonia levels. A finer time-line analysis would clarify this.

A recent report by Shih *et al.* (2008) confirmed the involvement of skin Rh proteins in ammonia excretion using Rhcg1 knockdown zebrafish larvae. In the present study there was an upregulation of Rhbg at 12 hours in the skin of NH₄HCO₃-infused fish, and an upregulation of both Rhbg and Rhcg1 in the skin of the catheterized controls (Fig. 4.6*B*). These skin Rh expression changes were less pronounced than those during HEA exposure (Nawata *et al.*, 2007) or Hepes exposure (Nawata and Wood, 2008) in trout, or HEA exposure in the mangrove killifish (Hung *et al.*, 2007) where Rhcg1 and/or Rhcg2 were also upregulated. This suggests that Rh gene responses in the skin may differ with treatment.

The decrease in Rhag mRNA in the erythrocytes of NH₄HCO₃-infused fish (Fig. 4.5) was also observed previously in erythrocytes of HEA- and Hepes-exposed fish (Nawata *et al.*, 2007; Nawata and Wood, 2008). Since the Rhag mRNA levels were actually upregulated in the catheterized controls, the downregulation that occurred after NH₄HCO₃ infusion was likely in direct response to the elevated plasma ammonia and not related to either the surgical procedures or infusion alone. We speculate that this may help protect erythrocyte respiratory functions against deleterious effects of high plasma ammonia, an area worthy of future study. The significance of the downregulation of Rhag when plasma ammonia levels are high is not clear but the fact that it was upregulated after the surgical procedure along with the other Rh mRNAs in the gill

suggests that is responding to plasma ammonia and that there may be a negative feedback response when ammonia levels reach a certain threshold.

4.5.4 Does cortisol play a role in Rh gene expression and ammonia excretion?

Cortisol treatment has been shown to upregulate the transcript levels of a number of genes in fish including the aforementioned NHE2 (Ivanis *et al.*, 2008) and several genes involved in osmoregulation (Kiilerich *et al.*, 2007; McCormick *et al.*, 2008) but a clear relationship between cortisol levels and Rh mRNA expression was not established in earlier *in vivo* experiments on rainbow trout (Nawata and Wood, 2008). However in *in vitro* experiments with cultured trout gill epithelia pre-exposed to a combination of ammonia and cortisol, there was an upregulation of both Rhbg and Rhcg2 mRNA, while treatment with ammonia or cortisol alone failed to produce a similar effect (Tsui *et al.*, 2009). This suggests that cortisol and elevated ammonia act synergistically, and indeed, there were synergistic effects on the ammonia permeability of the cultured epithelium. Whether or not cortisol or ammonia can independently induce changes in Rh transcript levels *in vivo* remains to be determined.

The time course of NH_4HCO_3 infusion used in the current study revealed a transient increase of cortisol at 6 hours (Fig. 4.3) and this cortisol surge may have contributed to the subsequent increase in Rh mRNA seen at 6 and 12 hours (Fig. 4.4). In the gulf toadfish, a similar transient surge in cortisol was found to be responsible for an increase in hepatic glutamine synthetase activity, mRNA, and protein (Hopkins *et al.*, 1995; Kong *et al.*, 2000). The transitory nature of cortisol increases could explain why a

128

clear correlation between elevated levels of plasma ammonia (or Rh mRNA) and cortisol were not detected earlier (Nawata and Wood, 2008) despite the apparent linear relationship between plasma ammonia and cortisol levels observed by Ortega et al. (2005). Also, the fact that chronically elevated levels of cortisol do not increase plasma ammonia or ammonia excretion rates (DeBoeck *et al.*, 2001; Hopkins *et al.*, 1995; McDonald and Wood, 2004), whereas acute exposure by injection does (Chan and Woo, 1978), reinforces the idea that pulses in cortisol are stimulatory in fish. Indeed, chronically elevated cortisol levels may have an autoregulatory effect, decreasing the affinity and number of glucocorticoid receptors (Shrimpton and Randall, 1994).

Taken together, these observations suggest that both pulses of plasma cortisol and elevated internal ammonia levels are involved in the regulation of Rh genes at the transcript level. Future studies should also investigate Rh changes in trout following feeding or exercise events since both cortisol (Bry, 1982; Milligan, 1996) and plasma ammonia and ammonia excretion rates (Bucking and Wood, 2008; Mommsen and Hochachka, 1988; Wicks and Randall, 2002a,b; Wood, 1988) are naturally elevated for short periods in these situations.

4.5.5 Conclusions

In summary, infusion of NH_4HCO_3 resulted in elevated plasma ammonia and increased ammonia excretion rates with patterns of Rh mRNA expression in trout tissues (erythrocytes, gill, and skin) similar to that seen when plasma ammonia levels were elevated after HEA exposure. Inclusion of a no-surgery control revealed that surgical

129

placement of the dorsal aortic catheter (and associated anaesthesia) resulted in an increase in ammonia excretion rate accompanied by the upregulation of all three Rh mRNAs in the gill. Whether or not the changes resulted from the anaesthesia itself, the surgery alone, or a combination of both these factors remains unknown. Similarly, results from NaCl infusion reflected changes from these surgical procedures as well as effects associated with the high rate of NaCl infusion alone. What is clear however, it is that any changes associated with NH₄HCO₃ infusion would have gone undetected if either the catheterized control or the NaCl infusion were used alone as a control. Finally, the results suggest that a transient surge in cortisol along with elevated plasma ammonia may be key components in the regulation of Rh genes and the subsequent control of ammonia transport in the rainbow trout.

4.6 FIGURES



Figure 4.1 The ammonia excretion rates (J_{Amm}) in rainbow trout infused with 140 mmol L⁻¹ NaCl or 140 mmol L⁻¹ NH₄HCO₃ over a course of 12 hours. The no-surgery control fish did not undergo experimental manipulation. The catheterized control fish underwent anaesthesia and surgery for catheter placement, but were not infused. The negative values indicate excretion into the water. Single asterisks represent values significantly different from the no-surgery control, crosses indicate significant difference from the catheterized control, and double asterisks indicate a significant difference between the two control values (P<0.05). Data are means ± s.e.m. (n=6-9).



Figure 4.2 Plasma total ammonia (T_{Amm}) in rainbow trout infused with 140 mmol L⁻¹ NaCl or 140 mmol L⁻¹ NH₄HCO₃ over a course of 12 hours. The no-surgery control fish did not undergo experimental manipulation. The catheterized control fish underwent anaesthesia and surgery for catheter placement, but were not infused. Asterisks represent plasma values significantly different from both control values. Controls were not significantly different from each other (P < 0.05). Data are means \pm s.e.m. (n=6).



Figure 4.3 Plasma cortisol levels during 12 hours of infusion with 140 mmol L⁻¹ NaCl or 140 mmol L⁻¹ NH₄HCO₃. The no-surgery control fish did not undergo experimental manipulation. The catheterized control fish underwent anaesthesia and surgery for catheter placement, but were not infused. Asterisk indicates significant difference from both control values. Controls were not significantly different from each other (P<0.05). Data are means ± s.e.m. (n=6).



Figure 4.4 Gill Rh mRNA expression in rainbow trout during 12 hours of infusion with 140 mmol L⁻¹ NaCl or 140 mmol L⁻¹ NH₄HCO₃. The no-surgery control fish did not undergo experimental manipulation. The catheterized control fish underwent anaesthesia and surgery for catheter placement, but were not infused. Expression data were normalized to ng total RNA concentration. Single asterisks indicate significant difference from the no-surgery control. Double asterisks indicate significant difference between the two control values. No significant differences were found between the catheterized control fish and both sets of infused fish (P<0.05). Data are means ± s.e.m. (n=6).



Figure 4.5 Erythrocyte Rhag mRNA expression in rainbow trout after 12 hours of infusion with 140 mmol L⁻¹ NaCl or 140 mmol L⁻¹ NH₄HCO₃. The no-surgery control fish did not undergo experimental manipulation. The catheterized control fish underwent anaesthesia and surgery for catheter placement, but were not infused. Expression data were normalized to ng total RNA concentration. The single asterisk indicates a significant difference from the catheterized control. Double asterisks indicate significant difference between the control values. There were no significant differences between the no-surgery control fish and both sets of infused fish (P<0.05). Data are means ± s.e.m. (n=6).



Figure 4.6 Rh mRNA expression in the (*A*) brain and (*B*) skin of rainbow trout after 12 hours of infusion with 140 mmol L⁻¹ NaCl or 140 mmol L⁻¹ NH₄HCO₃. The no-surgery control fish did not undergo experimental manipulation. The catheterized control fish underwent anaesthesia and surgery for catheter placement, but were not infused. Expression data were normalized to ng total RNA concentration. The single asterisk indicates a significant difference from the no-surgery control value. Double asterisks indicate significant difference between the control values. No significant differences were noted between the catheterized control fish and both sets of infused fish (*P*<0.05). Data are means \pm s.e.m. (*n*=6).



Figure 4.7 The effect of 12 hours of infusion with 140 mmol L⁻¹ NaCl or 140 mmol L⁻¹ NH₄HCO₃ on the mRNA expression of carbonic anhydrase (CA2), H⁺-ATPase, NHE2, and Na⁺/K⁺-ATPase α -1a (NKA) in the gills of rainbow trout. The no-surgery control fish did not undergo experimental manipulation. The catheterized control fish underwent anaesthesia and surgery for catheter placement, but were not infused. Expression data were normalized to ng total RNA concentration. Single asterisks indicate significant difference from the no-surgery control and crosses indicate a significant difference from the catheterized control value. Double asterisks indicate a significant difference between the control values (*P*<0.05). Data are means ± s.e.m. (*n*=6).

4.7 REFERENCES

- Biver, S., Belge, H., Bourgeois, S., Van Vooren, P., Nowik, M., Scohy, S., Houillier P., Szpirer, J., Szpirer, C., Wagner, C. A., Devuyst, O. and Marini, A. -M. (2008). A role for Rhesus factor Rhcg in renal ammonium excretion and male fertility. *Nature* 456, 339-343.
- Bry, C. (1982). Daily variations in plasma cortisol levels of individual female rainbow trout *Salmo gairdneri*: evidence for a post-feeding peak in well-adapted fish. *Gen. Comp. Endocrin.* 48, 462-468.
- Bucking, C. and Wood, C.M. (2008). The alkaline tide and ammonia excretion after voluntary feeding in freshwater rainbow trout. *J. Exp. Biol.* 211, 2533-2541.
- **Bustin, S. A.** (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrin.* 25,169–193.
- Bustin, S. A. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J. Mol. Endocrin.* 29, 23-39.
- Cameron, J. N. and Heisler, N. (1983). Studies of ammonia in the rainbow trout: physico-chemical parameters, acid-base behaviour and respiratory clearance. J. *Exp. Biol.* 105, 107–125.
- **Cartron, J. –P.** (2008). Blood group antigens structure and function: recent advances Hematology Education: the education program for the annual congress of the European Hematology Association 2, 158-174.
- Chan, D. K. A. and Woo, N. Y. S. (1978). Effect of cortisol on the metabolism of the eel, *Anguilla japonica. Gen. Comp. Endocrin.* 35, 205-215.
- **Constable, P. D.** (2003). Hyperchloremic acidosis: the classic examples of strong ion acidosis. *Anesth. Analg.* 96, 919-22.
- Curtis, B. J. and Wood, C. M. (1992). Renal and urinary bladder responses of trout to isosmotic NaCl and NaHCO₃ loading. *J. Exp. Biol.* 173, 181-203.
- **DeBoeck, G., Alsop, D. and Wood, C. M.** (2001). Cortisol effects on aerobic and anaerobic metabolism, nitrogen excretion, and whole-body composition in juvenile rainbow trout. *Physiol. Biochem. Zool.* 74, 858-868.
- Eddy, F. B. (2005). Ammonia in estuaries and effects on fish. J. Fish. Biol. 67, 1495-1513.

- Edwards, S. L., Wall, B. P., Morrison-Sheltar, A., Sligh, S., Weakley, J. C., Claiborne, J. B. (2005). The effect of environmental hypercapnia and salinity on the expression of NHE-like isoforms in the gills of a euryhaline fish (*Fundulus heteroclitus*). J. Exp. Zool. 303A, 464-475.
- Georgalis, T., Perry, S. F. and Gilmour, K. M. (2006). The role of branchial carbonic anhydrase in acid-base regulation in rainbow trout (*Oncorhynchus mykiss*). J. *Exp. Biol.* 209, 518-530.
- Gerloff, T., Geier, A., Stieger, B., Hagenbuch, B., Meier, P. J., Matern, S. and Gartung, C. (1999). Differential expression of basolateral and canalicular organic anion transporters during regeneration of rat liver. *Gastroenterology* 117, 1408-1415.
- **Goss, G. G. and Wood, C. M.** (1990). Na⁺ and Cl⁻ uptake kinetics, diffusive effluxes and acidic equivalent fluxes across the gills of rainbow trout II. Responses to bicarbonate infusion. *J. Exp. Biol.* 152, 549-571.
- Hopkins, T. E., Wood, C. M. and Walsh, P. J. (1995). Interactions of cortisol and nitrogen metabolism in the ureogenic gulf toadfish *Opsanus beta*. J. Exp. Biol. 198, 2229-2235.
- Houston, A. H. (1990). Blood and circulation. In *Methods for fish biology* (eds. C. B. Schreck and P. B. Moyle), pp 273-334. Bethesda MD: American Fisheries Society.
- Huang, C. –H. and Liu, P. Z. (2001). New insights into the Rh superfamily of genes and proteins in erythroid cells and nonerythroid tissues. *Blood Cells Mol. Dis.* 27, 90-101.
- Huang, C. -H. and Peng, J. (2005). Evolutionary conservation and diversification of Rh family genes and proteins. *Proc. Natl. Acad. Sci. USA* 102, 15512-1551.
- Hung, C. Y. C., Tsui, K. N. T., Wilson, J. M., Nawata, C. M., Wood, C. M. and Wright, P. A. (2007). Rhesus glycoprotein gene expression in the mangrove killifish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air. J. Exp. Biol. 210, 2419-2429.
- Hung, C. C., Nawata, C. M., Wood, C. M. and Wright, P. A. (2008). Rhesus glycoprotein urea transporter genes are expressed in early stages of development of rainbow trout (*Oncorhynchus mykiss*). J. Exp. Zool. 309A, 262-268.

Kiilerich, P., Kristiansen, K. and Madsen, S. S. (2007). Cortisol regulation of ion

transporter mRNA in Atlantic salmon gill and the effect of salinity on the signaling pathway. J. Endocrinol. 194, 417-427.

- Ivanis, G., Esbaugh, A. J. and Perry, S. F. (2008). Branchial expression and localization of SLC9A2 and SLC9A3 sodium/hydrogen exchangers and their possible role in acid–base in freshwater rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 211, 2467-2477.
- Iwama, G. K., McGeer, J. C. and Pawluk, M. P. (1989). The effects of five fish anaesthetics on acid-base balance, hematocrit, blood gases, cortisol, and adrenaline in rainbow trout. *Can. J. Zool.* 67, 2065–2073.
- Kim, H. Y., Baylis, C., Verlander, J. W., Han, K. H., Reungjui, S., Handlogten, M.
 E. and Weiner, I. D. (2007). Effect of reduced renal mass on renal ammonia transporter family RhC glycoprotein and Rh B glycoprotein, expression. Am. J. Physiol. 293, F1238-1247.
- Kong, H., Kahatapitiya, N., Kingsley, K., Salo, W. L., Anderson, P. M., Wang, Y. S. and Walsh, P. J. (2000). Induction of carbamoyl phosphate synthetase III and glutamine synthetase mRNA during confinement stress in gulf toadfish (*Opsanus beta*). J. Exp. Biol. 203, 311-320.
- Lin, D. W., Coleman, I. M., Hawley, S., Huang, C. Y., Dumpit, R., Gifford, D., Kezele, P., Hung, H., Knudsen, B. S., Kristal, A. R. and Nelson, P. S. (2006). Influence of surgical manipulation on prostate gene expression: implications for molecular correlates of treatment and disease prognosis. J. Clin. Oncol. 24, 3763-3770.
- McCormick, S. D., Regish, A., O'Dea, M. F. and Shrimpton, J. M. (2008). Are we missing a mineralocorticoid in teleost fish? Effects of cortisol, deoxycorticosterone and aldosterone on osmoregulation, gill Na⁺,K⁺-ATPase activity and isoform mRNA levels in Atlantic salmon. *Gen. Comp. Endocrinol.* 157, 35-40.
- McDonald, D. G. and Prior, E. T. (1988). Branchial mechanisms of ion and acid-base regulation in the freshwater rainbow trout, *Salmo gairdneri*. *Can. J. Zool.* 66, 2699–2708.
- McDonald, M. D. and Wood, C. M. (2004). The effect of chronic cortisol elevation on urea metabolism and excretion in the rainbow trout (*Oncorhynchus mykiss*). J. Comp. Physiol. B 174, 71-81.

McGeer, J. C. and Eddy, F. B. (1998). Ionic regulation and nitrogenous excretion in

Ph.D. Thesis - C. M. Nawata

rainbow trout exposed to buffered and unbuffered freshwater of pH 10.5. *Physiol. Zool.* 71, 179-190.

- Milligan, L. (1996). Metabolic recovery from exhaustive exercise in rainbow trout. *Comp. Biochem. Physiol.* 113A, 51-60.
- Mommsen, T. P. and Hochachka, P. W. (1988). The purine nucleotide cycle as two temporally separated metabolic units. *Metabolism* 37, 552-556.
- Mommsen, T. P. and Walsh, P. J. (1992). Biochemical and environmental perspectives nitrogen metabolism in fishes. *Experientia* 48, 583-593.
- Nakada, T., Hoshijima, K., Esaki, M., Nagayoshi, S., Kawakami, K. and Hirose, S. 2007a). Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. *Am. J. Physiol.* 293, R1743-1753.
- Nakada, T., Westhoff, C. M., Kato, A. and Hirose, S. (2007b). Ammonia secretion from fish gill depends on a set of Rh proteins. *FASEB J.* 21, 1067-1074.
- Nawata, C. M., Hung, C. C. Y., Tsui, T. K. N., Wilson, J. M., Wright, P. A. and Wood, C. M. (2007). Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H⁺-ATPase involvement. *Physiol. Genomics* 31, 463-474.
- Nawata, C. M. and Wood, C. M. (2008). The effects of CO₂ and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. *J. Exp. Biol.* 211, 3226-3236.
- **Ortega, V. A., Renner, K. J. and Bernier, N. J.** (2005). Appetite-suppressing effects of ammonia exposure in rainbow trout associated with regional and temporal activation of brain monoaminergic and CRF systems. *J. Exp. Biol.* 208, 1855-1866.
- **Perry, S. F., Beyers, M. L. and Johnson, D. A.** (2000). Cloning and molecular characterization of the trout (*Oncorhynchus mykiss*) vacuolar H⁺-ATPase B subunit. *J. Exp. Biol.* 203, 459-470.
- Perry, S. F., Rivero-Lopez, L., McNeill, B. and Wilson, J. (2006). Fooling a freshwater fish: how dietary salt transforms the rainbow trout gill into a seawater gill phenotype. *J. Exp. Biol.* 209, 4591-4596.
- Randall, D. J. and Tsui, T. K. N. (2002). Ammonia toxicity in fish. Mar. Poll. Bull. 45, 17-23.

- Salama, A., Morgan, I. J. and Wood, C. M. (1999). The linkage between sodium uptake and ammonia excretion in rainbow trout - kinetic analysis, the effects of (NH₄)₂ SO₄ and NH₄ HCO₃ infusion, and the influence of gill boundary layer pH. J. Exp. Biol. 202, 697-709.
- Scheingraber, S., Rehm, M., Schmisch, C. and Finsterer, U. (1999). Rapid saline infusion produces hyperchloremic acidosis in patients undergoing gynecologic surgery *Anesthesiology* 90, 1265-1270.
- Seshadri, R. M., Klein, J. D., Kozlowski, S., Sands, J. M., Kim, Y. H., Handlogten, M. E., Verlander, J. W. and Weiner, I. D. (2006). Renal expression of the ammonia transporters, Rhbg and Rhcg, in response to chronic metabolic acidosis. *Am. J. Physiol.* 290, F397-408.
- Shih, T. -H., Horng, J. -L., Hwang, P. -P. and Lin, L. -Y. (2008). Ammonia excretion by the skin of zebrafish (*Danio rerio*) larvae. Am. J. Physiol. 295, C1625-1632.
- Shrimpton, J. M. and Randall, D. J. (1994). Downregulation of corticosteroid receptors in gills of coho salmon due to stress and cortisol treatment Am. J. Physiol. 267, R432-438.
- Soivio, A. and Nynolm, K. (1975). A technique for repeated sampling of the blood of individual resting fish. J. Exp. Biol. 62, 207-217.
- Tashjian, D. and Hung, S. S. O. (2005). Noninvasive surgery techniques in fish research: A review on esophageal intubation, dorsal aorta cannulation, and urinary catheterization in sturgeon. In: Sakai Y, McVey JP, Jang D, McVey E, Caesar M (eds) Aquaculture and Pathobiology of Crustacean and Other Species. Proceedings, 32nd U.S.–Japan Meeting on Aquaculture, Davis, CA, USA, November 17–18, 2003. UJNR Technical Report 32. National Oceanic and Atmospheric Administration Research, Silver Spring, MD, USA, pp 131–142. Available online at http://www.lib.noaa.gov/japan/aquaculture/proceedings/report32/hung_corrected.pdf
- Tsui, T. K. N., Hung, C. Y. C., Nawata, C. M., Wilson, J. M., Wright, P. A. and Wood, C. M. (2009). Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical Na⁺/NH₄⁺ exchange complex. J. Exp. Biol. 212, 878-892.
- Verdouw, H., van Echteld, C. J. A. and Dekkers, E. M. J. (1978). Ammonia determinations based on indophenol formation with sodium salicylate. *Water Res.* 12, 399-402.

- Vermette, M. G. and Perry, S. F. (1987a). The effects of prolonged epinephrine infusion on the physiology of the rainbow trout, *Salmo gairdneri* I. Blood respiratory, acid-base and ionic states. J. Exp. Biol. 125, 235-253.
- Vermette, M. G. and Perry, S. F. (1987b). The effects of prolonged epinephrine infusion on the physiology of the rainbow trout, *Salmo gairdneri*. II. Branchial solute fluxes. *J. Exp. Biol.* 128, 255-267.
- Vermette, M.G. and Perry, S. F. (1987c). The effects of prolonged epinephrine infusion on the physiology of the rainbow trout, Salmo gairdneri. III. Renal ionic fluxes. J. Exp. Biol. 128, 269-285.
- Wicks, B. J. and Randall, D. J. (2002a). The effect of feeding and fasting on ammonia toxicity in juvenile rainbow trout, *Oncorhynchus mykiss. Aquat. Toxicol.* 59, 71-82.
- Wicks, B. J. and Randall, D. J. (2002b). The effect of sub-lethal ammonia exposure on fed and unfed rainbow trout: the role of glutamine in regulation of ammonia. *Comp. Biochem. Physiol. A* 132, 275-85.
- Wilkie, M. P. (2002). Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. J. Exp. Zool. 293, 284-301.
- Wilson, R. W., Wright, P. M., Munger, S. and Wood, C. M. (1994). Ammonia excretion in freshwater rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: lack of evidence for Na⁺/NH₄⁺ exchange. J. *Exp. Biol.* 191, 37–58.
- Wolf, K. (1963). Physiological salines for freshwater teleosts. *Prog. Fish. Cult.* 25, 135-140.
- Wood, C. M. (1988). Acid-base and ionic exchanges at gills and kidney after exhaustive exercise in the rainbow trout. *J. Exp. Biol.* 136, 461-481.
- Wood, C. M. (1993). Ammonia and urea metabolism and excretion. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 379-425. Boca Raton: CRC Press.
- Yesaki, T. Y. and Iwama, G. K. (1992). Survival, acid-base regulation, ion regulation, and ammonia excretion in rainbow trout in highly alkaline hard water. *Physiol. Zool.* 65, 763-787.
- Zhao, M., Chow, A., Powers, J., Fajardo, G. and Bernstein, D. (2004). Microarray analysis of gene expression after transverse aortic constriction in mice. *Physiol. Genomics* 19, 93-105.

CHAPTER 5

FUNCTIONAL CHARACTERIZATION OF RHESUS GLYCOPROTEINS FROM AN AMMONIOTELIC TELEOST, THE RAINBOW TROUT, USING OOCYTE EXPRESSION AND SIET ANALYSIS

5.1 ABSTRACT

Recent experimental evidence from rainbow trout suggests that gill ammonia transport may be mediated in part via Rhesus (Rh) glycoproteins. In this study we analyzed the transport properties of trout Rh proteins (Rhag, Rhbg1, Rhbg2, Rhcg1, Rhcg2, Rh30-like) expressed in Xenopus oocytes, using the radiolabeled ammonia analogue, $[^{14}C]$ methylamine, and the scanning ion electrode technique (SIET). All of the trout Rh proteins, except Rh30-like, facilitated methylamine uptake. Uptake was saturable with K_m values ranging from 4.6 - 8.9 mmol L⁻¹. Raising external pH from 7.5 to 8.5 resulted in 3-4 fold elevations in J_{max} values for methylamine; K_{m} values were unchanged when expressed as total or protonated methylamine. Efflux of methylamine was also facilitated in Rh-expressing oocytes. Efflux and influx rates were stimulated by a pH gradient, with higher rates observed with steeper H⁺ gradients. NH₄Cl inhibited methylamine uptake in oocytes expressing Rhbg1 or Rhcg2. When external pH was elevated from 7.5 to 8.5, the K_i for ammonia against methylamine transport was 35-40% lower when expressed as total ammonia or NH4⁺, but 5-6 fold higher when expressed as NH₃. With SIET we confirmed that ammonia uptake was facilitated by Rhag and Rhcg2, but not Rh30-like proteins. Ammonia uptake was saturable, with a comparable J_{max} but lower K_m value than for total or protonated methylamine. At low substrate concentrations, the ammonia uptake rate was greater than that of methylamine. The K_m for total ammonia (560 μ mol L⁻¹) lies within the physiological range for trout. The results are consistent with a model whereby NH₄⁺ initially binds, but NH₃ passes through the Rh channels. We propose that Rh glycoproteins in the trout gill are low affinity, high capacity ammonia transporters that exploit the favourable pH gradient formed by the acidified gill boundary layer in order to facilitate rapid ammonia efflux when plasma ammonia concentrations are elevated.

5.2 INTRODUCTION

While ammonia is an important nitrogen source for the growth of bacteria, fungi and plants, it is the major end product of nitrogen metabolism in ammoniotelic animals. Transport of ammonia across membranes is therefore essential for the maintenance of homeostasis in these organisms. The classical view has been that the lipid soluble gas phase (NH₃) of ammonia passes readily through membranes whereas the ionic phase (NH₄⁺) requires carriers in order to cross membranes (Kleiner, 1981). This view is now being challenged by the recent identification of genes for ammonia transporters in yeast (MEP) and plants (Amt), followed later by the discovery that Rhesus (Rh) blood group proteins are related to these transporters (Marini *et al.*, 1994, 1997; Ninnemann *et al.*, 1994).

The X-ray structure of the *Escherichia coli* ammonia transporter (AmtB) revealed that NH_3 and not NH_4^+ is the species that passes through the channel. NH_4^+ is deprotonated in the periplasmic vestibule of AmtB before NH_3 passes through the pore and reprotonates in the cytoplasmic vestibule (Khademi *et al.*, 2004; Zheng *et al.*, 2004). Comparison of the recently solved structure of the Rh protein (Rh50) from the bacteria *Nitrosomonas europaea* with AmtB, showed similarities in the pore but differences in the external vestibule which may reflect a lower affinity or a weaker sequestering capacity

McMaster - Biology

for NH_4^+ in the Rh proteins (Li *et al.*, 2007; Lupo *et al.*, 2007). Although a few reports have suggested that CO_2 could also pass through the Rh channels (Endeward *et al.*, 2007; Kustu and Inwood, 2006; Li *et al.*, 2007; Soupene *et al.*, 2002, 2004), the numerous functional studies that have been performed to date support the view that both Amt and Rh proteins facilitate ammonia transport (Javelle *et al.*, 2007).

In mammals, RhAG/Rhag proteins are mainly confined to erythrocytes but RhBG/Rhbg and RhCG/Rhcg are located in several key tissues related to ammonia metabolism such as the brain, liver, kidney and gastrointestinal tract (Handlogten *et al.*, 2005; Huang, 2008; Liu *et al.*, 2000, 2001). In fact, recent knock-down studies in mice revealed that Rhcg protein expression was necessary for renal ammonia excretion (Biver *et al.*, 2008; Lee *et al.*, 2009).

Unlike ureotelic mammals, most fish are ammoniotelic and excrete large amounts of ammonia, mostly through the gills rather than through the kidney. The first study that linked fish Rh proteins to ammonia excretion identified in pufferfish gills, apical Rhcg2 and basolateral Rhbg in the pavement cells, apical Rhcg1 in the mitochondria-rich cells, and apical and basolateral Rhag in the pillar cells (Nakada *et al.*, 2007a). Rhbg, Rhcg1, and Rhcg2 have also been identified in the gills and skin of the air-breathing mangrove killifish (Hung *et al.*, 2007). Thereafter, it was reported that Rhcg2 mRNA expression levels in the adult rainbow trout gill paralleled restoration of ammonia excretion in the face of elevated external ammonia (Nawata *et al.*, 2007) and levels of Rhcg2 mRNA in larval rainbow trout correlated with an increase in ammonia excretion rate over developmental time (Hung *et al.*, 2008). Similarly, Rhcg1 mRNA expression in larval

zebrafish coincided with increased ammonia excretion (Nakada *et al.*, 2007b) while knockdown of Rhag, Rhbg, and Rhcg1 in the same larval species led to a decrease in ammonia excretion (Braun *et al.*, 2009; Shih *et al.*, 2008). Also, an *in vitro* cultured gill epithelium system demonstrated that increased ammonia permeability caused by pre-exposure to elevated ammonia and cortisol, as well as the exposure to apical freshwater low in Na⁺, was associated with increased Rhcg2 mRNA (Tsui *et al.*, 2009). These data have been further examined in several recent reviews (Perry *et al.*, 2009; Weihrauch *et al.*, 2009; Wright and Wood, 2009).

Functional studies of Rh proteins have been hampered by the lack of a specific inhibitor as well as a long-lived radiotracer for ammonia. Researchers have therefore relied on the heterologous expression of Rh proteins in cells or cell-preparations in conjunction with the radiolabeled ammonia analogue, [¹⁴C]methylamine, to study Rh protein function. The first detailed study of Rh proteins in fish showed that *Xenopus* oocytes expressing pufferfish Rh proteins exhibited an increased uptake of methylamine (Nakada *et al.*, 2007a). Our goal was to further characterize the functional properties of these potentially important gill ammonia transporters of rainbow trout. Trout Rhag, Rhbg1, Rhbg2, Rhcg1, Rhcg2 and Rh30-like proteins were expressed in *Xenopus* oocytes and [¹⁴C]methylamine was used to characterize the transport properties of these proteins. In addition, we used the scanning ion electrode technique (SIET) (Ammann, 1986) to directly confirm that ammonia transport was also facilitated, and to characterize ammonia uptake kinetics in Rh-expressing oocytes and in H₂O-injected (control) oocytes.

5.3 MATERIALS AND METHODS

5.3.1 Reagents and solutions

All chemicals and reagents used in this study were obtained from Sigma (St. Louis, MO, USA) unless otherwise noted. The standard oocyte bath solution was ND96 containing (in mmol 1^{-1}): 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.5. Sterile ND96 for long-term storage of oocytes contained 2.5 mmol 1^{-1} sodium pyruvate, 1 mg ml⁻¹ penicillin-streptomycin and 50 µg ml⁻¹ gentamicin (Gibco, Long Island, NY, USA). Low K⁺ ND96 contained 0.2 mmol 1^{-1} KCl and Na⁺/K⁺-free ND96 contained 98 mmol 1^{-1} *N*-methyl-D-glucamine-Cl in substitution for NaCl and KCl. The acidification buffers adjusted to pH 6.8 or pH 6.4 contained (in mmol 1^{-1}): 55 NaCl, 60 sodium acetate, 1.8 CaCl₂, 1 MgCl₂, and 10 HEPES.

5.3.2 Plasmid constructs and cRNA synthesis

Rh cDNAs were isolated from rainbow trout gill and erythrocytes as previously described (Nawata *et al.*, 2007; Nawata and Wood, 2008) and amplified with high fidelity taq polymerase (Invitrogen, Burlington, ON, Canada) using primers (Table 5.1) flanking the coding region of each gene. Correct sequences were verified after cloning into a pGEM T-easy vector (Promega, Fisher Scientific, Nepean, ON, Canada). The Rh cDNAs were then subcloned by blunt-end ligation into the *XhoI* and *SpeI* restriction sites of a pXT7 vector containing *Xenopus* beta globin 3' and 5'-UTR sequences flanking the cloning site (courtesy of G. Goss, University of Alberta). In-frame insertion of cDNAs was confirmed by sequencing. Linearization with *SmaI* was followed by proteinase K

treatment (1 mg ml⁻¹, Invitrogen) and phenol/chloroform extraction. The linearized constructs were then transcribed and capped (Ambion, Austin, TX, USA) *in vitro* with T7 RNA polymerase (Fermentas, Burlington, ON, Canada). The resulting cRNAs were purified by phenol/chloroform extraction and quantified spectrophotometrically (Nanodrop, ND-1000, Wilmington, DE, USA) and assessed for quality on a 1% agarose gel.

5.3.3. Preparation of oocytes

Stage V-VI oocytes were collected from adult female *Xenopus* following an established protocol (Ceriotti and Colman, 1995). Briefly the frogs were anaesthetized in 0.1% MS222 for approximately 20 min. Excised ovarian tissue was placed in Ca²⁺-free ND96 solution containing collagenase (1 mg ml⁻¹) and gently agitated for 30 min. The oocytes were then rinsed three times in Ca²⁺-free ND96, three times with ND96, and then allowed to recover overnight at 18°C in sterile ND96. Frogs were humanely euthanized after the final oocyte collection. All procedures used were approved by the McMaster University Animal Research Ethics Board and are in accordance with the Guidelines of the Canadian Council on Animal Care.

5.3.4 Injection of oocytes

Oocytes isolated the previous day were injected with 38.6 nl of cRNA (0.5 ng nl⁻¹) for a total of 18.5 ng using a Nanoliter 2000 Injector (World Precision Instruments,

McMaster - Biology

Sarasota, FL, USA). Control oocytes were injected with 38.6 nl of RNase-free H₂O. Experiments were performed 3-5 days post-injection.

5.3.5 [¹⁴C]methylamine studies

Experiments were performed at room temperature in 200 µl of uptake buffer which contained: low K⁺ ND96, 0.5 µCi ml⁻¹ [¹⁴C]methylamine (Dupont, New England Nuclear, Boston, MA, USA), and 20 µmol 1⁻¹ of unlabeled methylamine. Incubation times ranged from 15 to 60 min depending on each experiment, described in more detail below. Three groups of three oocytes were assayed for each experimental point; each group was considered to represent one replicate. At the end of each assay, oocytes were washed three times with 2 ml of ice-cold, unlabeled uptake buffer and immediately solubilized in 200 µl of 5% SDS. Radioactivity was measured in 5 ml of Ultima-Gold AB scintillation cocktail (Perkin Elmer, Toronto, ON, Canada) by liquid scintillation counting (Tri-Carb 2900 TR; Perkin Elmer). H₂O-injected (control) oocytes were run in parallel in all assays. The focus of our study was on trout Rhcg2 since according to previous studies, mRNA levels of this protein responded the most to elevated levels of ammonia (e.g. Nawata et al., 2007; Tsui et al., 2009). Other trout Rh genes were included in the assays to evaluate whether the same principles applied, and to ensure that the protocols were working properly, but not all genes were used in all tests.

5.3.6 Methylamine kinetics

The methylamine uptake rates measured over 20 min incubation periods in 0.02, 0.2, 1, 2, 10, and 15 mmol l⁻¹concentrations of methylamine were used to determine the kinetic profile. Endogenous uptake rates in control oocytes were subtracted from the test oocyte uptake values. The concentration dependence of methylamine was described in terms of J_{max} and K_{m} values by using non-linear regression to fit the Michaelis-Menten equation to the experimental data using Sigma Plot version 10.0.

5.3.7 NH₄Cl inhibition

Total ammonia concentrations used for NH₄Cl inhibition studies were verified enzymatically by measuring the formation of L-glutamate catalyzed by L-glutamate dehydrogenase, an assay linear to 600 μ mol l⁻¹ with reproducibility of ± 5% (Riachem, Cliniqa Corp., CA, USA) and concentrations of NH₃ and NH₄⁺ were calculated using the Henderson-Hasselbalch equation with a pK_a value of 9.25 (Cameron and Heisler, 1983) for ammonia. A pK_a value of 10.66 (CRC Handbook of Chemistry and Physics, 2005) for methylamine was used to determine the unprotonated (MA) and protonated (MA⁺) fractions of the K_m values. The inhibition constant (K_i) of ammonia against methylamine uptake was determined using the equation:

$$K_{\rm i} = {\rm IC}_{50} / (1 + c / K_{\rm m})$$

where IC₅₀ is the concentration of NH₄Cl, NH₄⁺, or NH₃ that reduces the uptake by 50%, *c* is the substrate concentration, and $K_{\rm m}$ is the substrate concentration permitting half-maximal uptake of methylamine.

5.3.8 Measurements of ammonia uptake using the scanning ion electrode technique (SIET)

Transport of ammonia into or out of an oocyte produces gradients in NH_4^+ concentration in the unstirred layer adjacent to the oocyte surface. These gradients can be calculated from the voltages recorded by an NH_4^+ -selective microelectrode moved between two points within the unstirred layer. While the great majority (>95%) of the ammonia will exist as NH_4^+ at the pH value (7.5) set by the buffer in the present experiment, it may have moved across the oocyte membrane as NH_3 , or as NH_4^+ , or both. Thus the microelectrode can be used to measure "apparent NH_4^+ flux" which is approximately equivalent to total "ammonia flux", the term used here. Ammonia flux can then be calculated from the NH_4^+ concentration gradients measured in the unstirred layer using Fick's law, as described below. Measurement of fluxes in this way is the basis of the scanning ion electrode technique (SIET), which allows fluxes to be repeatedly measured in near real-time at multiple sites on the oocyte surface. Extensive descriptions of the use of SIET are reported in Rheault and O'Donnell (2004) and Donini and O'Donnell (2005).

SIET measurements were made using hardware from Applicable Electronics (Forestdale, MA, USA) and automated scanning electrode technique (ASET) software (version 2.0) from Science Wares Inc. (East Falmouth, MA, USA). Each oocyte was placed in a 35 mm diameter petri dish filled with 5 ml of Na^+/K^+ -free ND96. Reference electrodes were made from 10 cm lengths of 1.5 mm borosilicate glass capillaries that were bent at a 45° angle, 1–2 cm from the end, to facilitate placement in the sample dish.

McMaster - Biology

Capillaries had been filled with boiling 3 mol 1^{-1} KCl in 3% agar and were connected to the ground input of the Applicable Electronics amplifier through a Ag/AgCl half cell.

Micropipettes for NH4⁺-selective microelectrodes were made from 1.5 mm unfilamented borosilicate glass capillaries pulled on a Flaming-Brown P-97 pipette puller (Sutter Instruments, Novato, CA, USA) to tip diameters of 3 to 5 µm. The micropipettes were backfilled with 100 mM NH₄Cl and tip-filled with a 200 μ m long column of NH₄⁺ Ionophore I, Cocktail A (Fluka, Buchs, Switzerland). This ionophore is sensitive to interference from K⁺ and Na⁺ and the calibration and bathing solutions were therefore based on Na⁺/K⁺-free ND96. NH₄⁺-selective microelectrodes for use with SIET were calibrated in 0.1, 1 and 10 mmol $l^{-1} NH_4^+$ in Na⁺/K⁺-free ND96 resulting in a Nernstian slope of 57.2 \pm 0.3 mV log unit⁻¹ (*n*=7). The NH₄⁺-selective microelectrode was initially placed 5-10 µm from the surface of the oocyte. The microelectrode was then moved a further 50 µm away, perpendicular to the oocyte surface. The "wait" and "sample" periods at each limit of the 50 µm excursion distance were 5.5 and 0.5 s, respectively. Voltage differences across this excursion distance were measured three times at each of four sites located 25 µm apart over the surface of the oocyte. Voltage differences were corrected for electrode drift measured at a reference site 20 mm away from the oocyte. Voltage differences (ΔV) were converted to the corresponding NH₄⁺ concentration difference by the following equation (Donini and O'Donnell, 2005):

$$\Delta C = C_{\rm B} \times 10^{(\Delta V/S)} - C_{\rm B}$$

where ΔC is the NH₄⁺ concentration difference between the two limits of the excursion distance (µmol cm⁻³), $C_{\rm B}$ is the background NH₄⁺ concentration in the bathing medium,

 ΔV is the voltage gradient (mV), and S is the slope of the electrode between 0.1 and 1 mmol l^{-1} NH₄⁺. Concentration differences were used to determine the ammonia flux using Fick's law of diffusion:

$$J_{\rm Amm} = D_{\rm NH4}(\Delta C / \Delta X)$$

where J_{Amm} is the net flux in µmol cm⁻² s⁻¹, D_{NH4} is the diffusion coefficient of NH₄⁺ (2.09 x 10⁻⁵ cm² s⁻¹), ΔC is the NH₄⁺ concentration gradient and ΔX is the excursion distance between the two points (cm). Ammonia uptake rates were determined immediately after oocytes were exposed to 0.1, 0.3, 1, 3, and 10 mmol l⁻¹ NH₄Cl. Nonlinear regression to fit the Michaelis-Menten equation was used to determine the J_{max} and K_m values (Sigma Plot version 10.0). The endogenous uptake measured in the H₂Oinjected oocytes was not subtracted from the control uptake rate as it was in the methylamine uptake kinetic analysis.

5.3.9 Data analysis

All data shown are means \pm s.e.m with N = number of replicates or for SIET, N = number of oocytes. Statistical significance was determined by Student's unpaired *t*-test followed by Bonferonni adjustment using Systat version 10.0. α was set at 0.05.

5.4 RESULTS

5.4.1 Methylamine uptake

Methylamine uptake rates were measured in 20 μ mol l⁻¹ methylamine over a period of 60 min in control oocytes and in oocytes expressing Rhag, Rhcg2, Rhbg1, and

McMaster - Biology

Rh30-like proteins. The uptake rates of the Rh30-like-injected oocytes and the control oocytes were not significantly different from each other (Fig. 5.1). However, expression of Rhag, Rhbg1, or Rhcg2 enhanced the uptake rate when compared to those of the control and Rh30-like-expressing oocytes. Rhag-expressing oocytes maintained an uptake rate that was 6-fold greater that of the control oocytes throughout the time course. The rate in Rhbg1-expressing oocytes was 4.5-fold higher than the control oocytes at 10 min and 3.5-fold higher at 60 min. Similarly, the rate in Rhcg2-expressing oocytes was 4-fold higher than the control oocytes at 10 min and 3-fold higher at 60 min.

5.4.2 Kinetics of methylamine uptake

The rate of methylamine uptake was measured in oocytes expressing Rhag, Rhbg1, Rhbg2, Rhcg2 over increasing methylamine concentrations (0.02 - 15 mmol 1^{-1}) at pH 7.5. Endogenous uptake rates measured in control oocytes were subtracted from test oocyte uptake rates. Uptake rates were saturable as a function of methylamine concentration. The J_{max} values (in pmol/oocyte/min) were: 191.1 ± 36.0 (Rhag), 106.1 ± 15.0 (Rhbg1), 87.4 ± 11.3 (Rhbg2), and 194.7 ± 35.9 (Rhcg2), with the values for Rhag and Rhcg2 being significantly greater than for the other two. The respective concentrations permitting half-maximal uptake (K_m) were: 7.8 ± 3.4 (Rhag), 6.8 ± 2.4 (Rhbg1), 4.6 ± 1.7 (Rhbg2), and 8.9 ± 3.6 mmol 1^{-1} (Rhcg2), none of which were significantly different from the others (Fig. 5.2). To test the effect of pH on the uptake kinetics of methylamine, we performed the same test on Rhbg1 and Rhcg2-expressing oocytes, at pH 8.5. There was some variability in the responses at the highest methylamine concentrations tested, but overall the results again indicated saturating relationships (Fig. 5.3). Compared to the J_{max} values obtained at pH 7.5, the values at pH 8.5 increased significantly by about 4-fold in Rhbg1-expressing oocytes (440.8 ± 69.9 pmol/oocyte/min) and about 3-fold in Rhcg2-expressing oocytes (663.6 ± 102.7 pmol/oocyte/min), while the $K_{\rm m}$ values did not change significantly when expressed as total or protonated methylamine (Fig. 5.3; Table 5.2). However, $K_{\rm m}$ values increased 8-10 fold (significant for Rhcg2 only) when expressed as the unprotonated form (Table 2).

5.4.3 pH-sensitive transport

The apparent sensitivity of methylamine uptake to pH led us to a more in-depth investigation of this observation. Methylamine uptake rates in control oocytes and those expressing Rhag, Rhbg1, Rhbg2, Rhcg1, and Rhcg2, were measured for 60 min in uptake buffers at a substrate level of 20 μ mol l⁻¹ set at pH 6.5, 7.5, and 8.5. The substrate concentration of 20 μ mol l⁻¹ was chosen since saturation of transport did not occur over time at this concentration (see Fig. 5.1). Uptake rates of all Rh-expressing oocytes decreased at pH 6.5 and increased at pH 8.5 when compared to rates at pH 7.5 (Fig. 5.4). Notably, the rates in Rhag- and Rhcg2-expressing oocytes were 3.5-fold higher at pH 8.5 than the rates at pH 7.5. The rates were significantly higher at pH 7.5 than at pH 6.5 for Rhag-, Rhbg1-, Rhbg2-, and Rhcg2-expressing oocytes.

To further test the pH dependence of methylamine transport, Rhag-, Rhbg2-, and Rhcg2-expressing oocytes were acidified following established methods (Tsai *et al.*,

1995; Westhoff *et al.*, 2002). Oocytes were incubated in sodium acetate at pH 6.8 or pH 6.4 for 25 min, washed once in ice-cold unlabeled uptake buffer and transferred to radiolabeled [¹⁴C]methylamine uptake buffer (20 μ mol l⁻¹ methylamine, pH 7.5) for 15 min. Uptake rates from controls were subtracted from the rates measured in the acidified and untreated oocytes. Control oocytes had an average intracellular pH of 7.29 \pm 0.09 and therefore the intracellular to extracellular pH difference in untreated oocytes was approximately 0.2. Intracellular acidification at both pH 6.4 and 6.8 resulted in a 2-fold increase in uptake in Rhbg2 and Rhcg2-expressing oocytes and a 4-5 fold increase in uptake in Rhag-expressing oocytes, when compared to the rate in unacidified oocytes (Fig. 5.5). Additionally, the uptake rate in Rhag-expressing oocytes acidified at pH 6.4 was significantly higher than those acidified at pH 6.8.

5.4.4 Efflux of methylamine

To determine whether or not Rh proteins facilitate bi-directional methylamine transport, we measured the efflux of methylamine from control oocytes and Rhag-, Rhbg1-, Rhcg2-expressing oocytes. Oocytes were incubated in [¹⁴C]methylamine (20 μ mol l⁻¹) uptake buffer at pH 8.5 for 60 min and then quickly washed with ice-cold, unlabeled uptake buffer three times before being transferred into unlabeled uptake buffer without methylamine at pH 8.5 or pH 6.5 for 15 min. Radioactivity in the oocytes and buffer was counted separately. Efflux rates were expressed as the percent of the total initial radioactivity in the oocytes that appeared in the buffer during the 15 min efflux period. Control oocytes released a similar amount of methylamine (5% and 4%) at pH
McMaster - Biology

6.5 and 8.5 (Fig. 5.6). Effluxes from Rhag-, Rhbg1-, and Rhcg2-expressing oocytes at pH 6.5 (22%, 14%, and 17%, respectively) and at pH 8.5 (7%, 8%, and 9%, respectively) were significantly greater than those from the control oocytes. Additionally, significantly greater effluxes were observed at pH 6.5 compared to pH 8.5 in all the Rh-expressing oocytes.

5.4.5 Inhibition by NH₄Cl

Next, we examined the kinetics of methylamine uptake in the presence of NH₄Cl in order to characterize the substrate specificity of the Rh proteins. Methylamine uptake was measured in Rhbg1 and Rhcg2-expressing oocytes at a constant methylamine concentration of 20 µmol Γ^{-1} in the presence of increasing concentrations of total ammonia (80-3500 µmol Γ^{-1}) which takes into account the background total ammonia concentration of 80 µmol Γ^{-1} in the oocyte bath medium (Fig. 5.7). External buffer pH was set to either 7.5 or 8.5 and control oocyte uptake rates were subtracted from test oocyte uptake rates. The K_i values measured at pH 7.5 were similar for Rhbg1- and Rhcg2-expressing oocytes at 2.45 ± 0.31 and 2.53 ± 0.21 mmol Γ^{-1} , respectively (expressed as total ammonia). The values measured at pH 8.5 were also similar with 1.61 ± 0.04 mmol Γ^{-1} for Rhbg1 and 1.55 ± 0.06 mmol Γ^{-1} for Rhcg2 (Fig. 5.6). Compared to the values at pH 7.5, these K_i values at pH 8.5 were moderately reduced by 35-40% when expressed as total ammonia or NH₄⁺, but greatly raised by 5-6 fold when expressed as NH₃ (Table 2). Both of these changes were significant.

5.4.6 Ammonia uptake measured by SIET

We used SIET to further verify whether or not ammonia is a true substrate of the Rh transporters. Ammonia uptake rates were measured in control oocytes and oocytes expressing Rhag, Rhcg2, and Rh30-like proteins exposed to 100 µmol l⁻¹ NH₄Cl at pH 7.5. In Fig. 5.8., SIET rates have been expressed both in traditional units $(pmol/cm^2/s)$ and in pmol/oocyte/min by taking into account the surface area of the oocytes (~0.031 cm²). All oocytes facilitated ammonia uptake but the rates in Rhag- and Rhcg2expressing oocytes (18.5 \pm 1.1 and 17.4 \pm 0.5 pmol/oocyte/min, respectively) were significantly higher by 1.5-fold than the rate in control oocytes (12.0 \pm 0.6 pmol/oocyte/min). Notably, oocytes expressing Rh30-like protein exhibited no increase in ammonia uptake rate, consistent with the findings for methylamine uptake (Fig. 5.1). To compare ammonia uptake rates to those of methylamine, the $[^{14}C]$ methylamine uptake rates of H₂O-, Rhag-, Rhcg2-injected oocytes at a methylamine concentration of 100 μ mol l⁻¹ were calculated from the Michaelis-Menten curves generated earlier (Fig. 5.2). Methylamine uptake rates were much lower than the ammonia uptake rates (10-fold lower in H₂O-injected oocytes and about 4-fold lower in Rhag and Rhcg2-expressing oocytes).

5.4.7 Kinetics of ammonia uptake measured by SIET

Finally, we measured the kinetics of ammonia uptake using SIET. Oocytes expressing Rhcg2 and control oocytes were exposed to increasing concentrations of $NH_4Cl (0.1-10 \text{ mmol } l^{-1})$ at pH 7.5 (Fig. 5.9). Uptake of ammonia by Rhcg2-expressing

oocytes displayed saturation kinetics with a J_{max} of 63.3 ± 0.6 pmol/cm²/s, or 118 ± 1 pmol/oocyte/min. The latter was not significantly different from the J_{max} value measured earlier for methylamine (195 ± 36 pmol/oocyte/min; Fig. 5.2). K_{m} values (in mmol l⁻¹) were 0.56 ± 0.26 for NH₄Cl, 0.55 ± 0.26 for NH₄⁺, and 0.010 ± 0.005 for NH₃. These were much lower than the respective values for total and protonated methylamine, but comparable for unprotonated methylamine (Table 2). Uptake by control oocytes saturated much later with a J_{max} of 137.5 ± 17.3 pmol/cm²/s and K_{m} values (in mmol l⁻¹) of 5.6 ± 1.5, 5.5 ± 1.5, and 0.09 ± 0.03 for NH₄Cl, NH₄⁺, and NH₃ respectively. The J_{max} value (137.5 ± 17.3 pmol/cm²/s) was also significantly higher. There were no significant differences in the uptake rates between the control oocytes and the Rhcg2-expressing oocytes than in the control oocytes. At 10 mM, the uptake rate in Rhcg2-expressing oocytes was significantly lower (1.8 fold) than that in the control oocytes.

5.5 DISCUSSION

The recent addition of the Rh proteins to the ammonia transporter superfamily has sparked renewed interest in the area of ammonia transport in fish (Perry *et al.*, 2009; Weihrauch *et al.*, 2009; Wright and Wood, 2009). Indeed there is increasing evidence that Rh proteins are involved in the gill ammonia transport mechanism(s). We observed earlier that experimental elevations in plasma ammonia in rainbow trout resulted in enhanced ammonia excretion as well as an upregulation of gill Rhcg2 mRNA levels

McMaster - Biology

(Nawata *et al.*, 2007; Nawata and Wood, 2009). In light of these findings, the aim of our present study was to characterize the functional properties of trout Rh proteins in an effort to understand what role these proteins may play in gill ammonia transport.

With one exception (Rh30-like protein), all trout Rh proteins expressed in *Xenopus* oocytes facilitated uptake of the ammonia analogue, methylamine, with rates 3-6 fold greater than that seen in control oocytes (Fig. 5.1). The uptake was also saturable, suggesting a carrier-mediated process (Figs 5.2, 5.3). This is in accordance with previous studies which showed that human, murine, and pufferfish Rh proteins expressed in *Xenopus* oocytes also facilitated methylamine uptake (Ludewig, 2004; Mak *et al.*, 2006; Mayer *et al.*, 2006; Nakada *et al.*, 2007a; Westhoff *et al.*, 2002). Interestingly, all transport rates at 1 mmol 1^{-1} appeared to fall below the curve. Whether or not this is random variation or a true characteristic of the trout Rh proteins is not clear but this was not due to systematic error since experiments were conducted at different times with fresh solutions. However, it could represent an additional transport system of lower affinity and higher capacity superimposed on a higher affinity, lower capacity system. Further experiments using a series of low methylamine concentrations would be needed to evaluate this possibility.

More importantly for the first time in any system, we showed by direct measurement using SIET that these proteins also facilitated the uptake of ammonia, the purported natural substrate. Ammonia has also been demonstrated to be the natural substrate for RhAG expressed in MEP-deficient yeast (Marini *et al.*, 2000). At least for the one protein (Rhcg2) tested in detail with this more difficult approach, the uptake was

162

saturable with a K_m (560 µmol l⁻¹) within the physiological range for total ammonia in trout (Fig. 5.9), and considerably below the K_m for the analogue methylamine (8850 µmol l⁻¹; Fig. 5.2; Table 5.2). When compared at the same low substrate concentration, these proteins facilitated a greater transport rate of ammonia than of methylamine (Fig. 5.8). Furthermore NH₄Cl inhibited methylamine uptake with K_i values for total ammonia which were also considerably lower than the K_m values for methylamine. Overall, we believe these data provide strong evidence that these Rh proteins are important in facilitating ammonia transport across cell membranes, such as those in the gill epithelium.

The trout Rh30-like protein did not enhance methylamine uptake (Fig. 5.1) or ammonia uptake (Fig. 5.8). Similarly, human Rh30 proteins (RhD and RhCE) which are present in the erythrocyte membrane do not facilitate methylamine or ammonia transport (Ripoche *et al.*, 2004; Westhoff and Wylie, 2006). Homology modeling of the human Rh proteins and AmtB has shown that several residues in the channel differ between AmtB/RhAG/RhBG/RhCG and the Rh30 (RhD/RhCE) proteins, and could possibly explain the difference in transport properties (Zidi-Yahiaoui *et al.*, 2009).

There was enhanced uptake of methylamine into Rh-expressing oocytes at an external alkaline pH and a reduction in uptake rate at an external acidic pH (Fig. 5.5). The same effect of pH on uptake rates has been seen in studies on human RhAG, RhBG, RhCG (Ludewig, 2004; Mayer *et al.*, 2006; Westhoff *et al.*, 2002), murine Rhcg and Rhbg (Mak *et al.*, 2006), as well as AmtB (Javelle *et al.*, 2005). At least for Rhag, it appeared that the transport rate was not only sensitive to the direction of the pH gradient,

163

but also to the magnitude of the gradient with greater uptake observed at pH 6.4 than at pH 6.8 (Fig. 5.4). A simple explanation for these observations is that the transport rate depends upon the concentration of the unprotonated species (MA) since a one-unit pH change would increase or decrease the MA concentration by 10-fold. However, the situation is not that simple. If the transport rate depended only on the concentration of MA, we would expect to see approximately equivalent increases and decreases in rate, since these assays were run at a total methylamine concentration of 20 µmol l⁻¹, right at the bottom of the kinetic uptake curves (Fig. 5.2). However, despite a 10-fold decrease in MA from pH 7.5 to pH 6.5 and 10-fold increase in MA from pH 7.5 to pH 8.5, the uptake rates observed in Rh-expressing oocytes did not change by a similar fold. Rather, the changes were in the order of 3-5 fold per pH unit (Fig. 5.4). The explanation for this likely lies in the fact that the position of the kinetic curves was not constant, but also shifted with pH (Fig. 5.3). Indeed, based on the observed changes in the K_m and J_{max} values (Fig. 5.3, Table 5.2), we can calculate for Rhbg1 and Rhcg2 that at a total methylamine concentration of 20 µmol 1⁻¹, the transport rate would have increased by 3-5 fold as pH was increased from 7.5 to 8.5, exactly as was observed (Fig. 5.3). It is important to note that this conclusion is independent of whether the substrate is considered to be MA or MA⁺. If MA is the true substrate, the fact that the increase in transport rate was only 3-5 fold rather than 10 fold is because the affinity decreased markedly at higher pH (i.e. K_m expressed as MA increased 6-10 fold; Table 2). If MA⁺ is the true substrate, then K_m did not change appreciably at higher pH (Table 2), and the fact that transport rate increases 3-5 fold is entirely due to the observed increase in J_{max} .

It is unclear why K_m should change with pH, so a conservative conclusion is that MA⁺ is the species that binds initially to the transporter, but the transport itself is sensitive to a pH gradient.

Similar conclusions may be drawn from the inhibition studies. When pH was increased from 7.5 to 8.5, the K_i for NH₃ increased 5-6 fold, whereas the K_i for NH₄⁺ changed only moderately (decreases of 35-40%). While the K_i values for NH₄⁺ were lower than the K_m values of MA⁺, the K_i values for NH₃ were higher than the K_m values of MA (Table 2). Why the natural substrate should have a higher K_i than the analogue K_m , and why the value should change greatly with pH is again unclear, but supports the conclusion that the protonated species binds to the transporter. Importantly, however, this does not indicate whether it is the protonated or unprotonated form that is actually transported.

Overall, this argues against NH₃ as the species binding to the Rh proteins, in accordance with conclusions made previously regarding mammalian RhAG, Rhbg and Rhcg (Ludewig, 2004; Mak *et al.*, 2006; Mayer *et al.*, 2006; Westhoff *et al.*, 2002). Indeed in a physiological context this is reasonable, considering that the majority of ammonia (>95%) in fish plasma is present in the protonated form (Wood, 1993). If this is the case, then why does the pH gradient have a substantial influence on Rh protein-mediated methylamine transport in the oocyte expression system (Figs 5.3, 5.4, 5.5, and 5.6)? Similarly, why does the pH gradient influence ammonia efflux across cultured branchial epithelia *in vitro* (Kelly and Wood, 2001) and trout gills *in vivo* (Wright and Wood, 1985; Wilson et al., 1994; Wright et al., 1986, 1989)? We suggest that this is

because the actual species moving through the Rh channel is the unprotonated form, such that both the upstream de-protonation reaction and the downstream re-protonation reaction ("acid trapping") are a function of the ambient pH. This is almost impossible to distinguish from NH_4^+/H^+ exchange, and functionally the two processes would be the same in their net effect (Ludewig, 2004) and therefore we cannot rule out the possibility that NH_4^+ or that both NH_3 and NH_4^+ pass through the channel. Indeed there is considerable discordance in the conclusions made from the numerous functional studies on Rh proteins with respect to the mechanism involved (for summary, see Javelle *et al.*, 2007).

Not only was methylamine uptake facilitated in Rh-expressing oocytes, but enhanced efflux was also observed, and this was similarly dependent upon a pH gradient with the greatest efflux rate observed at a low external pH (Fig. 5.5). Bi-directionality has also demonstrated in yeast expressing RhAG and RhCG (Marini *et al.*, 2000; Mayer *et al.*, 2006; Westhoff *et al.*, 2004) or Rh50 from *N. europaea* (Weidinger *et al.*, 2007). This property would be particularly beneficial for erythrocytes. Rhag in erythrocytes could facilitate ammonia loading from the tissues and the subsequent unloading of ammonia at the gill where it can ultimately be eliminated into the external water, as depicted in the model of Wright and Wood (2009). A bi-directional function however, appears counterintuitive in the gill where unidirectional flow or excretion of ammonia is necessary. Nevertheless, the data of Tsui *et al.* (2009) suggest that the upregulation of Rh proteins in the cultured trout gill epithelium *in vitro* conferred a bi-directional increase in ammonia permeability. An acidified gill boundary layer has long been thought to aid in ammonia excretion *in vivo* (Wilson *et al.*, 1994; Wright *et al.*, 1986, 1989). This property would also create a favourable pH gradient for optimal transport by the Rh proteins and facilitate vectorial ammonia movement out of the gill. At the same time, this acidified layer would form an inwardly unfavourable H^+ gradient that could potentially slow the influx of ammonia into the gill through the Rh channels when external ammonia concentrations are elevated. In fact, we demonstrated earlier that when the acidified boundary layer in trout was abolished with HEPES, there was a down-regulation of Rhcg2 mRNA in the gill and a reduction in ammonia excretion (Nawata and Wood, 2008). Therefore one possible regulatory mechanism conferring unidirectionality in trout gill Rh proteins may be the pH or a H^+ gradient. Models have been recently proposed wherein functional coupling of Rh proteins to apical H^+ -ATPase pumps and/or Na⁺/H⁺ exchange mechanisms facilitate ammonia trapping in an acidic gill boundary layer (Tsui *et al.*, 2009; Wright and Wood, 2009).

Amt and Rh proteins share a common ancestor and coexist in many organisms, but higher vertebrates have retained only the Rh genes. This suggests that the Amt and Rh proteins have evolved different functions (Huang, 2008). Indeed structural studies revealed differences between the Rh and Amt proteins, especially in the external vestibule where critical residues thought to be essential for NH_4^+ binding are lacking or are not conserved in the Rh proteins (Lupo *et al.*, 2007; Zidi-Yahiaoui *et al.*, 2009). These differences may reflect different ammonia transport requirements. Bacteria need to assimilate ammonia from very low micromolar environmental concentrations, thus necessitating a high affinity NH_4^+ trapping mechanism. Mammals and fish, on the other hand, must dispose of ammonia, which is normally present in the millimolar range in the mammalian renal system (Knepper *et al.*, 1989) and in the high micromolar range in fish plasma (Wood, 1993), and therefore high affinity NH_4^+ trapping would be less critical. In fact, AmtB proteins are only induced in bacteria when external ammonia concentrations are limiting (Javelle *et al.*, 2004). However in the rainbow trout, Rh transcripts were detectable under control conditions but were upregulated when plasma ammonia concentrations were elevated (Nawata *et al.*, 2007; Nawata and Wood, 2009). These differences are also reflected in the different binding affinities for methylamine. For the trout Rh proteins the K_m values ranged from 4.6 to 8.9 mmol l⁻¹, whereas the K_m for AmtB was reported to be 200 µmol l⁻¹ (Merrick *et al.*, 2001).

The concentration of NH₄Cl required to inhibit the uptake of methylamine was similar for both Rhbg and Rhcg2 (2.5 mmol l⁻¹ at pH 7.5 and 1.6 mmol l⁻¹ at pH 8.5) (Table 2). This suggests that these two proteins have a similar affinity for ammonia. Murine Rhbg however, had a much higher affinity for both methylamine and ammonia than Rhcg, with K_i values for NH₄Cl of 0.5 mmol l⁻¹ for Rhbg vs. 2.9 mmol l⁻¹ for Rhcg (Mak *et al.*, 2006). It was proposed that in the mammalian kidney, a higher affinity basolateral Rhbg and a lower affinity apical Rhcg in the collecting duct could facilitate the vectorial transport of ammonia from the interstitium to the lumen where there is an increasing ammonia gradient (Mak *et al.* 2006; Westhoff and Wylie, 2006). In fish, it is not certain whether all species and all types of gill cells have an apical Rhcg and a basolateral Rhbg. Although this is the case in pufferfish gill pavement cells, it is not the case in pufferfish gill mitochondria-rich cells (Nakada *et al.*, 2007a). Moreover, it was

reported recently that Rhcg is present both apically and basolaterally in the mouse kidney (Kim *et al.*, 2009). In the trout gill, favourable plasma-to-water ammonia and pH gradients, plus the high intracellular ammonia levels and low extracellular ammonia levels (Wood, 1993), may be more important in regulating unidirectional flow than the differential binding affinities of basolateral and apical Rh proteins. Furthermore, we still cannot rule out passive diffusion of ammonia out of the gill as a significant mode of excretion under basal conditions. Indeed evidence from studies on the cultured trout gill epithelium indicates that there is a large diffusive component to ammonia excretion (Tsui *et al.*, 2009). The relatively high K_m values of the trout Rh proteins suggest that these are low-affinity, high capacity transporters that would function optimally when plasma ammonia concentrations are elevated. In support of this idea, it has been proposed that above a certain threshold concentration of plasma ammonia (200 µmol l^{-1}), a carrier-mediated process replaces passive ammonia transport out of the gill (Heisler, 1990).

The difficulty and discrepancies in determining how Rh proteins transport ammonia may be inherent in the different methodologies used and in the case of native *Xenopus* oocytes, a unique handling of ammonia. Although there are exceptions (e.g., Kikeri *et al.*, 1989; Waisbren *et al.*, 1994), most eukaryotic cells are more permeable to NH₃ than NH₄⁺. These cells, when exposed to ammonia, display a classic biphasic rise and fall of intracellular pH (pH_i) (basis of the pre-pulse method) caused by the influx of NH₃ followed by a lower influx of NH₄⁺ (Boron and DeWeer, 1976). *Xenopus* oocytes are exceptional in that exposure to high ammonia concentrations (≥ 1 mmol l⁻¹) leads to a paradoxical fall in pH_i, depolarization, and an inward current (Bakouh *et al.*, 2004; Boldt *et al.*, 2003; Burckhardt and Burckhardt, 1997; Burckhardt and Fromter, 1992). These changes are thought to be caused by an initial rapid diffusion of NH₃ into the oocyte that causes an alkalinization close to the oocyte surface which subsequently activates nonselective cation channels in which NH_4^+ could enter (Boldt *et al.*, 2003; Cougnon *et al.*, 1996). Low concentrations of ammonia (<1 mmol 1⁻¹) however, cause little change in pH_i and no inwardly induced current (Bakouh *et al.*, 2004; Holm *et al.*, 2005; Mayer *et al.*, 2006).

We measured the uptake of ammonia into Rhcg2-expressing oocytes using SIET and observed saturation uptake kinetics with a K_m for NH₄Cl of 560 µmol l⁻¹ (Fig. 5.9). The early saturation of uptake in Rhcg2-expressing oocytes compared to the control oocytes suggests that the rapid influx of ammonia mediated by Rhcg2 (at 1 mmol l⁻¹) resulted in the rapid accumulation of intracellular ammonia, which likely reduced the gradient for further uptake at higher external ammonia concentrations. Also, since Rh proteins function bi-directionally, efflux may have been enhanced when intracellular ammonia levels became elevated. Endogenous NH₄⁺ uptake pathways, triggered by external ammonia concentrations >1 mmol l⁻¹, likely explain why the ammonia uptake rate into the control oocytes was similar to that of the Rhcg2-expressing oocytes at 3 mmol l⁻¹, and surpassed the rate in Rhcg2-expressing oocytes at 10 mmol l⁻¹.

Although control oocytes demonstrated endogenous uptake of ammonia, the Rhcg2-expressing oocytes had a greater affinity for ammonia and facilitated uptake more rapidly at a lower concentration. Plasma ammonia concentrations in fasted fish are typically under 500 μ mol l⁻¹ and closer to 100-200 μ mol l⁻¹ (Wood, 1993) but

170

McMaster - Biology

postprandially, levels can increase more than 3-fold (Bucking and Wood, 2008; Wicks and Randall, 2002). Concentrations approaching 2 mmol 1^{-1} in salmonids cause toxicity (Lumsden *et al.*, 1993) and it was around this concentration (between 1-2 mmol 1^{-1}) when ammonia uptake by Rhcg2 started to saturate.

Rhcg2-mediated ammonia influx followed the same kinetic profile as that reported for RhCG-expressing oocytes, where inward currents saturated as a function of ammonia concentration with a K_m of 468 µmol l⁻¹ for NH₄Cl (Bakouh et al., 2004). While these K_m values fall within the physiological range for trout, the K_i values for methylamine inhibition by NH₄Cl measured in this study as well as in previous reports (Ludewig, 2004; Mak et al., 2006; Mayer et al., 2006) seem high (2-3 mmol l⁻¹), and the values also differed moderately between pH 7.5 and pH 8.5 (Table 2). Inhibition studies may be confounded by the endogenous uptake of ammonia by Xenopus oocytes, which appeared to be greater than the endogenous uptake of methylamine (see Fig. 5.8). Our results suggested that when the concentrations were low (0.1-0.3 mmol l⁻¹), ammonia entered the oocytes mainly via an endogenous pathway(s), whereas higher concentrations (1-10 mmol 1⁻¹) were Rh-mediated (Fig. 5.9). Therefore low concentrations of NH₄Cl would be relatively ineffective at reducing Rh-mediated methylamine uptake. Additionally, application of high NH₄Cl concentrations acidify Xenopus oocytes (Bakouh et al., 2004; Burckhardt and Fromter, 1992; Cougnon et al., 1996; Nakhoul et al., 2005), a factor that would further stimulate methylamine uptake. The result would be an underestimation of the ability of NH₄Cl to inhibit methylamine uptake.

It was concluded recently, based on pH measurements on the oocyte surface, that NH_3 rather than NH_4^+ fluxes predominate in native oocytes and that expression of AmtB in oocytes enhances these NH_3 fluxes (Musa-Aziz *et al.*, 2009). Although it is still premature to make definitive conclusions about the transport mechanism of Rh proteins, the evidence seems to point more in favour of NH_3 rather than NH_4^+ transport through these channels. One possible interpretation of the dependence of transport on the pH gradient is that uptake is mediated by an exchange of NH_4^+ with H^+ , but a diffusion trapping mechanism is equally plausible. Regardless, both mechanisms are chemically equivalent to NH_3 uptake. We suggest that NH_4^+ is deprotonated before NH_3 enters the Rh channel, however we cannot rule-out the possibility that trout Rh proteins function as electroneutral NH_4^+/H^+ exchangers as suggested for the mammalian Rh proteins (Ludewig, 2004; Mak *et al.*, 2006). Our understanding of trout Rh protein function is far from complete, and structural studies would be informative.

The functional characteristics of trout Rh proteins we observed in this study agree well with the findings reported for humans, mice, and pufferfish. Trout Rh proteins facilitated the movement of both methylamine and ammonia across the *Xenopus* oocyte membrane and the rates were dependent upon the concentration of the protonated species as well as upon the pH gradient. Therefore, the mechanism may involve binding of NH_4^+ , but transport of NH_3 . Using SIET, we obtained a K_m of 560 µmol l⁻¹ for ammonia uptake in Rhcg2-expressing oocytes, a value that lies within the physiological range for trout. This suggests that Rhcg2 is a low affinity, high capacity ammonia transporter that could exploit the acidified gill boundary layer and facilitate rapid efflux of ammonia from the trout gill when plasma ammonia levels are elevated. Basal plasma ammonia levels on the other hand, are likely maintained by passive diffusion of NH_3 out of the gill and Rh proteins may have a lesser role under these conditions.

5.6 TABLES AND FIGURES

Name	Forward / reverse sequence (5'-3')	Accession #
Rhag	ggagactattaccacaagcc / ctcactttcccatctctagc	EF667352
Rhbg1	gaccaactcatgtgtcagcttgag / gctgccacatcctggttgtac	EF051113
Rhbg2	cgacaacgacttttactaccgc / gtacaaccaggatgtggcagc	EU660221
Rhcg1	gccgtctttctccataaggcacc / ccagcaggagtccgtgtaggataggacc	DQ431244
Rhcg2	gtacttactcagcctccacc / gagtgcggttgtctgttgg	AY619986
Rh30-like	gacattccggtttcgcgtag / gattggttcattgctctcctgac	EF062577

Table 5.1 Primer list for cloning

			K _m		Ki		
	pН	MA/MA ⁺	MA ⁺	MA	NH ₃ /NH ₄ ⁺	NH4 ⁺	NH ₃
		$(\mu mol l^{-1})$	$(\mu mol l^{-1})$	$(\mu mol l^{-1})$	(µmol 1 ⁻¹)	$(\mu mol l^{-1})$	$(\mu mol l^{-1})$
Rhbg1	7.5	6822.0±	6817.3 ±	4.7 ±	2447.5 ±	2414.0 ±	38.1 ±
		2368.8	2367.2	1.6	30.5	30.5	30.5
	8.5	$4659.8 \pm$	$4627.8 \pm$	32.0 ±	$1607.1 \pm$	$1382.1 \pm$	$218.4 \pm$
		2089.3	2075.0	14.4	4.3 *	4.3 *	4.3 *
Rhcg2	7.5	$8849.9 \pm$	$8843.8 \pm$	6.1 ±	$2524.8 \pm$	$2478.7 \pm$	39.2 ±
		3600.9	3598.4	2.5	20.5	20.5	20.5
	8.5	$8566.5 \pm$	$8507.6 \pm$	58.9 ±	$1552.8 \pm$	$1340.9 \pm$	212.1 ±
		2969.1	2948.7	20.4 *	5.8 *	5.8 *	5.8 *

Table 5.2 Protonated and unprotonated fractions of K_m for methylamine and K_i values for ammonia as an inhibitor of methylamine uptake at pH 7.5 and 8.5. Asterisks represent significant differences from the corresponding pH 7.5 values. Means \pm s.e.m. (n = 3) for groups of 3 oocytes.



Figure 5.1 Rh-facilitated methylamine uptake. Time course of [¹⁴C]methylamine uptake (pmol/oocyte) measured in Rhag-, Rhbg1-, Rhcg2-, Rh30-like-, and H₂O-injected control oocytes at pH 7.5. Uptake in Rh30-like-injected oocytes was not significantly different from that of control oocytes. In each case, the concentration of methylamine was 20 μ mol l⁻¹. Data shown are means \pm s.e.m. (*n*=3) for groups of 3 oocytes.



Figure 5.2 Methylamine uptake kinetics at pH 7.5. Uptake rates (pmol/oocyte/min) of [¹⁴C]methylamine in Rhag-, Rhbg1-, Rhbg2- and Rhcg2-expressing oocytes were measured over a concentration range of 0.02-15 mmol l⁻¹ methylamine set at pH 7.5. H₂O-injected control oocytes were run in parallel and control uptake values have been subtracted from test oocyte values. Values are means \pm s.e.m. (*n*=3) for groups of 3 oocytes.



Figure 5.3 Methylamine uptake kinetics at pH 8.5. Uptake rates (pmol/oocyte/min) of $[^{14}C]$ methylamine in Rhbg1- and Rhcg2-expressing oocytes were measured over a concentration range of 0.02-15 mmol I^{-1} methylamine set at pH 8.5. H₂O-injected control oocytes were run in parallel and control uptake values have been subtracted from test oocyte values. Dashed lines represent the corresponding uptake rates at pH 7.5. Values are means \pm s.e.m. (*n*=3) for groups of 3 oocytes.



Figure 5.4 The effect of external pH on methylamine uptake. [¹⁴C]methylamine uptake rates (pmol/oocyte/min) were measured in H₂O-, Rhag-, Rhbg1-, Rhbg2-, Rhcg1-, and Rhcg2-expressing oocytes for 60 min at an external pH of 6.5, 7.5, and 8.5. In each case, the concentration of methylamine was 20 μ mol l⁻¹. Asterisks represent significant differences between the rates at pH 8.5 and those at pH 6.5 and pH 7.5. Crosses represent significant differences between pH 6.5 and pH 7.5. Values are means ± s.e.m. (*N*=3) for groups of 3 oocytes.



Figure 5.5 The effect of intracellular acidification on methylamine uptake. [¹⁴C]methylamine uptake (pmol/oocyte/min) was measured in Rhag-, Rhbg2-, Rhcg2-expressing oocytes that were acidified in sodium acetate (pH 6.4 or 6.8) for 25 min. Untreated oocytes (intracellular pH 7.29 ± 0.09) were not incubated in sodium acetate. In each case, the concentration of methylamine was 20 µmol l⁻¹. Asterisks indicate a significant increase in uptake rates in the acidified oocytes compared to the untreated oocytes. The cross indicates a significant difference between the pH 6.4 and pH 6.8 uptake rates in Rhag-expressing oocytes. H₂O-injected control oocytes were run in parallel and control uptake values have been subtracted from test oocyte values. Values are means \pm s.e.m. (*N*=3) for groups of 3 oocytes.



Figure 5.6 Methylamine efflux. H₂O-, Rhag-, Rhbg-, and Rhcg2-injected oocytes were incubated for 60 min in buffer containing 20 μ mol 1⁻¹ [¹⁴C]methylamine at pH 8.5. Oocytes were then washed and added to fresh buffer containing 0 μ mol 1⁻¹ methylamine at pH 6.5 and 8.5. Radioactivity in the buffer and oocytes was counted separately. Results are expressed as the percent radioactivity that appeared in the buffer after 15 min. Asterisks indicate significantly lower methylamine release from H₂O-injected oocytes compared to Rhag-, Rhbg1- and Rhcg2-injected oocytes. Crosses indicate significantly lower efflux at pH 8.5 compared to pH 6.5. Values are means ± s.e.m. (*n*=3) for groups of 3 oocytes.



Figure 5.7 Inhibition of methylamine uptake with NH₄Cl. [¹⁴C]methylamine concentration was held constant at 20 µmol 1⁻¹. Uptake rates (pmol/oocye/min) of Rhbg1- and Rhcg2-expressing oocytes were measured in the presence NH₄Cl ranging from 80-3500 µmol 1⁻¹ at an external pH of 7.5 and 8.5. H₂O-injected control oocytes were run in parallel and control uptake values have been subtracted from test oocyte values. Dashed lines represent the IC₅₀ values. Corresponding K_i values are listed in Table 5.2. Values are means ± s.e.m. (*n*=3) for groups of 3 oocytes.



Figure 5.8 Ammonia uptake. The uptake rate of ammonia in pmol/cm²/s (left axis) and pmol/oocyte/min (right axis) measured with the scanning ion electrode technique (SIET) in H₂O-, Rhag-, Rhcg2-, and Rh30-like-injected oocytes exposed to 100 μ mol l⁻¹ NH₄Cl. Asterisks indicate significant differences from the ammonia uptake rates in H₂O-injected oocytes. Data are means ± s.e.m. (*n*=6-10). Inset hatched bars represent corresponding [¹⁴C]methylamine uptake rates of H₂O-, Rhag-, Rhcg2-, Rhag-, Rhcg2-injected oocytes at a methylamine concentration of 100 μ mol l⁻¹.



Figure 5.9 Kinetics of ammonia uptake. The uptake of ammonia $(pmol/cm^2/s)$ was measured with the scanning ion electrode technique (SIET) in Rhcg2-, and H₂O-injected oocytes over the concentration range of 0.1-10 mmol 1⁻¹ NH₄Cl. Asterisks represent significant differences between the uptake rates in the H₂O-injected oocytes and the Rhcg2-expressing oocytes at the corresponding NH₄Cl concentration. Data are means \pm s.e.m. (*n*=5-8).

5.7 REFERENCES

- Ammann, D. (1986). Ion-Selective Microelectrodes. Principles, Design and Application. Berlin, Heidelberg, New York, Tokyo: Springer-Verlag.
- Bakouh, N., Benjelloun, F., Hulin, P., Brouillard, F., Edelman, A., Cherif-Zahar, B.
 and Planelles G. (2004). NH₃ is involved in the NH₄⁺ transport induced by the functional expression of the human Rh C glycoprotein. J. Biol. Chem. 279, 15975-15983.
- Biver, S., Belge, H., Bourgeois, S., Van Vooren, P., Nowik, M., Scohy, S., Houillier,
 P., Szpirer, J., Szpirer, C., Wagner, C. A., Devuyst, O. and Marini, A. –M. (2008). A role for Rhesus factor Rhcg in renal ammonium excretion and male fertility. Nature 456, 339-343.
- **Boldt, M., Burckhardt, G. and Burckhardt, B. -C.** (2003). NH₄⁺ conductance in *Xenopus laevis* oocytes. III. Effect of NH₃. *Eur J. Physiol.* 446, 652-657.
- Boron, W.F. and De Weer, P. (1976). Intracellular pH transients in squid giant axons caused by CO₂, NH₃, and metabolic inhibitors. *J. Gen. Physiol.* 67, 91-112.
- Braun, M. H., Steele, M., Ekker, M. and Perry S. F. (2009). Nitrogen excretion in developing zebrafish (*Danio rerio*): A role for Rh proteins and urea transporters. *Am. J. Physiol.* 296, F994-1005.
- Bucking, C. and Wood, C.M. (2008). The alkaline tide and ammonia excretion after voluntary feeding in freshwater rainbow trout. *J. Exp. Biol.* 211, 2533-2541.
- **Burckhardt, B.-C. and Burckhardt, G.** NH₄⁺ conductance in *Xenopus laevis* oocytes. I. Basic observations. *Eur. J. Physiol.* 434, 306-312.
- **Burckhardt, B.-C. and Fromter, E.** (1992). Pathways of NH₃/NH₄⁺ permeation across *Xenopus laevis* oocyte cell membrane. *Eur. J. Physiol.* 420, 83-86.
- Cameron, J. N. and Heisler, N. (1983). Studies of ammonia in the rainbow trout: physico-chemical parameters, acid-base behaviour and respiratory clearance. J. Exp. Biol. 105, 107-125.
- Ceriotti, A. and Colman, A. (1995). mRNA translation in *Xenopus* oocytes. In *Methods in Molecular Biology*, Vol. 37. (ed. M. J. Tymms), pp. 151-178. Totowa: Humana Press Inc.
- Cougnon, M., Bouyer, P., Hulin, P., Anagnostopoulos, T. and Planelles, G. (1996).

Further investigation of ionic diffusive properties and of NH_4^+ pathways in *Xenopus laevis* oocyte cell membrane. *Eur. J. Physiol.* 431, 658-667.

- **CRC Handbook of Chemistry and Physics** (2005). 86th edition. (ed. D. R. Lide). 2616 pp. CRC Press.
- **Donini, A. and O'Donnell, M. J.** (2005). Analysis of Na⁺, Cl⁻, K⁺, H⁺ and NH₄⁺ concentration gradients adjacent to the surface of anal papillae of the mosquito *Aedes aegypti*: application of self-referencing ion-selective microelectrodes. *J. Exp. Biol.* 208, 603-610.
- Endeward, V., Cartron, J. -P., Ripoche, P., and Gros, G. (2007). RhAG protein of the complex is a CO₂ channel in the human red cell membrane. FASEB J. 22, 64-73.
- Handlogten, M. E., Hong, S. -P., Zhang, L., Vander, A. W., Steinbaum, M. L., Campbell-Thompson, M. and Weiner, I. D. (2005). Expression of the ammonia transporter proteins Rh B glycoprotein and Rh C glycoproteins in the intestinal tract. Am. J. Physiol. 288, G1036-1047.
- Heisler, N. (1990). Mechanisms of ammonia elimination in fishes. In Animal Nutrition and Transport Processes. 2. Transport, Respiration, and Excretion: Comparative and Environmental Aspects (ed, J.-P,Truchot and B. Lahlou), pp. 137-151. Basel: Karger.
- Holm, L. M., Jahn, T. P., Moller, A. K., Schjoerring, J. K., Ferri, D., Klaerke, D. A. and Zeuthen, T. (2005). NH₃ and NH₄⁺ permeability in aquaporin-expressing *Xenopus* oocytes. *Eur. J. Physiol.* 450, 415-428.
- Huang, C. -H. (2008). Molecular origin and variability of the Rh gene family: an overview of evolution, genetics and function. *Haematology Education: the Education Programme for the Annual Congress of the European Hematology Association* 2, 149-157.
- Hung, C. Y. C., Tsui, K. N. T., Wilson J. M., Nawata M., Wood C. M. and Wright P. A. (2007) Molecular cloning, characterization and tissue distribution of the Rhesus Glycoproteins Rhbg, Rhcg1 and Rhcg2 in the Mangrove killifish *Rivulus marmoratus* exposed to elevated environmental ammonia levels. J. Exp. Biol. 210, 2419-2429.
- Hung, C. Y. C., Nawata, C. M, Wood, C. M. and Wright, P. A. (2008). Rhesus glycoprotein and urea transporter genes are expressed in early stages of development of rainbow trout (*Oncorhynchus mykiss*). J. Exp. Zool. 309A, 262-268.

- Javelle, A., Lupo, D., Li, X. D., Merrick, M., Chami, M., Ripoche, P. and Winkler, F. K. (2007). Structural and mechanistic aspects of Amt/Rh proteins. J. Struct. Biol. 158, 472-481.
- Javelle, A., Severi, A., Thornton, J. and Merrick M. (2004). Ammonium sensing in Escherichia coli. J. Biol. Chem. 279, 8530-8538.
- Javelle, A., Thomas, G., Marnini, A. -M., Kramer, R. and Merrick M. (2005). *In vivo* functional characterization of the *Escherichia coli* ammonium channel AmtB: evidence for metabolic coupling of AmtB to glutamine synthetase. *Biochem. J.* 390, 215-222.
- Kelly, S. P. and Wood, C. M. (2001) The cultured branchial epithelium of the rainbow trout as a model for diffusive fluxes of ammonia across the fish gill. *J. Exp. Biol.* 204, 4115-4124.
- Khademi, S., O'Connell III, J., Remis, J., Robles-Colmenares, Y., Mierke, L. J. W. and Stroud, R. M. (2004). Mechanism of ammonia transport by Amt/MEP/Rh: structure of AmtB at 1.35 Å. *Science* 305, 1587-1594.
- Kikeri, D., Sun, A., Zeidel, M. L., and Hebert, S. C. (1989). Cell membranes impermeable to NH₃. *Nature* 339, 478-480.
- Kim, H. -Y., Verlander, J. W., Bishop, J. M., Cain, B. D., Han, K. -H., Igarashi, P., Lee, H. -W., Handlogten, M. E. and Weiner, I. D. (2009). Basolateral expression of the ammonia transporter family member Rh C glycoprotein in the mouse kidney. Am. J. Physiol. 296, F543-555.
- **Kleiner, D.** (1981). The transport of NH₃ and NH₄⁺ across biological membranes. *Biochim. Biophys. Acta*. 639, 41-52.
- Knepper, M. A., Packer, R. and Good, D.W. (1989). Ammonium transport in the kidney. *Physiol. Rev.* 69, 179-249.
- Kustu, S. and Inwood, W. (2006). Biological gas channels for NH₃ and CO₂: evidence that Rh (Rhesus) proteins are CO₂ channels. *Transfus. Clin. Biol.* 13, 103-110.
- Lee, H. -W., Verlander, J. W., Bishop, J. M., Igarashi, P., Handlogten, M. E. and Weiner, I. D. (2009). Collecting duct-specific Rh C glycoprotein deletion alters basal and acidosis-stimulated renal ammonia excretion. Am. J. Physiol. 296, F1364-1375.
- Li, X., Jayachandran, S., Nguyen, H. H. T. and Chan, M. (2007). Structure of the

Nitrosomonas europaea Rh protein. Proc. Natl. Acad. Sci. USA 104, 19279-19284.

- Liu, Z., Chen, Y., Mo, R., Hui, C., Cheng, J. -F., Mohandas, N. and Huang, C. -H. (2000). Characterization of human RhCG and mouse Rhcg as novel nonerythroid Rh glycoprotein homologues predominantly expressed in kidney and testis. *J. Biol. Chem.* 275, 25641-25651.
- Liu, Z., Peng, J., Mo, R., Hui, C. and Huang, C. -H. (2001). Rh type B glycoprotein is a new member of the Rh superfamily and a putative ammonia transporter in mammals. J. Biol. Chem. 276, 1424-1438.
- Ludewig, U. (2004). Electroneutral ammonium transport by basolateral rhesus B glycoprotein. J. Physiol. 559, 751-759.
- Lumsden, J. S., Wright, P., Derksen, J., Byrne, P. J. and Ferguson, H. W. (1993). Paralysis in farmed Arctic char (*Salvelinus alpinus*) associated with ammonia toxicity. *Vet. Rec.* 133, 422-423.
- Lupo, D., Li, X. D., Curand, A., Tomikazi, T., Cherif-Zahar, B., Matassi, G., Merrick, M. and Winkler, F. K. (2007). The 1.3Å resolution structure of *Nitrosomonas europaea* Rh50 and mechanistic implications for NH₃ transport by Rhesus family proteins. *Proc. Natl. Acad. Sci. USA* 104, 19303-19308.
- Mak, D. O. D., Dang, B., Weiner I. D., Foskett, J. K. and Westhoff, C. M. (2006). Characterization of ammonia transport by the kidney Rh glycoproteins RhBG and RhCG. *Am. J. Physiol.* 290, F297-305.
- Marini, A. M., Matassi, G., Raynal, V., André, B., Cartron, J. –P. and Cherif-Zahar, B. (2000). The human Rhesus-associated RhAG protein and a kidney homolgue promote ammonium transport in yeast. *Nat. Genet.* 26, 341-344.
- Marini, A. -M., Urrestarazu, A., Beauwens, R. and André, B. (1997). The Rh (Rhesus) blood group polypeptides are related to NH₄⁺ transporters. *Trends Biochem. Sci.* 22, 460-461.
- Marini, A. -M., Vissers, S., Urrestarazu, A. and André B. (1994). Cloning and expression of the MEP1 gene encoding an ammonium transporter of *Saccharomyces cerevisiae*. *EMBO J*. 13, 3456-3463.
- Mayer, M., Schaaf, G., Mouro, I., Lopez., Colin, C., Neumann, P., Cartron, J. -P. and Ludewig, U. (2006). Different transport mechanisms in plant and human AMT/Rh-type ammonium transporters. J. Gen. Physiol. 127, 133-144.

- Merrick, M., Blakey, D., Coutts, G. and Thomas, G. (2001). Bacterial ammonium transport proteins: structure and function. In *Nitrogen fixation: global perspectives : proceedings of the 13th International Congress on Nitrogen Fixation, Hamilton, Ontario, Canada, 2-7 July 2001.* (eds. T. M. Finan, M. R., O'Brian, D. B. Layzell, J. K. Vassey, W. Newton). pp. 223-227. Great Britain: CABI Publishing.
- Musa-Aziz, R., Jiang, L., Chen, L. -M., Behar, K. L. and Boron, W. F. (2009). Concentration-dependent effects on intracellular and surface pH of exposing *Xenopus* oocytes to solutions containing NH₃/NH₄⁺. *J. Membrane Biol.* 228, 15-31.
- Nakada, T., Westhoff, C. M., Kato, A. and Hirose, S. (2007a). Ammonia secretion from fish gill depends on a set of Rh proteins. *FASEB J.* 21, 1067-1074.
- Nakada, T., Hoshijima, K., Esaki, M., Nagayoshi, S., Kawakami, K. and Hirose, S. (2007b). Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. Am. J. Physiol. 293, R1743-1753.
- Nakhoul, N. L., DeJong, H., Abdulnour-Nakhoul, S. M., Boulpaep, E. L., Hering-Smith, K. and Hamm, L. L. (2005). Characteristics of renal Rhbg as an NH₄⁺ transporter. Am. J. Physiol. 288, F170-181.
- Nawata, C. M., Hung, C. C. Y., Tsui, T. K. N., Wilson, J. M., Wright, P. A. and Wood, C. M. (2007). Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H⁺-ATPase involvement. *Physiol. Genomics* 31, 463-474.
- Nawata, C. M. and Wood, C. M. (2008). The effects of CO₂ and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. *J. Exp. Biol.* 211, 3226-3236.
- Nawata, C. M. and Wood, C. M. (2009). mRNA analysis of the physiological responses to ammonia infusion in rainbow trout. J. Comp. Physiol. B. 179, 799-810.
- Ninnemann, O., Jauniaux, J. -C. and Frommer, W. B. (1994). Identification of a high affinity NH₄⁺ transporter from plants. *EMBO J.* 13, 3464-3471.
- Perry, S. F., Esbaugh A., Braun, M. and Glimour, K. M. (2009). Gas transport and gill function in water-breathing fish. In *Cardio-Respiratory Control in Vertebrates.* (eds. M. L. Glass and S. C. Woods), pp. 5-42. Berlin: Springer-Verlag.

- **Rheault, M. R. and O'Donnell, M. J.** (2004). Organic cation transport by Malpighian tubules of *Drosophila melanogaster:* Application of two novel electrophysiological methods. *J. Exp. Biol.* 207, 2173-2184.
- **Ripoche, P., Bertrand, O., Gane, P., Birkenmeier, C., Colin, Y., and Cartron, J. -P.** (2004). Human Rhesus-associated glycoprotein mediates facilitated transport of NH₃ into red blood cells. *Proc. Natl. Acad. Sci.* 101, 17222-17227.
- Shih, T. -H., Horng, J. -L., Hwang, P. -P. and Lin, L. -Y. (2008) Ammonia excretion by the skin of zebrafish (*Danio rerio*) larvae. *Am. J. Physiol.* 295, C1625-1632.
- Soupene, E., Inwood, W. and Kustu, S. (2004). Lack of the Rhesus protein Rh1 impairs growth of the green alga *Chlamydomonas reinhardtii* at high CO₂. *Proc. Natl. Acad. Sci. USA* 101, 7787-7792.
- Soupene, E., King, N., Field, E. Liu, P., Niyogi, K. K., Huang, C. H. and Kustu, S. (2002). Rhesus expression in a green alga is regulated by CO₂. *Proc. Natl. Acad. Sci. USA* 99, 7769-7773.
- **Tsai, T. D., Shuck, M. E., Thompson, D. P., Bienkowski, M. J. and Lee, K. S.** (1995). Intracellular H⁺ inhibits a cloned rat kidney outer medulla K⁺ channel expressed in *Xenopus* oocytes. *Am. J. Physiol.* 268, C1173-1178.
- Tsui, T. K. N., Hung, C. Y. C., Nawata, C. M., Wilson, J. M., Wright, P. A. and Wood, C. M. (2009). Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical Na⁺/NH₄⁺ exchange complex. J. Exp. Biol. 212, 878-892.
- Weidinger, K., Neuhauser, B., Gilch, S., Ludewig, U., Meyer, O. and Schmidt, I. (2007). Functional and physiological evidence for a Rhesus-type ammonia transporter in *Nitrosomonas europaea*. *FEMS Microbiol. Lett.* 273, 260-267.
- Weihrauch, D., Wilkie, M. P. and Walsh, P. J. (2009). Ammonia and urea transporters in gill of fish and aquatic crustaceans. J. Exp. Biol. 212, 1716-1730.
- Westhoff, C. M., Ferri-Jacobia, M., Mak, D. O. D. and Foskett, J. K. (2002). Identification of the erythrocyte Rh blood group glycoprotein as a mammalian ammonium transporter. J. Biol. Chem. 277, 12499-12502.
- Westhoff, C. M., Siegel, D. L., Burd, C. G. and Foskett, J. K. (2004). Mechanism of genetic complementation of ammonium transport in yeast by human erythrocyte Rh-associated glycoprotein. J. Biol. Chem. 279, 17443-17448.

Westhoff, C. M. and Wylie, D. E. (2006). Transport characteristics of mammalian Rh

and Rh glycoproteins expressed in heterologous systems. *Transfus. Clin. Biol.* 13, 132-138.

- Wicks, B. J. and Randall, D. J. (2002). The effect of feeding and fasting on ammonia toxicity in juvenile rainbow trout, *Oncorhynchus mykiss. Aquatic Toxicol.* 59, 71-82.
- Wilson, R. W., Wright, P. M., Munger, S. and Wood, C. M. (1994). Ammonia excretion in fresh water rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: lack of evidence for Na⁺/NH₄⁺ exchange. *J. Exp. Biol.* 191, 37-58.
- Wood, C. M. (1993). Ammonia and urea metabolism and excretion. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 379-425. Boca Raton: CRC Press.
- Wright, P. A., Heming, T. and Randall, D. J. (1986). Downstream pH changes in water flowing over the gills of rainbow trout. J. Exp. Biol. 126, 499-512.
- Wright, P. A., Randall, D. J. and Perry, S. F. (1989). Fish gill boundary layer: a site of linkage between carbon dioxide and ammonia excretion. J. Comp. Physiol. 158, 627-635.
- Wright, P. A. and Wood, C. M. (1985). An analysis of branchial ammonia excretion in the freshwater rainbow trout: effects of environmental pH change and sodium uptake blockade. J. Exp. Biol. 114, 329-353.
- Wright, P. A. and Wood, C. M. (2009). A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. *J. Exp. Biol.* 212, 2302-2312.
- Zheng, L., Kostrewa, D., Berneche, S., Winkler, F. K. and Li, X. -D. (2004). The mechanism of ammonia transport based on the crystal structure of AmtB of *Escherichia coli. Proc. Natl. Acad. Sci.* 101, 17090-17095.
- Zidi-Yahiaoui, N, Callebaut, I., Genetet, S., Van Kim, C. L., Cartron, J. –P., Colin, Y., Ripoche, P. and Mouro-Chanteloup, I. (2009). Functional analysis of human RhCG: a comparison with *E. coli* ammonium transporter reveals similarities in the pore and differences in the vestibule. *Am. J. Physiol.* 297, C537-547.

CHAPTER 6

DISCUSSION

192

6.1 Preface

The involvement of Rh proteins as potential modulators of gill ammonia excretion was analyzed in the freshwater rainbow trout and five hypotheses were tested. The initial hypothesis was that a number of Rh genes are present in the trout gill as well as in other tissues. Cloning several Rh cDNAs from the trout gill and determining their tissue distribution evaluated this hypothesis. Following this positive result, a second hypothesis was that these Rh genes would respond to experimental manipulations that elevated the plasma ammonia concentrations. Therefore Rh mRNA expression levels, as well as ammonia excretion rates were monitored following exposure to HEA and ammonia infusion. A third hypothesis was that other transporters and enzymes thought to be involved in the ammonia excretion mechanism in the trout gill would also respond to ammonia loading. This was tested by measuring the mRNA levels of these proteins in the ammonia-loading experiments mentioned above. A fourth hypothesis postulated that Rh proteins would also respond to an increase in external CO₂. Rh mRNA levels were therefore measured in tissues after trout were exposed to environmental hypercapnia. The final hypothesis was that trout Rh proteins function as ammonia transporters by binding NH4⁺ and conducting NH3. This was tested in *Xenopus* oocytes expressing trout Rh proteins using [14C]methylamine to characterize the transport of the ammonia analogue methylamine, and SIET to measure ammonia transport. The major conclusions are highlighted below.

6.2 Many Rh genes are present in the rainbow trout

As detailed in Chapters 2 and 3, a total of ten Rh cDNA sequences were identified in the trout tissues. Ancient and recent genome duplication events in salmonids (Allendorf and Thorgaard 1984; Wittbrodt *et al.*, 1998) may provide at least a partial explanation, but the reason for the seeming redundancy of these Rh genes in trout is unknown. Interestingly, only fish have two Rhcg genes (Huang, 2008). Rhbg1 had the broadest tissue distribution, but all of the Rh cDNAs were present in the gill, except for Rhag and Rh30-like which may be restricted to the RBCs. A similar pattern of tissue distribution was reported in the mangrove killifish (Hung *et al.*, 2007), but in the pufferfish, these genes were restricted to cells in the gill epithelium and in the erythroid tissues (Nakada *et al.*, 2007a).

6.3 Trout Rh mRNA levels change in response to both HEA and ammonia infusion

Exposure to HEA (Chapter 2) resulted in an initial net uptake of ammonia into the fish that resulted in an elevated level of plasma ammonia. This was followed by the subsequent recovery and enhancement in the rate of ammonia excretion after 12 hours. Likewise, ammonia infusion (Chapter 4), which quickly elevated the plasma ammonia, was accompanied by an increased ammonia excretion rate. In both cases, the mRNA expression levels of Rhcg2 were significantly elevated in the gill. Rhbg1 mRNA was also upregulated in the pavement cell fraction, but only after 48 hours of HEA and was similarly upregulated with ammonia infusion. mRNA levels of Rhcg1 did not change in response to HEA but were upregulated after ammonia infusion. Overall Rhcg2 in the gill
appeared to be the most sensitive gene, responding quickly to elevations in plasma ammonia, whereas Rhbg and Rhcg1 responded later and only when levels were very high. The expression of Rhbg, Rhcg1, and Rhcg2 mRNA was greatest in the pavement cell fraction of the gill epithelium. Since the pavement cells constitute the greatest proportion of cells in the branchial epithelium (Evans *et al.*, 2005; Perry, 1998; Wilson and Laurent, 2002), they would likely be the powerhouses of ammonia excretion in the freshwater trout gill.

6.4 Other trout gill proteins also respond to elevated plasma ammonia

As discussed in Chapter 1, other proteins in the gill are thought to be involved in the ammonia transport mechanism, either directly or indirectly. This thesis focused on four particular proteins: H⁺-ATPase, NHE2, NKA, and cytoplasmic carbonic anhydrase (CA2). The transcript levels of all of these proteins changed in response to high plasma ammonia as described in more detail below.

6.4.1 Importance of H^+ -ATPase and an acidified gill boundary layer

It has long been thought that a gill boundary layer, acidified by H⁺-ATPase and/or the hydration of CO₂, facilitated ammonia excretion (Lin and Randall, 1990; Lin *et al.*, 1994; Wright *et al.*, 1986, 1989). Results from Chapters 2, 3, and 4 confirmed a role for H⁺-ATPase and an acidified gill boundary layer. Upregulated H⁺-ATPase mRNA levels and enzyme activity accompanied the increase in plasma ammonia, enhanced ammonia excretion, and upregulated Rhcg2 mRNA levels when plasma ammonia was elevated by HEA exposure. The same upregulation of H⁺-ATPase mRNA coincided with the upregulated Rhcg2 mRNA and elevated plasma ammonia and ammonia excretion rates achieved after ammonia infusion. Elimination of the acidified boundary layer by Hepes resulted in the exact opposite effect. Ammonia excretion was reduced and both H⁺-ATPase and Rhcg2 mRNA levels were downregulated.

6.4.2 NHE2 and NKA may be involved in the ammonia excretion mechanism

No changes in NHE2 mRNA expression were observed in whole gill or in the pavement and MR gill cell fractions after HEA exposure (Chapter 2) however, after ammonia infusion, NHE2 mRNA levels were elevated (Chapter 4). Tsui *et al.* (2009) reported that in a cultured gill cell preparation, NHE2 transcripts were elevated after pre-exposure to very high (2 mmol L^{-1}) basolateral NH₄Cl concentrations. This reinforces the possibility that NHE2 may have role in the ammonia transport mechanism, especially after plasma ammonia reaches very high levels as occurred after ammonia infusion.

HEA exposure did not result in changes in NKA at the transcript or activity level in whole gill samples, however there was an upregulation of NKA mRNA in the pavement cell fraction after 48 hours (Chapter 2). Similarly, after ammonia infusion, NKA mRNA levels were upregulated (Chapter 4). Therefore, NKA may also have a role during the ammonia excretion process, again when the plasma ammonia levels are highly elevated. However, whether this transporter is directly involved (i.e. replacement of K⁺ with NH₄⁺) or whether it is upregulated in association with increased NHE2 activity and an accompanying increase in Na⁺ uptake, is unclear.

6.4.3 Carbonic anhydrase (CA2) is downregulated during HEA

HEA exposure resulted in the downregulation of CA2 mRNA and activity levels (Chapter 2). This same response by CA2 mRNA was observed in a cultured trout gill cell system after pre-exposure to high basolateral ammonia (Tsui et al., 2009). Ammonia infusion however, did not result in a change in CA2 mRNA levels (Chapter 4). The surgical procedure itself that was used to infuse ammonia resulted in an upregulation of CA2 mRNA, and therefore it is possible that the true response by CA2 was masked by the procedure. The downregulation of CA2 is difficult to interpret. If ammonia is deprotonated before passing through the Rh channels as is the case with AmtB, then elevated plasma ammonia levels would result in an accumulation of excess of intracellular H⁺ ions as NH₃ passes through the apical Rh channel. Therefore, the lowered abundance and activity of CA2 suggests that this enzyme is no longer the only source of H^+ ions for the H^+ -ATPase, but that the excess H^+ ions produced after NH_4^+ deprotonation are also fueling the H⁺-ATPase and/or a exiting via an apical NHE. Wright and Wood (2009) however, suggest that because approximately 10-fold more CO₂ is excreted across the gill than ammonia, it is the CO₂ hydration reaction that fuels the H⁺-ATPase and therefore the downregulation of CA2 prevents intracellular acidification. Furthermore, there is now evidence (Hirata et al., 2003; Perry et al., 2003; Scott et al., 2005; Parks et al., 2007) that a Na⁺/HCO₃⁻ co-transporter (NBC1), fuelled by CA2, could function as a "3 $HCO_3^- + 1 Na^+$ " transporter removing HCO_3^- and Na^+ from the gill cell into the plasma across the basolateral membrane, in addition to the traditional role of NKA in this regard. Exit of intracellular HCO_3^- is equivalent to H^+ entry in acid-base

terms, so again, downregulation of CA2 would limit intracellular acidification. Clearly, more research is needed to determine the role of CA2 when plasma ammonia levels are elevated.

6.5 Trout Rh mRNA levels do not change in response to high external CO₂

External hypercapnia lowered the water pH and enhanced the ammonia excretion rate (Chapter 3). As a result, plasma ammonia was maintained at low levels and no changes in Rh mRNA levels were noted. Hepes buffering reduced the acidification caused by the external hypercapnia but also eliminated the acidified gill boundary layer (Chapter 3). There was decreased ammonia excretion and elevated plasma ammonia with a concurrent downregulation of Rhcg2 in the gill and upregulation of Rhcg2 in the skin after exposure to Hepes during both normocapnia and hypercapnia. These changes in Rh mRNA levels appeared to reflect a response to the high plasma ammonia rather than to high CO₂. The mRNA expression levels of other gill proteins, specifically the upregulation of NHE2 and CA2, reflected a response to an acidosis. Overall the results supported the conclusion that trout Rh proteins do not function as CO₂ gas conduits. However, because Rhag mRNA levels in the RBCs decreased during normocapnia and Hepes exposure, but increased during hypercapnia and Hepes exposure, the possibility that Rhag responds to both ammonia and CO₂ cannot be ruled out.

6.6 Trout Rh proteins bind NH4⁺ but transport NH3

Measurements of ammonia uptake by heterologously expressed proteins in Xenopus oocytes are difficult due to a lack of either a long-lived radiotracer for ammonia or probes to measure intracellular ammonia. Therefore, [14C]methylamine is most commonly used to study the transport properties of Rh proteins expressed in oocytes. SIET allows for sensitive and direct measurements of ammonia flux and this technique was employed in addition to $[^{14}C]$ methylamine in the study of Chapter 5. Oocytes expressing trout Rh proteins facilitated the saturable transport of methylamine. This transport was sensitive to a pH gradient and to the concentration of the protonated species. These trout Rh proteins also facilitated the transport of ammonia. Notably, both the affinities and transport rates for ammonia were higher than those for methylamine. Ammonia uptake by Rhcg2-expressing oocytes was saturable with a K_m of 560 µmol L⁻¹, a value that lies within the physiological range for trout. Together the results of these experiments indicate that trout Rh proteins are low affinity, high capacity ammonia transporters that function optimally when plasma ammonia levels are elevated. Binding and deprotonation of NH₄⁺ likely occurs before NH₃ passes through the Rh channel and the presence of an acidified gill boundary layer provides a favourable pH gradient that allows for the rapid transport of ammonia out of the gill.

6.7 Other studies on the involvement of Rh proteins in ammonia transport in fish

The discovery of an Rh-like ammonia transporter in the gills of the shore crab (*Carcinus maenas*) (Weihrauch *et al.*, 2004), followed by a comprehensive phylogenetic study of Rh genes by Huang and Peng in 2005, stimulated a number of other recent

investigations into the role of Rh proteins as ammonia transporters in fish. A brief overview these studies is presented in the following sections.

6.7.1 More evidence for Na⁺ involvement in ammonia excretion

As mentioned in Chapter 1, a long-standing debate is whether or not Na⁺ uptake is linked to ammonia excretion in freshwater fish gills. Two recent studies have provided further evidence for this linkage. Nakada et al. (2007b) reported that Rhcg1 mRNA was upregulated in zebrafish larvae exposed to low external Na⁺ conditions. Similarly, in a cultured trout gill preparation, pre-exposure to low apical Na⁺ resulted in an upregulation of Rhcg2 mRNA (Tsui et al., 2009). In the model proposed in Chapter 2, an apical Rhcg2 and H⁺-ATPase function cooperatively such that each NH₃ molecule which passes out of the apical Rh channel, combines with a H⁺ ion released from the H⁺-ATPase to form NH4⁺. Upregulation of Rhcg2 would result in an increase in NH3 efflux with a concomitant increase in H⁺-ATPase activity and production of NH₄⁺. This would provide the driving force necessary for Na⁺ uptake through Na⁺ channels and may explain why ammonia loading, which stimulated ammonia excretion, also stimulated Na⁺ uptake in many studies (Maetz and Garcia Romeu, 1964; Salama et al., 1999: Wilson et al., 1994). Tsui et al. (2009) also observed that pre-treatment of the cultured trout gill epithelium with specific inhibitors of H⁺-ATPase, NHE2, and Na⁺ channels resulted in an inhibition of ammonia excretion. They therefore proposed a model linking Na⁺ uptake with ammonia excretion via an apical Na⁺/NH₄⁺ exchange complex consisting of different transporters (Rh proteins, H⁺-ATPase, NHE2, and Na⁺ channels) functioning together.

6.7.2 The linkage between Rh proteins and H^+ -ATPase in zebrafish larvae

Using *in situ* hybridization and immunostaining, Nakada *et al.* (2007b) localized Rhcg1 to the apical region of a subpopulation of vacuolar-type H^+ -ATPase mitochondriarich cells (HRCs) on the yolk sac of zebrafish larvae 3 days post fertilization (dpf) and in the HRCs in the gills of 4-5 dpf larvae. Subsequently, Shih *et al.* (2008) showed with SIET that both ammonia and H^+ excretion occurred from these HRCs of the yolk sac skin in the zebrafish larvae. Inhibition of H^+ -ATPase with bafilomycin or morpholino knockdown of Rhcg1 reduced ammonia efflux, indicating a coupling of ammonia efflux to H^+ ion release.

6.7.3 Rh proteins in the early life stages of fish

Apart from the studies performed on zebrafish larvae described above, two additional studies have examined Rh genes and proteins in the early life stages of fish. In 2008, Hung *et al.* reported that Rhbg, Rhcg1, and Rhcg2 mRNA were all expressed in rainbow trout embryos and that mRNA expression levels of Rhcg2 correlated with an increase in ammonia excretion rates over developmental time. Later, Braun *et al.* (2009) used morpholino knockdown experiments to show that each of the proteins, Rhag, Rhbg, and Rhcg1 were essential to normal ammonia excretion in zebrafish larvae.

6.7.4 Amphibious air-breathing fish

Rh proteins have also been identified in two amphibious air-breathing fish: the mangrove killifish (*Kryptolebias marmoratus*) (Hung *et al.*, 2007) and the Asian

weatherloach (Misgurnus anguillicaudatus) (Moreira-Silva et al., 2009). In the latter, Rhcg1 was co-localized with H⁺-ATPase in the gill and the mRNA levels of Rhcg1 increased with aerial exposure. The importance of an acidified gill boundary layer was also demonstrated in the weatherloach after inhibition of H⁺-ATPase with bafilomycin treatment decreased net ammonia flux and increased plasma ammonia levels. Although Hung et al. (2007) did not measure gill Rh mRNA levels after aerial exposure, Rhcg1 and Rhcg2 mRNA expression in the skin of the mangrove killifish increased with this treatment. It was reported earlier that ammonia was volatilized from the skin surface of the mangrove killifish (Frick and Wright, 2002; Litwiller et al., 2006) and therefore it was proposed that Rh proteins in the skin might be mediating this process. In their review, Weihrauch et al. (2009) incorporated Rhcg1 and Rhcg2 proteins into a model of ammonia volatilization from the cutaneous surface of the mangrove killifish during air exposure. Included in the model are the basolateral entry of NH₃ via Rhbg with the additional entry of NH_4^+ to generate the high intracellular ammonia and P_{NH3} levels necessary to facilitate NH₃ transport to the skin surface.

6.7.5 Marine elasmobranch

Urea is retained in the tissues of elasmobranchs at high concentrations for osmotic water retention (Anderson 2001; Ballantyne 1997; Perlman and Goldstein 1998). Recently, Anderson *et al.* (2009) used quantitative real-time PCR to demonstrate that Rhbg was present in the intestine, rectal gland and kidney of the little skate (*Leucoraja erinacea*). It has been proposed that a significant amount of urea may be reabsorbed

across the intestinal epithelia in elasmobranch fish (Wood *et al.*, 2007). However because the relative mRNA expression of the urea transporter was several-fold lower than that of Rhbg in the little skate tissues, Anderson *et al.* (2009) hypothesized that ureolytic bacteria hydrolyze urea to ammonia in the gut such that these fish are forced to reabsorb nitrogen in the form of ammonia *via* Rhbg.

6.8 Overall implications of work

This thesis reports research that has identified Rh proteins as additional components to the gill ammonia excretion mechanism of rainbow trout. We can now revise the model of ammonia transport at the trout gill to include these transporters (Fig. 6.1). In particular, Rhcg2 in the pavement cells, together with H⁺-ATPase that acidifies the gill boundary layer, appear to be key players. Ammonia enters the cell *via* a basolateral Rhbg1 and/or Rhbg2 and exits the cell *via* an apical Rhcg2. A H⁺ ion is stripped off NH₄⁺ as it enters Rhcg2 so that only NH₃ passes through the channel. In the gill boundary layer, NH₃ combines with a H⁺ ion released from H⁺-ATPase and/or NHE2 and forms NH₄⁺. Basolateral NBC1 and NKA may also be recruited into this process to facilitate the simultaneous uptake of Na⁺. As low affinity high capacity ammonia transporters, Rh proteins would facilitate the rapid transport of ammonia out of the gill by exploiting the favourable pH gradient formed by the acidified gill boundary. Rh proteins would function optimally when plasma ammonia levels are elevated whereas basal levels of plasma ammonia are likely maintained mainly by the passive diffusion of NH₃.

6.9 Future directions

Although we can now include Rh proteins in the model of trout gill ammonia excretion, results from this thesis work have also generated several questions that remain to be answered. Described below are three topics that would be of particular interest for further investigation.

6.9.1 Localization and distribution of trout Rh proteins

In the revised model of trout gill ammonia transport, it is assumed that Rhcg2 is limited to the apical region while Rhbg1/Rhbg2 is located in the basolateral region of the pavement cells. This assumption is based on the immunohistochemical study by Nakada *et al.* (2007a) that localized Rhcg2 to the apical side and Rhbg to the basolateral region of the pufferfish pavement cells. It is not certain however, whether or not the same distribution of Rh proteins occurs in all fish species (Wright and Wood, 2009). Therefore, trout Rh antibody production followed by immunohistochemical analyses of the gill would be informative. Trout Rh specific antibodies would also be useful to determine levels of protein changes in response to elevated plasma ammonia and to verify whether or not these changes correlate with mRNA expression levels.

6.9.2 A role for trout skin in ammonia excretion?

Although fish skin is thought to have a minimal role in ammonia excretion in freshwater fish (Wood, 1993), Shih *et al.* (2008) recently reported that Rhcg1 in the skin of freshwater zebrafish larvae facilitated ammonia excretion. Results obtained from this

204

thesis work also provide evidence that Rh-mediated ammonia transport may occur in the skin of the adult freshwater trout. Both the presence of Rh mRNA in the skin and the observation that expression levels of these correlated with elevations in plasma ammonia suggest that they may be facilitating ammonia passage across the skin. The skin may be an alternative route for ammonia elimination especially when ammonia excretion is blocked at the gill by HEA or well-buffered water (e.g. Hepes treatment), however studies are needed to confirm this possibility. Divided chambers used to isolate the gills from the rest of the fish body (e.g. Ip *et al.*, 2004; Sayer and Davenport, 1987) or Ussing chambers would be useful for clarifying this.

6.9.3 Signaling mechanism of trout Rh genes

The regulatory mechanism of Rh protein expression is currently unknown. Cortisol has been shown to regulate the transcript levels of a number of genes (e.g. Ivanis *et al.*, 2008; Kiilerich *et al.*, 2007; McCormick *et al.*, 2008). As noted in Chapter 4, infusion of ammonia resulted in a cortisol surge followed later by the upregulation of Rh mRNA in the gill. Plasma cortisol levels also increase markedly during HEA exposure in trout (Ortega *et al.*, 2005; Tsui *et al.*, 2009). A transient surge in cortisol was associated with an increase in the activity, mRNA, and protein levels of glutamine synthetase in the liver of the gulf toadfish (Hopkins *et al.*, 1995; Kong *et al.*, 2000). Similarly, cultured trout gill epithelia pre-exposed to cortisol and ammonia responded with upregulations of Rhbg and Rhcg2 mRNA levels. (Tsui *et al.*, 2009). Very recently, two glucocorticoid response elements (GRE) were identified in the promoter region of gulf toadfish

glutamine synthetase (Esbaugh and Walsh, 2009). Therefore, it would be interesting to determine whether or not GREs are similarly present in Rh genes and this could be accomplished by analyzing the promoter regions of the corresponding genomic DNA sequences.



Figure 6.1 Proposed model of ammonia excretion in the pavement cell of the freshwater rainbow trout gill.

REFERENCES

(for Chapters 1 and 6)

- Allendorf, F. W. and Thorgaard, G. H. (1984). Tetraploidy and the evolution of salmonid fishes. In *Evolutionary Genetics of Fishes* (ed. B. J. Turner), pp.1-46. New York: Plenum Press.
- Anderson, P. M. (2001) Urea and glutamine synthesis: Environmental influences on nitrogen excretion. In *Nitrogen Excretion* (eds. P. A. Wright and P. M. Anderson), pp. 239-278. San Diego: Academic Press.
- Anderson, W. G., Dasiewicz, P. J., Liban, S., Ryan, C., Taylor, J. R., Grosell, M. and Weihrauch, D. (2009) Gastro-intestinal handling of water and solutes in three species of elasmobranch fish, the whitespotted bamboo shark, *Chiloscyllium plagiosum*, little skate, *Leucoraja erinacea* and the clear nose skate *Raja eglanteria*, *Comp. Biochem. Physiol.* doi:10.1016/j.cbpa.2009.09.020.
- Avella, M. and Bornancin, M. (1989). A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). J. Exp. Biol. 142, 155-175.
- Bakouh, N., Benjelloun, F., Hulin, P., Brouillard, F., Edelman, A., Cherif-Zahar, B. and Planelles G. (2004). NH₃ is involved in the NH₄⁺ transport induced by the functional expression of the human Rh C glycoprotein. J. Biol. Chem. 279, 15975-15983.
- Ballantyne, J. S. (1997). Jaws: the inside story: the metabolism of elasmobranch fishes. *Comp. Biochem. Physiol.* 118B, 703–742.
- **Balm, P., Goosen, N., van de Rijke, S. and Bonga, S. W.** (1988). Characterization of transport Na⁺-ATPases in gills of freshwater tilapia. Evidence for branchial Na⁺/H⁺ (-NH₄⁺), ATPase activity in fish gills. *Fish Physiol. Biochem.* 5, 31-38.
- Biver, S., Belge, H., Bourgeois, S., Van Vooren, P., Nowik, M., Scohy, S., Houillier,
 P., Szpirer, J., Szpirer, C., Wagner, C. A., Devuyst, O. and Marini, A. –M. (2008). A role for Rhesus factor Rhcg in renal ammonium excretion and male fertility. *Nature* 456, 339-343.
- **Boldt, M., Burckhardt, G. and Burckhardt, B. C.** (2003). NH₄⁺ conductance in *Xenopus laevis* oocytes. III. Effect of NH₃. *Eur. J. Physiol.* 446, 652-657.
- Braun, M. H., Steele, M., Ekker, M. and Perry S. F. (2009). Nitrogen excretion in developing zebrafish (*Danio rerio*): A role for Rh proteins and urea transporters. *Am. J. Physiol.* 296, F994-1005.
- Brockway, D. R. (1950). Metabolic products and their effects. *Progve. Fish. Cult.* 12, 127-129.

- Brown, A. C. N., Hallouane, D., Mawbt, W. J., Karet, F. E., Saleem, M. A., Howie, A. J. and Toyes, A. M. (2009). RhCG is the major putative ammonia transporter expressed in the human kidney, and RhBG is not expressed at detectable levels. *Am. J. Physiol.* 296, F1279-1290.
- **Burckhardt, B. -C. and Fromter, E.** (1992). Pathways of NH₃/NH₄⁺ permeation across *Xenopus laevis* oocyte cell membrane. *Eur. J. Physiol.* 420, 83-86.
- Callebaut, I., Dulin, F., Bertrand, O., Ripoche, P., Mouro, I., Colin, Y., Mornon, J. P., and Cartron, J. -P. (2006). Hydrophobic cluster analysis and modeling of the human Rh protein three-dimensional structures. *Transfus. Clin. Biol.* 13, 70-84.
- Cameron, J. N. and Heisler, N. (1983). Studies of ammonia in the trout: physicochemical parameters, acid-base behaviour and respiratory clearance. *J Exp Biol* 105, 107-125.
- Chambrey, R., Goossens, D., Bourgeois, S., Picard, N., Bloch-Faure, M., Leviel, F., Geoffroy, V., Cambillau, M., Colin, Y., Paillard, M., Houillier, P., Carton, J. –P. and Eladari, D. (2005). Genetic ablation of Rhbg in the mouse does not impair renal ammonium excretion. Am. J. Physiol. 289, F1281-1290.
- Cherif-Zahar, B., Durand, A., Schmidt, I., Hamdaoui, N. Matic, I., Merrick, M. and Matassi, G. (2007). Evolution and functional characterization of the RH50 gene from the ammonia-oxidizing bacterium *Nitrosomonas europaea*. J. Bacteriol. 189, 9090-9100.
- Conroy, M. J., Bullough, P. A., Merrick, M. and Avent, N. D. (2005). Modelling the human rhesus proteins: implications for structure and function. *Br. J. Haematol.* 131, 543-551.
- **Cougnon, M., Bouyer, P., Hulin, P., Anagnostopoulos, T. and Planelles, G.** (1996). Further investigation of ionic diffusive properties and of NH₄⁺ pathways in *Xenopus laevis* oocyte cell membrane. *Eur. J. Physiol.* 431, 658-667.
- **De Vooys, G. G. N.** (1968). Formation and excretion of ammonia in Teleostei: I. excretion of ammonia through the gills. *Arch. Physiol. Biochem.* 76, 268-272.
- Edwards, S. L., Wall, B. P., Morrison-Sheltar, A., Sligh, S., Weakley, J. C. and Claiborne, J. B. (2005). The effect of environmental hypercapnia and salinity on the expression of NHE-like isoforms in the gills of a euryhaline fish (*Fundulus heteroclitus*). J. Exp. Zool. 303A, 464-475, 2005.

Endeward, V., Cartron, J.-P., Ripoche, G. and Gros, G. (2006). Red cell membrane

CO₂ permeability in normal human blood and in blood deficient in various blood groups, and effect of DIDS. *Transfus. Clin. Biol.* 13, 123-127.

- Endeward, V., Cartron, J. -P., Ripoche, P., and Gros, G. (2007). RhAG protein of the complex is a CO₂ channel in the human red cell membrane. *FASEB J.* 22, 64-73.
- Esbaugh, A. J. and Walsh, P. J. (2009). Identification of two glucocorticoid response elements in the promoter region of the ubiquitous isoform of glutamine synthetase in gulf toadfish, *Opsanus beta. Am. J. Physiol.* 297, R1075-1081.
- Evans, D. H. and Cameron, J. N. (1986). Gill ammonia transport. J. Exp. Zool. 239, 1-23.
- **Evans, D. H., Piermarini, P. M. and Choe, K. P.** (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* 85, 97-177.
- Frick, N. T. and Wright, P. A. (2002). Nitrogen metabolism and excretion in the mangrove killifish *Rivulus marmoratus* II. Significant ammonia volatilization in a teleost during air-exposure. *J. Exp. Biol.* 205, 91-100.
- Fromm, P. O. and Gillette, J. R. (1968). Effect of ambient ammonia on blood ammonia and nitrogen excretion of rainbow trout (*Salmo gairdneri*). Comp. Biochem. Physiol. 26, 887-896.
- Handlogten, M. E., Hong, S. -P., Zhang, L., Vander, A. W., Steinbaum, M. L., Campbell-Thompson, M. and Weiner, I. D. (2005). Expression of the ammonia transporter proteins Rh B glycoprotein and Rh C glycoproteins in the intestinal tract. Am. J. Physiol. 288, G1036-1047.
- Hargreaves, J. A. (1998). Nitrogen biogeochemistry of aquaculture ponds. *Aquaculture* 166, 181-212.
- Heisler, N. (1990). Mechanisms of ammonia elimination in fishes. In Animal Nutrition and Transport Processes. 2. Transport, Respiration, and Excretion: Comparative and Environmental Aspects (eds. J. -P,Truchot and B. Lahlou), pp. 137-151. Basel: Karger.
- Henry, R. P. and Heming, T. A. (1998). Carbonic anhydrase and respiratory gas exchange. In *Fish Respiration, Fish Physiology Vol. 17* (eds. S. F. Perry and B. L. Tufts), pp. 75-111. San Diego: Academic Press.

- Hirata, T., Kaneko, T., Ono, T., Nakazato, T., Furukawa, N., Hasegawa, S.,
 Wakabayashi, S., Shigekawa, M., Chang, M. H., Romero, M. F. and Hirose,
 S. (2003). Mechanism of acid adaptation of a fish living in a pH 3.5 lake. Am. J. Physiol. 284, R1199-1212.
- Hopkins, T. E., Wood, C. M. and Walsh, P. J. (1995). Interactions of cortisol and nitrogen metabolism in the ureogenic gulf toadfish *Opsanus beta*. J. Exp. Biol. 198, 2229-2235.
- Huang, C. -H. (2008). Molecular origin and variability of the Rh gene family: an overview of evolution, genetics and function. *Haematology Education: the Education Programme for the Annual Congress of the European Hematology Association* 2, 149-157.
- Huang, C. -H. and Peng, J. (2005). Evolutionary conservation and diversification of Rh family genes and proteins. *Proc. Natl. Acad. Sci. USA* 102, 15512-15517.
- Hung, C. Y. C., Tsui, K. N. T., Wilson J. M., Nawata M., Wood C. M. and Wright P. A. (2007). Molecular cloning, characterization and tissue distribution of the Rhesus Glycoproteins Rhbg, Rhcg1 and Rhcg2 in the Mangrove killifish *Rivulus marmoratus* exposed to elevated environmental ammonia levels. J. Exp. Biol. 210, 2419-2429.
- Hung, C. Y. C., Nawata, C. M, Wood, C. M. and Wright, P. A. (2008). Rhesus glycoprotein and urea transporter genes are expressed in early stages of development of rainbow trout (*Oncorhynchus mykiss*). J. Exp. Zool. 309A, 262-268.
- Ip, Y. K., Randall, D. J., Kok, T. K. T., Barzaghi, C., Wright, P. A., Ballantyne, J. S., Wilson, J. M. and Chew, S. F. (2004). The giant mudskipper *Periophthalmodon* schlosseri facilitates active NH₄⁺ excretion by increasing acid excretion and decreasing NH₃ permeability in the skin. J. Exp. Biol. 207, 787-801.
- Javelle, A., Lupo, D., Li, X. D., Merrick, M., Chami, M., Ripoche, P. and Winkler, F. K. (2007). Structural and mechanistic aspects of Amt/Rh proteins. J. Struct. Biol. 158, 472-481.
- Jordan, T. E. and Weller, D. E. (1996). Human contributions to terrestrial nitrogen flux. *Bioscience* 46, 655-664.
- Kelly, S. P. and Wood, C. M. (2001). The cultured branchial epithelium of the rainbow trout as a model for diffusive fluxes of ammonia across the fish gill. *J. Exp. Biol.* 204, 4115-4124.

- Kerstetter, T. H., Kirschner, L. B. and Rafuse, D. D. (1970). On the mechanism of sodium ion transport by the irrigated gills of rainbow trout (*Salmo gairdneri*). J. *Gen. Physiol.* 56, 342-359.
- Khademi, S., O'Connell III, J., Remis, J., Robles-Colmenares, Y., Mierke, L. J. W. and Stroud, R. M. (2004). Mechanism of ammonia transport by Amt/MEP/Rh: structure of AmtB at 1.35 Å. *Science* 305, 1587-1594.
- Kiilerich, P., Kristiansen, K. and Madsen, S. S. (2007). Cortisol regulation of ion transporter mRNA in Atlantic salmon gill and the effect of salinity on the signaling pathway. J. Endocrinol. 194, 417-427.
- Kim, H. -Y., Verlander, J. W., Bishop, J. M., Cain, B. D., Han, K. -H., Igarashi, P., Lee, H. -W., Handlogten, M. E. and Weiner, I. D. (2009). Basolateral expression of the ammonia transporter family member Rh C glycoprotein in the mouse kidney. Am. J. Physiol. 296, F543-555.
- Kirschner, L. B., Greenwald, L. and Kerstetter, T. H. (1973). Effect of amiloride on sodium transport across body surfaces of fresh water animals. *Am. J. Physiol.* 224, 832-837.
- Kitano, Y. and Saitou, N. (2000). Evolutionary history of the *Rh* blood group-related genes in vertebrates. *Immunogenetics* 51, 856-862.
- **Kleiner, D.** (1981). The transport of NH₃ and NH₄⁺ across biological membranes. *Biochim. Biophys. Acta.* 639, 41-52.
- Kong, H., Kahatapitiya, N., Kingsley, K., Salo, W. L., Anderson, P. M., Wang, Y. S. and Walsh, P. J. (2000). Induction of carbamoyl phosphate synthetase III and glutamine synthetase mRNA during confinement stress in gulf toadfish (*Opsanus beta*). J. Exp. Biol. 203, 311-320.
- **Krogh, A.** (1938). The active absorption of ions in some freshwater animals. *Z. vergl. Physiol.* 25, 335-350.
- Ivanis, G., Esbaugh, A. J. and Perry, S. F. (2008). Branchial expression and localization of SLC9A2 and SLC9A3 sodium/hydrogen exchangers and their possible role in acid–base in freshwater rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 211, 2467-2477.
- Larmoyeux, J. D. and Piper, R. G. (1973). Effects of water reuse on rainbow trout in hatcheries. *Progve. Fish. Cult.* 35, 2-8.
- Lee, H. -W., Verlander, J. W., Bishop, J. M., Igarashi, P., Handlogten, M. E. and

Weiner, I. D. (2009). Collecting duct-specific Rh C glycoprotein deletion alters basal and acidosis-stimulated renal ammonia excretion. *Am. J. Physiol.* 296, F1364-1375.

- Li, X., Jayachandran, S., Nguyen, H. H. T. and Chan, M. (2007). Structure of the *Nitrosomonas europaea* Rh protein. *Proc. Natl. Acad. Sci. USA* 104, 19279-19284.
- Lin, H., Pfeiffer, D. C., Wayne, V. A., Pan, J. and Randall, D. J. (1994). Immunolocalization of H⁺-ATPase in the gill epithelia of rainbow trout. *J Exp Biol* 195, 169-183.
- Lin, H. and Randall, D. J. (1990). The effect of varying water pH on the acidification of expired water in rainbow trout. J. Exp. Biol. 141, 14-160.
- Lin, H. and Randall, D. J. (1991). Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. J. Exp. Biol. 161, 119-134.
- Litwiller, S. L., O'Donnell, M. J. and Wright, P. A. (2006). Rapid increase in the partial pressure of NH₃ on the cutaneous surface of air-exposed mangrove killifish, *Rivulus marmoratus*. J. Exp. Biol. 209, 1737-1745.
- Liu, Z., Chen, Y., Mo, R., Hui, C., Cheng, J. -F., Mohandas, N. and Huang, C. -H. (2000). Characterization of human RhCG and mouse Rhcg as novel nonerythroid Rh glycoprotein homologues predominantly expressed in kidney and testis. J. Biol. Chem. 275, 25641-25651.
- Liu, Z., Peng, J., Mo, R., Hui, C. and Huang, C. -H. (2001). Rh type B glycoprotein is a new member of the Rh superfamily and a putative ammonia transporter in mammals. J. Biol. Chem. 276, 1424-1438.
- Lupo, D., Li, X. D., Curand, A., Tomikazi, T., Cherif-Zahar, B., Matassi, G., Merrick, M. and Winkler, F. K. (2007). The 1.3Å resolution structure of *Nitrosomonas europaea* Rh50 and mechanistic implications for NH₃ transport by Rhesus family proteins. *Proc. Natl. Acad. Sci. USA* 104, 19303-19308.
- **Maetz, J. and Garcia-Romeu, F.** (1964). The mechanism of sodium and chloride uptake by the gills of a freshwater fish. *Carassius auratus*. II. Evidence for NH₄⁺/Na⁺ and HCO₃⁻/Cl⁻ exchanges. *J. Gen. Physiol.* 47, 1209-1227.
- Marini, A. –M., Boeckstaens, M., Benjelloun, F., Chérif-Zahar, B. and André, B. (2006). Structural involvement in substrate recognition of an essential aspartate residue conserved in Mep/Amt and Rh-type ammonium transporters. *Curr. Genet.* 49, 364-374.

- Marini, A. M., Matassi, G., Raynal, V., André, B., Cartron, J. –P. and Cherif-Zahar, B. (2000). The human Rhesus-associated RhAG protein and a kidney homolgue promote ammonium transport in yeast. *Nat. Genet.* 26, 341-344.
- Marini, A. -M., Urrestarazu, A., Beauwens, R. and André, B. (1997). The Rh (Rhesus) blood group polypeptides are related to NH₄⁺ transporters. *Trends Biochem. Sci.* 22, 460-461.
- Marini, A. -M., Vissers, S., Urrestarazu, A. and André B. (1994). Cloning and expression of the MEP1 gene encoding an ammonium transporter of *Saccharomyces cerevisiae*. *EMBO J.* 13, 3456-3463.
- **Marshall, W. S.** (2002). Na⁺, Cl⁻, Ca²⁺ and Zn²⁺ transport by fish gills: retrospective review and prospective synthesis. *J. Exp. Zool.* 293, 264-283.
- McCormick, S. D., Regish, A., O'Dea, M. F. and Shrimpton, J. M. (2008). Are we missing a mineralocorticoid in teleost fish? Effects of cortisol, deoxycorticosterone and aldosterone on osmoregulation, gill Na⁺,K⁺-ATPase activity and isoform mRNA levels in Atlantic salmon. *Gen. Comp. Endocrinol.* 157, 35-40.
- McGeer, J. C. and Eddy, F. B. (1998). Ionic regulation and nitrogenous excretion in rainbow trout exposed to buffered and unbuffered fresh water of pH 10.5. *Physiol. Zool.* 71, 179-190.
- Moreira-Silva, J., Tsui, T. K. N., Coimbra, J., Vijayan, M. M., Ip, Y. K. and Wilson, J. M. (2010). Branchial ammonia excretion in the Asian weatherloach *Misgurnus* anguillicaudatus, Comp. Biochem. Physiol. C. 151, 40-50.
- Nakada, T., Westhoff, C. M., Kato, A. and Hirose, S. (2007a). Ammonia secretion from fish gill depends on a set of Rh proteins. *FASEB J.* 21, 1067-1074.
- Nakada, T., Hoshijima, K., Esaki, M., Nagayoshi, S., Kawakami, K. and Hirose, S. (2007b). Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. Am. J. Physiol. 293, R1743-1753.
- Nawata, C. M., Hung, C. C. Y., Tsui, T. K. N., Wilson, J. M., Wright, P. A. and Wood, C. M. (2007). Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H⁺-ATPase involvement. *Physiol. Genomics* 31, 463-474.
- Nawata, C. M. and Wood, C. M. (2008). The effects of CO₂ and external buffering on

ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. *J. Exp. Biol.* 211, 3226-3236.

- Nawata, C. M. and Wood, C. M. (2009). mRNA analysis of the physiological responses to ammonia infusion in rainbow trout. *J. Comp. Physiol. B.* 179, 799-810.
- Nawata, C. M., Wood, C. M. and O'Donnell, M. J. (2009). Functional characterization of Rhesus glycoproteins from an ammoniotelic teleost, the rainbow trout, using oocyte expression and SIET analysis. (Submitted).
- Ninnemann, O., Jauniaux, J. -C. and Frommer, W. B. (1994). Identification of a high affinity NH₄⁺ transporter from plants. *EMBO J.* 13, 3464-3471.
- Ortega, V. A., Renner, K. J., and Bernier, N. J. (2005) Appetite-suppressing effects of ammonia exposure in rainbow trout associated with regional and temporal activation of brain monoaminergic and CRF systems. J. Exp. Biol. 208, 1855-1866.
- **Parks, S. K., Tresguerres, M. and Goss, G. G.** (2007). Interactions between Na⁺ channels and Na⁺-HCO₃⁻ cotransporters in the freshwater gill MR cell: a model for transepithelial Na⁺ uptake. *Am. J. Physiol.* 292, C935-944.
- Parks, S. K., Tresguerres, M. and Goss, G. G. (2008). Theoretical considerations underlying Na⁺ uptake mechanisms in freshwater fishes. *Comp. Biochem. Physiol.* 148C, 411-418.
- Perlman, D. F. and Goldstein, L. (1998). Nitrogen metabolism. In *Physiology of Elasmobranch Fishes* (ed. T. J. Shuttleworth), pp. 253–275. Springer: Berlin.
- Perry, S. F. (1986). Carbon dioxide excretion in fishes. Can. J. Zool. 64, 565-572.
- Perry, S. F. (1998). Relationships between branchial chloride cells and gas transfer in freshwater fish. Comp. Biochem. Physiol. 119A, 9-16.
- Perry, S. F., Esbaugh A., Braun, M. and Glimour, K. M. (2009). Gas transport and gill function in water-breathing fish. In *Cardio-Respiratory Control in Vertebrates*. (eds. M. L. Glass and S.C. Woods), pp. 5-42. Berlin: Springer-Verlag.
- Perry, S. F., Furimsky, M., Bayaa, M., Georgalis, T., Shahsavarani, A., Nickerson, J. G. and Moon, T. W. (2003). Integrated responses of Na⁺/HCO₃⁻ cotransporters and V-type H⁺-ATPases in the fish gill and kidney during respiratory acidosis. *Biochim. Biophys. Acta* 30, 175-184.

- **Perry, S. F. and Gilmour, K. M.** (2006). Acid-base balance and CO₂ excretion in fish: unanswered questions and emerging models. *Resp. Physiol. Neuro.* 154, 199-215.
- **Potts, W. T. W.** (1994). Kinetics of Na⁺ uptake in fresh water animals: a comparison of ion-exchange and proton pump hypotheses. *Am. J. Physiol.* 266, R315-320.
- Rahim, S. M., Delaunoy, J. P. and Laurent, P. (1988). Identification and immunocytochemical localization of two different carbonic anhydrase isoenzymes in teleostean fish erythrocytes and gill epithelia. *Histochemistry* 89, 451-459.
- **Ripoche, P., Goossens, D., Devuyst, O., Gane, P., Colin, Y., Verkman, A. S. and Cartron, J. - P.** (2006). Role of RhAG and AQP1 in NH₃ and CO₂ gas transport in red cell ghosts: a stopped-flow analysis. *Transfus. Clin. Biol.* 13, 117-122.
- Salama, A., Morgan, I. J. and Wood, C. M. (1999). The linkage between Na⁺ uptake and ammonia excretion in rainbow trout kinetic analysis, the effects of (NH₄)₂SO₄ and NH₄HCO₃ infusion and the influence of gill boundary layer pH. *J. Exp. Biol.* 202, 697-709.
- Sayer, M. D. J. and Davenport, J. (1987). The relative importance of the gills to ammonia and urea excretion in five seawater and one freshwater teleost species. *J. Fish Biol.* 31, 561-570.
- Scott, G. R., Claiborne, J. B., Edwards, S. L. Schulte, P. M. and Wood, C. M. (2005) Gene expression after freshwater transfer in gills and opercular epithelia of killifish: Insight into divergent mechanisms of ion transport. J. Exp. Biol. 208, 2719-2729.
- Seshadri, R., Klein, J. D., Kozlowski, S., Sands, J. M., Kim, Y. H., Handlogten, M. E., Verlander, J. M. and Weiner, I. D. (2006a). Renal expression of the ammonia transporters, Rhbg and Rhcg, in response to chronic metabolic acidosis. *Am. J. Physiol.* 290, F397-408.
- Seshadri, R. M., Klein, J. D., Smith, T., Sands, J. M., Handlogten, M. E., Verlander, J. W. and Weiner, I. D. (2006b). Changes in subcellular distribution of the ammonia transporter, Rhcg, in response to chronic metabolic acidosis. Am. J. Physiol. 290, F1443-1452.
- Shih, T. -H., Horng, J. -L., Hwang, P. -P. and Lin, L. -Y. (2008) Ammonia excretion by the skin of zebrafish (*Danio rerio*) larvae. *Am. J. Physiol.* 295, C1625-1632.
- Smart, G. (1976). The effect of ammonia exposure on gill structure of the rainbow trout (*Salmo gairneri*). J. Fish Biol. 8, 471-475.

- Smith, H. W. (1929). The excretion of ammonia and urea by the gills of fish. J. Biol. Chem. 81, 727-742.
- Soupene, E., Inwood, W. and Kustu, S. (2004). Lack of the Rhesus protein Rh1 impairs growth of the green alga *Chlamydomonas reinhardtii* at high CO₂. *Proc. Natl. Acad. Sci. USA* 101, 7787-7792.
- Soupene, E., King, N., Field, E. Liu, P., Niyogi, K. K., Huang, C. H. and Kustu, S. (2002). Rhesus expression in a green alga is regulated by CO₂. *Proc. Natl. Acad. Sci. USA* 99, 7769-7773.
- **Towle, D. W. and Holleland, T.** (1987). Ammonium ion substitutes for K⁺ in ATPdependent Na⁺ transport by basolateral membrane vesicles. *Am. J. Physiol.* 252, R479-489.
- Tsui, T. K. N., Hung, C. Y. C., Nawata, C. M., Wilson, J. M., Wright, P. A. and Wood, C. M. (2009). Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical Na⁺/NH₄⁺ exchange complex. J. Exp. Biol. 212, 878-892.
- Walsh, P. J. (1998). Nitrogen excretion and metabolism. In *The Physiology of Fishes*, 2nd edn. (ed. D. H. Evans), pp. 199-214. Boca Raton, FL: CRC Press.
- Weidinger, K., Neuhauser, B., Gilch, S., Ludewig, U., Meyer, O. and Schmidt, I. (2007). Functional and physiological evidence for a Rhesus-type ammonia transporter in *Nitrosomonas europaea*. *FEMS Microbiol. Lett.* 273, 260-267.
- Weihrauch, D., Morris, S. and Towle, D. W. (2004) Ammonia excretion in aquatic and terrestrial crabs. *J Exp Biol* 207, 4491-4504.
- Weihrauch, D., Wilkie, M. P. and Walsh, P. J. (2009). Ammonia and urea transporters in gill of fish and aquatic crustaceans. J. Exp. Biol. 212, 1716-1730.
- Weiner, I. D. and Hamm, L. L. (2006). Molecular mechanisms of renal ammonia transport. Ann. Rev. Physiol. 69, 317-340.
- Wilkie, M. P. (1997). Mechanisms of ammonia excretion across fish gills. *Comp. Biochem. Physiol.* 118A, 39-50.
- Wilkie, M. P. (2002). Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. J. Exp. Zool. 293, 284-301.

Wilson, J. M., Iwata, K., Iwama, G. K. and Randall, D. J. (1998). Inhibition of

ammonia excretion and production in rainbow trout during severe alkaline exposure. Comp. Biochem. Physiol. B. 121, 99-109.

- Wilson, J. M. and Laurent, P. (2002). Fish gill morphology: inside out. J. Exp. Zool. 293, 192-213.
- Wilson, R. W., Wright, P. M., Munger, S. and Wood, C. M. (1994). Ammonia excretion in fresh water rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: lack of evidence for Na⁺/NH₄⁺ exchange. *J. Exp. Biol.* 191, 37-58.
- Wittbrodt, J., Meyer, A. and Schartl, M. (1998). More genes in fish? *BioEssays* 20, 511-515.
- Wood, C. M. (1993). Ammonia and urea metabolism and excretion. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 379-425. Boca Raton: CRC Press.
- Wood, C. M., Bucking, C., Fitzpatrick, J. and Nadella, S. (2007). The alkaline tide goes out and the nitrogen stays in after feeding in the dogfish shark, *Squalus acanthias. Respir. Physiol. Neurobiol.* 159, 163-170.
- Wright, P. A., Heming, T. and Randall, D. J. (1986). Downstream pH changes in water flowing over the gills of rainbow trout. *J. Exp. Biol.* 126, 499-512.
- Wright, P. A., Randall, D. J. and Perry, S. F. (1989). Fish gill boundary layer: a site of linkage between carbon dioxide and ammonia excretion. J. Comp. Physiol. 158, 627-635.
- Wright, P. A. and Wood, C.M. (1985). An analysis of branchial ammonia excretion in the freshwater rainbow trout: effects of environmental pH change and sodium uptake blockade. *J. Exp. Biol.* 114, 329-353.
- Wright, P. A. and Wood, C. M. (2009). A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. *J. Exp. Biol.* 212, 2302-2312.
- Yesaki, T. Y. and Iwama, G. K. (1992). Survival, acid-base regulation, ion regulation, and ammonia excretion in rainbow trout in highly alkaline hard water. *Physiol. Zool.* 64, 763-787.
- Zheng, L., Kostrewa, D., Berneche, S., Winkler, F. K. and Li, X. -D. (2004). The mechanism of ammonia transport based on the crystal structure of AmtB of *Escherichia coli*. *Proc. Natl. Acad. Sci.* 101, 17090-17095.

Zidi-Yahiaoui, N., Callebaut, I., Genetet, S., Van Kim, C. L., Cartron, J. -P., Colin,

Y., Ripoche, P. and Mouro-Chanteloup, I. (2009). Functional analysis of human RhCG: a comparison with *E. coli* ammonium transporter reveals similarities in the pore and differences in the vestibule. *Am. J. Physiol.* 297, C537-547.