**Anuran adaptations to climatic stress:
Immune responses and the SMAD family in the wood frog,
Rana sylvatica, and the African clawed frog, *Xenopus laevis***

by

HELEN A. HOLDEN

B. Sc. Honours – Carleton University, 2009

**A THESIS SUBMITTED TO THE FACULTY OF GRADUATE AND POSTDOCTORAL AFFAIRS IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF**

MASTER OF SCIENCE

DEPARTMENT OF BIOLOGY

CARLETON UNIVERSITY

OTTAWA, ONTARIO, CANADA

© COPYRIGHT 2011

Helen A. Holden

Library and Archives
Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file *Votre référence*
ISBN: 978-0-494-83134-2
Our file *Notre référence*
ISBN: 978-0-494-83134-2

NOTICE:

The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protègent cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

The undersigned hereby recommend to the Faculty of Graduate and Postdoctoral Affairs
acceptance of the thesis entitled:

“Anuran adaptations to climatic stress:
Immune responses and the SMAD family in the
wood frog, *Rana sylvatica*, and the African clawed frog, *Xenopus laevis*”

submitted by

HELEN A. HOLDEN

B.Sc. Honours – Carleton University, 2009

In partial fulfillment of the requirements for the degree of Master of Science

Chair, Biology Department

Thesis Supervisor

Carleton University
2011

ABSTRACT

The wood frog, *Rana sylvatica*, survives freezing over winter. The African clawed frog, *Xenopus laevis*, withstands substantial dehydration seasonally. The effects of environment on these frogs' immunity were investigated with a focus on antimicrobial peptides. Expression of brevinin-1SY was analyzed during freezing, dehydration, anoxia, and development in *R. sylvatica*. Brevinin-1SY responded differently to each stress, suggesting environmentally regulated expression. Upregulation of hepcidin mRNA was demonstrated during dehydration in *X. laevis* liver, as were hepcidin agonists, STAT 3 and cMYC. Alternatively, hepcidin antagonizing TGF- β -mediated SMADs were downregulated and the BMP-mediated SMADs, promoters of hepcidin expression, did not change. Molecular controls of *X. laevis* skeletal muscle growth were also explored during dehydration. Myostatin, a muscle growth antagonist, was downregulated during dehydration, whereas cMYC, a muscle growth agonizer, and GLUT 4, a glucose transporter, were upregulated; differential control of SMADs was documented. The data suggest that, during estivation, muscle growth signals are promoted.

ACKNOWLEDGMENTS

I would like to take this opportunity to formally thank both my supervisor Dr. KB Storey, and his wife, Jan Storey, for the knowledge, patience, and continuous support they have provided me over the past three plus years. I am so grateful for the opportunities to conduct this research, explore my passions, and grow as a scientist and individual. With your guidance and support, I have developed numerous lifelong skills including a few highly specialized ones like wrangling *Xenopi* and siphoning tadpole water! I would also like to emphasize my great appreciation for Jan Storey's enduring patience and assistance in the preparation of this thesis and her thorough reviews and edits of my works that have allowed me to better express myself in the scientific field.

I would like to thank all my fellow Storey lab members past, present, and honorary. Your guidance, insights, and above all friendships have enriched my time inside and outside the lab and made for the best memories. And, a special thank you to Katrina and Shannon for being such excellent buddies – PCR was never as much fun as when we got to share that prime 12 o'clock time block.

A big thank you to my family for your love, encouragement, and great confidence in my abilities (despite being quite sure you have no idea what I am doing with 'those proteins') and to Mike for always being there for me, for all the wonderful dinners I came home to, and for knowing just how to make me laugh.

And finally, a salute to all the wee froggies who made the greatest sacrifice to my research, thank you.

TABLE OF CONTENTS

Title Page	i
Acceptance Sheet	ii
Abstract	iii
Acknowledgments	iv
Table of Contents	v
List of Abbreviations	vi
List of Figures	x
List of Appendices	xii
Chapter 1 General Introduction	1
Chapter 2 Developmental and environmental impacts on brevinin-1SY expression in the wood frog, <i>Rana sylvatica</i>	19
Chapter 3 Hepcidin antimicrobial peptides from the African clawed frog, <i>Xenopus laevis</i>	49
Chapter 4 Expression of SMAD transcription factors in the skeletal muscle of the African clawed frog, <i>Xenopus laevis</i>	100
Chapter 5 General Discussion	128
Appendices	142
References	145

LIST OF ABBREVIATIONS

Akt	protein kinase B
AMP	antimicrobial peptide
ANOVA	analysis of variance
APR	acute phase response
APS	ammonium persulphate
ATP	adenosine triphosphate
BHK	baby hamster kidney
BLAST	basic local alignment search tool
BMP	bone morphogenetic protein
bp	base pair
BWC _i	initial body water content
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
cDNA	complementary deoxyribonucleic acid
co-SMAD	common partner SMAD
CREB	cAMP response element binding protein
DEPC	diethylpyrocarbonate
dNTPs	deoxynucleotide triphosphate
ddH ₂ O	double distilled H ₂ O
DTT	dithiolthreitol
EDTA	ethylenediamine tetraacetic acid
EMSA	electrophoretic mobility shift assay

ERK	extracellular signal-regulated kinases
E2F	E2 promoter binding factor
FoxO	forkhead box, class O protein
GDF	growth differentiation factor
GLUT4	glucose transporter 4
gp130	glucoprotein 130
GSK-3 β	glycogen synthase kinase-3 β
HEPES	N- (2-hydroxyethyl) piperazine-N'- (2-ethanesulfonic acid)
HepG2	human hepatocellular liver carcinoma
HJV	hemojuvelin
HRP	horseradish peroxidase
IGF-1	insulin-like growth factor 1
IL6	interleukin 6
INA	ice nucleating active
I-SMAD	inhibitory SMAD
JAKs	Janus kinases
JNK	c-Jun N-terminal kinase
kDa	kilodalton
MAPK	mitogen-activated protein kinase
M _d	mass during dehydration
MEF2	myocyte enhancer factor-2
MH	mad homology
M _i	initial mass

MODS	multiple organ dysfunction syndrome
MRD	metabolic rate depression
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
Oligo-dT	oligodeoxythymidylic acid
PAGE	polyacrylamide gel electrophoresis
P-cMYC	phosphorylated cMYC
PCR	polymerase chain reaction
PGLa	peptide with amino-terminal glycine & carboxy-terminal leucinamide
PI3K	phosphatidylinositol-3-OH kinase
PKAc	cAMP-activated protein kinase
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
poly d(I-C)	poly-deoxy-inosinic-deoxy-cytidylic acid
P-SMAD	phosphorylated SMAD
P-STAT	phosphorylated STAT
PVA	polyvinyl acetate
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
rRNA	ribosomal RNA
R-SMAD	receptor regulated SMAD
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate

SEM	standard error of the mean
SMAD	small mothers against decapentaplegic
SNK	Student-Newman-Keuls
SOCS3	suppressor of cytokine signaling 3
STAT 3	signal transducers and activators of transcription 3
TAE	tris-acetate-ethylenediamine tetraacetic acid buffer
Temed	N,N,N',N'-tetramethylethylenediamine
TBE	tris/borate/EDTA
TBST	tris-buffered saline containing Tween-20
TGF- β	transforming growth factor- β
TH	thyroid hormone
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet

LIST OF FIGURES

FIG. 2.01	Partial nucleotide sequence of <i>R. sylvatica</i> brevinin-1SY and comparison to other frog species.	43
FIG. 2.02	Partial nucleotide and deduced amino acid sequence of <i>R. sylvatica</i> brevinin-1SY.	44
FIG. 2.03	Effects of freezing (24 h), anoxia (24 h), and dehydration (40% total body water lost) on the mRNA expression of brevinin-1SY as determined by RT-PCR.	46
FIG. 2.04	Effects of growth and metamorphosis through Gosner Stages 14-20, 21-25, 26-30, 31-35, 36-41, 42-43, and 44-45 on the mRNA expression of brevinin-1SY in <i>R. sylvatica</i> as determined by RT-PCR.	47
FIG. 2.05	Partial nucleotide and deduced amino acid sequence of <i>R. sylvatica</i> unidentified mRNA.	48
FIG. 3.01	Molecular events outlining the impact of the SMAD signalling pathway on liver cells.	53
FIG. 3.02	Partial nucleotide and deduced amino acid sequence of <i>X. laevis</i> hepcidin I.	77
FIG. 3.03	Partial nucleotide and deduced amino acid sequence of <i>X. laevis</i> hepcidin II.	79
FIG. 3.04	Effects of medium and high dehydration on the mRNA expression of hepcidin I and hepcidin II in <i>X. laevis</i> liver.	81
FIG. 3.05	Partial nucleotide and deduced amino acid sequence of <i>X. laevis</i> SMAD 2.	82
FIG. 3.06	Comparison of the partial African clawed frog (<i>X. laevis</i>) SMAD 2 protein sequence with the corresponding region in SMAD 2 from other animals.	84
FIG. 3.07	Effects of medium and high dehydration on the mRNA and protein expression of SMAD 2 in <i>X. laevis</i> muscle.	85

FIG. 3.08	Effects of medium and high dehydration on the mRNA and protein expression of SMAD 3 in <i>X. laevis</i> liver.	86
FIG. 3.09	Partial nucleotide and deduced amino acid sequence of <i>X. laevis</i> SMAD 4.	87
FIG. 3.10	Comparison of the partial African clawed frog (<i>X. laevis</i>) SMAD 4 protein sequence with the corresponding region in SMAD4 from other animals.	89
FIG. 3.11	Effects of medium and high dehydration on the mRNA and protein expression of SMAD 4 in <i>X. laevis</i> liver.	90
FIG. 3.12	Partial nucleotide and deduced amino acid sequence of <i>X. laevis</i> SMAD 5.	91
FIG. 3.13	Comparison of the partial African clawed frog (<i>X. laevis</i>) SMAD 5 protein sequence with the corresponding region in SMAD 5 from other animals.	93
FIG. 3.14	Effects of medium and high dehydration on the mRNA and protein expression of SMAD 5 in <i>X. laevis</i> liver.	94
FIG. 3.15	Effects of medium and high dehydration on the protein expression of SMAD 6 in <i>X. laevis</i> liver.	95
FIG. 3.16	Effects of medium and high dehydration on the protein expression of STAT 3 in <i>X. laevis</i> liver.	95
FIG. 3.17	Partial nucleotide and deduced amino acid sequence of <i>X. laevis</i> cMYC.	96
FIG. 3.18	Comparison of the partial African clawed frog (<i>X. laevis</i>) cMYC protein sequence with the corresponding region in cMYC from other animals.	98
FIG. 3.19	Effects of medium and high dehydration on the mRNA and protein expression of cMYC in <i>X. laevis</i> liver.	99
FIG. 4.01	Molecular events outlining the impact of the SMAD signalling pathway on skeletal muscle cells.	104

FIG. 4.02	Effects of medium and high dehydration on the mRNA and protein expression of SMAD 2 in <i>X. laevis</i> muscle.	118
FIG. 4.03	Effects of medium and high dehydration on the mRNA and protein expression of SMAD 3 in <i>X. laevis</i> muscle.	119
FIG. 4.04	Partial nucleotide and deduced amino acid sequence of <i>X. laevis</i> myostatin.	120
FIG. 4.05	Comparison of the partial African clawed frog (<i>X. laevis</i>) myostatin protein sequence with the corresponding region in myostatin from other animals.	122
FIG. 4.06	Effects of medium and high dehydration on the mRNA and protein expression of myostatin in <i>X. laevis</i> muscle.	123
FIG. 4.07	Effects of medium and high dehydration on the mRNA and protein expression of cMYC in <i>X. laevis</i> muscle.	124
FIG. 4.08	Effects of medium and high dehydration on the mRNA and protein expression of SMAD 5 in <i>X. laevis</i> muscle.	125
FIG. 4.09	Effects of medium and high dehydration on the protein expression of SMAD 6 in <i>X. laevis</i> muscle.	126
FIG. 4.10	Effects of medium and high dehydration on the protein expression of GLUT4 protein in <i>X. laevis</i> muscle as determined by Western blotting.	126
FIG. 4.11	Effects of medium and high dehydration on the mRNA and protein expression of SMAD 4 in <i>X. laevis</i> muscle.	127

LIST OF APPENDICES

Appendix A	List of publications	143
Appendix B	Communications at scientific meetings	144

CHAPTER 1

General Introduction

Amphibians have inhabited the Earth since an estimated 350 million years ago (Sodhi *et al.*, 2008). Despite the adverse conditions faced throughout these many years, their species have thrived on all continents except Antarctica (Duellman & Trueb, 1986). Some amphibians are found in hot areas of the world and face desiccation, while others are found in the north and are challenged by subzero temperatures (Clark *et al.*, 1997). In order to overcome the challenges associated with their environments, including changes in temperature, water, oxygen, and food availability, these animals utilize biochemical adaptations to survive.

Adaptations to Arid Conditions

Anurans have successfully distributed themselves in arid deserts, despite the fact that their highly water permeable integument predisposes them to rapid dehydration and generally requires them to live in moist or wet environments so they may hydrate themselves transcutaneously (Bentley & Yorio, 1979; Clark *et al.*, 1997). This has led to a suite of physiological, biochemical, and behavioural adaptations that allow anurans to tolerate the widest variation in body water content and body fluid ionic strength and osmolality of any vertebrate group (Churchill & Storey, 1994b).

Under arid conditions and high temperatures, a number of species facing food and water shortage will enter a state of dormancy termed estivation, during which the animal is inactive and fasts. Estivation can be a short term adaptation, but is more commonly used to survive through long dry seasons that are incompatible with active life and, for some species, can stretch for more than 2 years (Wilz & Heldmaier, 2000). The main criteria for anuran survival during estivation are water retention and sufficient fuel

reserves. Behavioural water conservation strategies include the selection of sheltered sites in which to estivate that minimize water loss (and also limit exposure to the elements and predators), adoption of the water holding posture, and apnoic breathing patterns.

Estivation is also accompanied by (a) the accumulation of osmolytes in the body fluids that raise total osmolality and act colligatively to prevent water loss, (b) the stabilization of organ hydration at the expense of water from extra-organ fluid spaces and large amounts of water stored in the bladder, and (c) the aerobic oxidation of lipid reserves to produce water metabolically (for reviews: Shoemaker *et al.*, 1992; Pinder *et al.*, 1992; Storey, 2002). Estivation is prefaced by ravenous eating to accumulate a large reserve of endogenous fuel and, as mentioned, lipids (and some proteins) are the principal fuel sources during estivation (Seymour, 1973; Hermes-Lima *et al.*, 2001). To conserve fuel, metabolic rate falls during estivation to only 10-30% of the resting metabolic rate of control animals (for review: Storey, 2002). Upon leaving estivation animals are faced with oxidative stress as a result of a rapid increase in oxygen uptake and consumption. Reactive oxygen species are dealt with by increased activities of antioxidant enzymes (Grundy & Storey, 1998).

The African clawed frog, *Xenopus laevis*, is native to sub-Saharan Africa, where it is almost exclusively aquatic, inhabiting wet environments such as swamps, lakes, and rivers and rarely venturing onto land (Tinsley & Kobel, 1996). Their environment can change rapidly during the dry season when water sources evaporate and vegetation and wildlife patterns change in the area. The frogs respond to these seasonally arid conditions in one of two ways (Alexander & Bellerby, 1938). When stressed by low water, frogs will undertake nocturnal migrations overland to find a new body of water or, when this is

not possible, they dig into the cooler, damper subsoil of their evaporating body of water and enter a state of estivation (Alexander & Bellerby, 1938). Both these options require tolerance of substantial desiccation (to 35% of total body water lost) (Romsper, 1975). Anuran tolerance to dehydration is generally positively correlated with a more terrestrial life-style, making the dehydration capacity of the aquatic *X. laevis* intriguing to study (Hillman, 1980).

Unlike many other estivating frog species, *X. laevis* has a very small bladder and cannot rely on its stored water when under dehydration stress (Calamita *et al.*, 1994; Feder & Burggren, 1992). Therefore, to resist water loss under desiccating conditions, *Xenopus* accumulate osmolytes in their plasma and tissues to provide colligative resistance to water loss. These include amino acids, ammonia (2-3 fold increase) and urea (15-20 fold increase) (Balinsky *et al.*, 1967). Urea accumulation is achieved via increased biosynthesis (due to increased protein catabolism) coupled with reduced renal clearance of nitrogenous wastes via antidiuresis (McBean & Goldstein, 1970a,b; Funkhouser & Goldstein, 1973). Supporting this, and despite an overall strong metabolic rate depression during estivation, activities of urea cycle enzymes are increased due to the enhanced transcription of the genes encoding these enzymes (Janssens & Cohen, 1968; Storey & Storey, 2010).

Adaptations to Subzero Temperatures

Amphibians in northern climates are generally forced to hibernate on land or underwater during the winter months (Tattersall & Ultsch, 2008). Those that overwinter aquatically must withstand prolonged bouts of hypoxia while sealed under ice. Those that overwinter terrestrially are faced with desiccation and freezing temperatures. One mode

of terrestrial hibernation used by toads, terrestrial salamanders, and some frogs is to overwinter below the frost line in a moist microenvironment (Tattersall & Ultsch, 2008). The overwintering strategy of the wood frog *Rana sylvatica*, whose geographic range extends from Alaska through Canada to the Great Lakes onto Labrador, has been a subject of high interest and study since 1982 when it was reported that this species tolerated freezing of 65-70% of its total body water during hibernation on the forest floor (Behler & King, 1979; Schmid, 1982). Several other terrestrially hibernating amphibian and reptile species have since been identified that survive whole body freezing during the winter; however, *R. sylvatica* remains the primary model organism for studies of vertebrate freeze-tolerance.

The unique physiology of wood frogs allows them to undergo whole body freezing and maintain a frozen state without bodily harm until warmer temperatures allow them to thaw and resume active functions. However, subzero temperatures can be damaging and/or lethal to intolerant animals. This is because of the multiple stresses that arise from tissue freezing. Freeze damage occurs from (i) physical damage to subcellular machinery and tissues imparted by the formation and growth of ice crystals, (ii) ice surges that result from flash freezing following supercooling, (iii) extensive dehydration as a result of extracellular ice formation, (iv) ischemia from the solidification of blood, and (v) the loss of vital, muscle-based physiological responses such as breathing and heartbeat (for review: Storey & Storey, 2004a). The thaw process can be equally damaging since reperfusion can result in injury due to oxidative stress; more importantly though, thawing requires the coordinated reactivation of vital functions.

Wood frogs employ a number of complex physiological and biochemical adaptations in order to survive freezing. They avoid extensive supercooling by initiating freezing at high subzero temperatures (-0.5 to -3°C) by inoculative freezing on the skin (Layne *et al.*, 1990; Costanzo *et al.*, 1999). The propagation of ice inwards through the frog's skin pores inoculates freezing internally and the frog freezes asymmetrically from the outside in (Rubinsky *et al.*, 1994). Ice nucleating proteins in wood frog plasma may also play a role in ice propagation (Wolanczyk *et al.*, 1990a,b; Storey *et al.*, 1992). A slow rate of ice formation (typically $<5\%$ of total body water per hour) allows the frog sufficient time to make the metabolic adjustments that will allow it to survive freezing (Layne & Lee, 1987).

Dehydration of the frog's body due to evaporation across the skin as well water loss from cells into extracellular ice masses are both consequences of freezing. Evaporative water loss can be minimized by the selection of a humid or moist hibernaculum under organic leaf litter on the forest floor (Light, 1991; Churchill & Storey, 1993). To prevent intracellular freezing and to colligatively limit the amount of intracellular water that is lost into extracellular ice masses, frogs synthesize the cryoprotectant glucose from glycogen stores in the liver and deliver glucose in high concentration to all tissues via the blood (Storey & Storey, 1986). More recently, urea has also been identified as an osmoprotectant and cryoprotectant in the wood frog (Costanzo & Lee, 2005). Cyroprotectant synthesis commences upon ice nucleation on the skin and glucose is rapidly distributed via the circulation due to cardio-acceleration that is also triggered when freezing begins (Storey & Storey, 1985; Layne *et al.*, 1989). This response highlights the importance of instigating ice nucleating at high subzero

temperatures so as to provide ample time to distribute glucose. Blood glucose levels can increase 60-fold during freezing, from about 5 mM glucose in controls to 300 mM glucose during freezing (Storey & Storey, 1986). Because of their high glucose content, the heart and liver are the last organs to freeze, and the first organs to revive during thaw (Rubinsky *et al.*, 1994). Without oxygen, all metabolism in the frozen state is limited to anaerobic pathways (glycolysis), resulting in substantially decreased levels of available ATP. In order to maintain the freeze-tolerant state, metabolic regulation is employed.

The endurance of *R. sylvatica* to freezing stems from its high capacity to tolerate water loss and to survive anoxia. In fact, most biochemical changes that occur during freezing can be categorized as responsive to either the anoxia (representing freeze-induced ischemia) or dehydration (representing freeze-induced cell volume reduction) component. Only a few appear to be truly unique to freezing such as the production of ice nucleating proteins (Wolanczyk *et al.*, 1990a,b; Storey *et al.*, 1992) and the up-regulation of three novel genes (*li16*, *fr10*, *fr47*) that are implicated in freezing survival but have no homologues in gene/protein databases (Cai & Storey, 1997; McNally *et al.*, 2002, 2003).

Various adaptations for dehydration were previously discussed with respect to the estivating frog, *X. laevis*. Terrestrially hibernating frogs, including the wood frog, can also become dehydrated, usually in late fall and winter. With the ability to tolerate up to 50-60% of total body water loss, *R. sylvatica* are actually more dehydration hardy than *X. laevis*, but the mechanisms used in survival remain the same (Churchill & Storey, 1993; Muir *et al.*, 2007). These include water-conserving behaviours, physiological adaptations, and the conservation of energy by reducing metabolic rate (Muir *et al.*,

2007). Responding to dehydration by building up osmolytes in the body fluid is a universal strategy to resist water loss. In *R. sylvatica*, dehydration stimulates liver glucogenolysis just as rapidly and elevates blood glucose to the same levels as freezing. Therefore, the cryoprotectant response is proposed to have evolved from a pre-existing adaptation to dehydration (Churchill & Storey, 1994b). Glucose accounts for about 3% of blood osmolyte pressure and is secondary to the mobilization of urea. The elevation of urea during dehydration is thought to play an important role in depressing metabolism, perhaps by regulating key enzymes (Muir *et al.*, 2007).

Naturally, many ranid frogs tolerate prolonged hypoxia or short bouts (several days) of anoxia in the winter as they hibernate aquatically in ice-covered waters that contain low levels of dissolved oxygen (Tattersall & Ultsch, 2008). Unlike many of its North American ranid counterparts, *R. sylvatica* hibernate terrestrially (Collins & Lewis, 1979; Light, 1991). Still, wood frogs experience levels of hypoxia during dehydration or diving and, during freezing, complete anoxia occur (Tattersall & Boutilier, 1999). During hypoxia, frogs preferentially locate low, above zero temperatures to lower their metabolic rate and postpone anaerobiosis (Tattersall & Boutilier, 1997). Without ample oxygen, glucose stores are rapidly used in anaerobic respiration and the differential regulation of proteins has been shown to increase the glycolytic capacity of wood frog organs (Wu *et al.*, 2009). During hypoxia/anoxia, metabolic regulation is used to depress demands to meet ATP supply and protective measures against reoxygenation injury are put in place (West & Boutilier, 1998).

Metabolic regulation during environmental stress

Hypometabolism accompanies estivation/dehydration, freezing, and anoxia and is crucial in allowing survival under inhospitable conditions since it extends the time that the animal can survive and resist stressful conditions using only endogenous fuel reserves (for review: Storey & Storey 2004b). Hypometabolism is achieved via a reorganization of energy-consuming processes whereby most physiological and biochemical processes are suppressed or turned off (typically at least 70-80%) during environmental stress, including all non-essential gene transcription and protein synthesis (for review: Storey & Storey, 2010). Energy is reprioritized to vital cell functions and preservation strategies are implemented to promote survival over the prolonged period of dormancy (for review: Storey & Storey, 2004a). Metabolic rate depression (MRD) is partly achieved due to the reduced muscular energy demands; dormant animals are immobilized, heart and breathing rate are greatly reduced, and no feeding/digestion occurs (for review: Storey & Storey, 2004b). However, the intrinsic regulation of cellular metabolism is also necessary and is used to coordinate the differential gene expression needed to survive during dormancy. The molecular mechanisms behind this continue to be elucidated. One trend found is the role of reversible phosphorylation in changing the activity of proteins/enzymes during dormancy. Phosphorylation has been found to play a role in control over changes in fuel metabolism and the suppression of transcription and protein synthesis. Despite MRD, selective upregulation of some genes and proteins key to cell survival have been found.

The immune system during environmental stress

The immune system is placed in the precarious position of being a large network of effector molecules that is energetically costly but also important in maintaining good health (for review: Buttgereit *et al.*, 2000). Animals have been found to divert energy from other physiological processes in order to mount an immune challenge, even in the absence of the energetic constraints of dormancy (Wolowczuk *et al.*, 2008; DiAngelo *et al.*, 2009). How do dormant animals control present or potential infections and reduce the associated costs to do this during hypometabolism? Alternatively, how would the cost of maintaining a functional immune system in the absence of infection weigh against that of the infection itself? In either case, precious resources are diverted or traded off against other functions.

During the inhospitable conditions that force animals into dormancy, the biomass of microbes is often lowered and there is a change in microbe community patterns (for review: Schimel *et al.*, 2007). One adaptive tactic during a lowered risk of infection is to reduce the investment in the immune system to a minimum. Hibernation in mammals may be a potential example of this, as reductions in many different types of immune molecules occur during torpor (for review: Bouma *et al.*, 2010). However, theoretical work maintains that a partially effective immunity can achieve the greatest fitness when balancing the costs of the immune system (Behnke *et al.*, 1992).

Surviving environmental stress is known to require changes to physiological and biochemical processes, however, very little research has investigated how the immune system responds to environmental stress in amphibians. Despite being in a vulnerable

hypometabolic state under conditions of hot, dry summers or cold winters, *X. laevis* and *R. sylvatica* return to a normal active state once environmental conditions become favourable again. This favours a hypothesis that even during MRD, some immune effector molecules may be essentially maintained or even up-regulated.

Amphibians are protected by both an innate and an adaptive immune system. The innate immune system provides the first line of defence against pathogens. It is made up of non-specific effector molecules which are constitutively present and available to combat pathogens but it does not lead to lasting immunity. The adaptive immune system provides a specific immune response to a given pathogen that has bypassed the innate immune system. Antibodies are the best known effector molecules of the adaptive immune system and these are developed during the lifetime of the host in response to the infections encountered. Even after an infection has passed, some of the specific antibodies remain in the host's system and provide lasting immunity (immunological memory). The adaptive immune system is slow to develop in ectothermic vertebrates and requires the circulation of adaptive immune molecules through the blood and lymph. This circulation is mediated by breathing in frogs, since the lymphoid organ is located dorsally on top of the lungs (Hedrick *et al.*, 2007). Being ectothermic, the frog's ability to mount an immune defence is highly dependent on environmental temperature, and can be compromised at cooler temperatures (Raffel *et al.*, 2006; Rohr & Raffel, 2010). Hence, freeze tolerant species such as *R. sylvatica* that spend the winter at very low temperatures (ranging between about +5 and -5°C) probably experience strong thermal suppression of their immune responses. Freezing of body fluids and the accompanying cessation of breathing and circulation would also disrupt/halt the capacity

to distribute blood-borne elements of the adaptive immune system to sites of infection in frozen animals. Furthermore, the energetic costs of an adaptive immune defence might be too high for frogs in a hypometabolic state, whether it is high temperature estivation or low temperature freezing. Indeed, it has been proposed that, under environmental stress conditions that induce hypometabolism, frogs would depend more on a heightened competence of the constitutively expressed innate immune system and the maintenance of its nonspecific effector molecules in an infectious environment than on acquired facets of adaptive immunity (Bonneaud *et al*, 2003; McNamara & Buchanan, 2005).

Antimicrobial Peptides

Hypothesis 1

Antimicrobial peptides, a key element of the innate immune response, are differentially up-regulated in frog tissues during environmental stress-induced hypometabolism.

To explore the idea that aspects of the innate immune system are heightened during dormancy, the regulation of antimicrobial peptides (AMPs) were investigated in response to freezing, anoxia, dehydration and metamorphosis in *R. sylvatica* (Chapter 2) and dehydration in *X. laevis* (Chapter 3). Studies focused on brevinin-1SY in wood frogs since this is the sole skin AMP that was detected previously in wood frogs (Matutte *et al.*, 2000) and on hepcidin in African clawed frogs, a liver AMP and iron regulatory hormone that is broadly distributed in vertebrates.

Microorganisms can enter the body through contact with external or internal epithelial surfaces. AMPs are molecules of the innate immune system that are expressed in both external and internal tissues, including the skin and mucosal membranes of the respiratory and gastrointestinal tract, and are effective against pathogens such as bacteria, fungi, and viruses (for review: Zasloff, 1992). Therefore, AMPs provide the first line of defense against microbes in the environment. AMPs are found in organisms from flies to humans, but the most abundant reported source of AMPs is from anurans (for review: Rinaldi *et al.*, 2002). AMPs are small in size (typically 10-46 amino acids) and therefore it has been argued that they are less energetically costly to synthesize than other immune effectors (for review: Moret *et al.*, 2003). These molecules are synthesized in a prepropeptide form and are stored in a propeptide form, making them readily available should infection occur, without the need to distribute them (although it has been shown that some circulating cells do secrete AMPs). The prepropeptides consist of a conserved signal sequence and acidic propeptide; these portions are cleaved off before or at the time of secretion to release the variable, C-terminal, mature, active peptides that are different from species to species (for review: Amiche *et al.*, 1999). The proteolytic cascades that lead to AMP cleavage are still being elucidated.

Mature peptide regions of AMPs are commonly modified post-translationally by amidation and glycosylation and less commonly by halogenation and phosphorylation (for review: Andreu & Rivas, 1998). Most skin AMPs are linear, cationic, amphipathic helical peptides, but another group is cyclic and contains one or multiple disulphide bonds (for review: Andreu & Rivas, 1998). These characteristics allow AMPs to bind to, and permeabilize the structure of target microbial membranes causing cell lysis or

perhaps transverse into the microbial cell and bind intracellular targets (for reviews: Rinaldi, 2002; Rollins-Smith *et al.*, 2005). Since animals enduring environmental stress have limited energy resources, AMPs may provide the necessary host defense during a weakened state.

SMAD Transcription Factors and Liver Immunity

Hypothesis 2

SMAD transcription factors, regulators of hepcidin gene expression, will be differentially regulated in *X. laevis* liver in response to dehydration stress to allow hepcidin expression to be enhanced.

The SMAD family of transcription factors have long been known to propagate signals regulating inflammation (for review: Hedger *et al.*, 2011). One of their roles is to balance immune responses, including the control of immune suppression (for review: Yoshimura & Muto, 2011); this makes SMAD action an intriguing molecular regulatory mechanism to examine with respect to immunity during hypometabolism. The liver is an active site of immunity, synthesizing and housing a preponderance of innate immune molecules as compared with other organs (for review: Mehal *et al.*, 2001). In the liver, SMAD signalling is a well-established key pathway controlling the activation of hepcidin expression (for review: Malyszko *et al.*, 2009). Hepcidin is an antimicrobial peptide produced primarily in hepatocytes that has a dual function of regulating iron homeostasis, another important facet of immunity. To explore how the innate immune system is

regulated in hypometabolism, Chapter 3 investigates the expression of hepcidin and the responses of the SMAD transcription factor family to dehydration in *X. laevis* liver.

SMAD Transcription Factors and Muscle Growth

Hypothesis 3

SMAD transcription factors in skeletal muscle, regulators of important muscle specific genes, will be differentially expressed in *X. laevis* muscle in response to dehydration stress to protect muscles from atrophy.

In skeletal muscle, the SMAD family of transcription factors propagate signals regulating muscle ontogeny and physiology (for review: Kollias & McDermott, 2008). In particular, the SMAD pathway opposes the muscle growth that is stimulated by the mammalian target of rapamycin (mTOR) pathway (for review: Wackerhage & Ratkevicius, 2008). The anabolic and catabolic pathways in muscle are balanced in a way that allows a plastic response by muscles to environmental stimuli. Muscle wasting typically occurs as a consequence of extended periods of disuse or unmet energy demands, and can occur during disease (cachexia) and aging (sarcopenia) (for review: Glass & Roubenoff, 2010). However, animals that undergo natural periods of muscle disuse, such as estivators, do not experience these same levels of muscle atrophy despite extended periods of dormancy (for review: Hudson & Franklin, 2002b). Therefore, skeletal muscle must undergo molecular changes in order to resist atrophy during estivation. To explore how the muscle mass is regulated in hypometabolism, Chapter 4 investigates the responses of SMADs to dehydration in *X. laevis* skeletal muscle.

SMAD Family of Transcription Factors

The genes regulated by the SMAD family of transcription factors are vast, and include functions such immunity, growth, and proliferation, whose regulation is likely changed substantially during hypometabolism. The SMAD pathway is initiated via a ligand of the Transforming Growth Factor- β (TGF- β) superfamily. The TGF- β superfamily consists of secreted cell-to-cell signalling cytokines of which the bone morphogenetic protein (BMP), TGF- β , and activins are the best known families (Kloos *et al.*, 2002). Ligand binding causes the hetero-oligomerization of two distantly related type I and type II transmembrane serine/threonine kinase receptors (Souchelnytskyi *et al.*, 1997). The type II receptor autophosphorylates and transphosphorylates the type I receptors; the type I receptor in turn phosphorylates the C-terminal tails of the receptor-regulated SMAD transcription factor family, activating them (Nakayama *et al.*, 2001).

The SMAD family of transcription factors consists of 8 proteins that are evolutionarily conserved in function and regulation. The homologues *Mothers against decapentaplegic* (*Mad*) and *sma* were first identified through genetic studies in *Drosophila melanogaster* and *Caenorhabditis elegans*, respectively, and gave rise to the naming of the vertebrate equivalents as ‘SMADs’ (Sekelsky *et al.*, 1995; Derynck & Zhang, 1996). SMADs 1-5 and 8 share highly similar N-terminal domains termed Mad homology (MH) 1, and all SMADs share a highly conserved C-terminal MH2 domain; the domains are separated by a variable proline-rich spacer sequence (Souchelnytskyi, 1997; Liu *et al.*, 1996). Based on structure and function, the eight known SMADs are divided into three classes: receptor regulated SMADs (R-SMADs), common partner SMADs (co-SMADs), and inhibitory SMADs (I-SMADs) (Miyazono, 2000).

Activated TGF- β superfamily type I kinase receptors phosphorylate R-SMADs at C-terminal Ser-Ser-X-Ser motifs. R-SMADs 1, 5, and 8 are phosphorylated in response to BMP signalling, whereas R-SMADs 2 and 3 are phosphorylated downstream of TGF- β and activin signalling. Phosphorylated R-SMADs dissociate from the TGF- β receptors and oligomerize with co-SMADs (SMAD 4). Unphosphorylated SMAD proteins routinely translocate in and out of the nucleus, but a phospho-R-SMAD:SMAD 4 complex translocates into and accumulates in the nucleus. Phosphorylated R-SMADs can regulate target gene expression because SMAD 4 acts as a coactivator and stabilizes the interaction of R-SMADs with DNA and two other essential activators: CREB binding protein (CBP) and p300 (Derynck & Zhang, 2003; Janknecht *et al.*, 1998; Topper *et al.*, 1998). Thus, SMAD proteins are critical in propagating TGF- β superfamily signals from outside the cell to transcriptional responses within the nucleus. Nuclear dephosphorylation renders SMADs transcriptionally inactive and transports them back to the cytoplasm.

The I-SMADs (SMADs 6 and 7) antagonize TGF- β and BMP signalling by competing for and forming a stable complex with the activated type I receptor; this association blocks access to the R-SMADs, preventing their phosphorylation and activation. SMAD 6 inhibits TGF- β /activin receptors, whereas SMAD 7 inhibits both BMP and TGF- β /activin receptors (Hayashi *et al.*, 1997; Hata *et al.*, 1998). The transcription of I-SMADs is rapidly and directly induced by TGF- β /activin and BMP signalling, providing a mechanism of negative feedback that regulates SMAD signalling (Nakao *et al.*, 1997; Afrakhte *et al.*, 1998; Takase *et al.*, 1998).

Despite the few numbers of SMADs in comparison to their many ligands, there is considerable context dependent transcriptional regulation versatility in this pathway.

SMAD oligomeric complexes and a coordination with many sequence-specific transcription factors help to achieve their signalling diversity. In addition, it is becoming increasingly clear that the TGF- β superfamily ligands activate many other signalling pathways besides the SMADs. While some of the resulting responses are SMAD-independent, others help mediate the SMAD response (for review: Derynck & Zhang, 2003).

The regulation of SMADs within the context of liver immunity and muscle plasticity in *X. laevis* is examined in Chapters 3 and 4, respectively.

CHAPTER 2

**Developmental and environmental impacts on brevinin-1SY
expression in the wood frog, *Rana sylvatica***

INTRODUCTION

The wood frog, *Rana sylvatica*, is one of just a few terrestrially hibernating amphibians that can survive whole body freezing during the winter. Freezing survival requires many changes to physiological and biochemical processes. The onset of freezing (signalled by ice nucleation on the skin) initiates the synthesis of high concentrations of glucose from hepatic glycogen stores (Storey & Storey, 1985). The skin is typically the first tissue to experience freezing and other environmental stresses, yet the consequential impacts on gene expression in this tissue have received very little attention.

In frogs, the skin plays an important role in immune defence. The dermal granular glands synthesize and store antimicrobial peptides (AMPs) that are effector molecules of the innate immune system. These are secreted onto the surface of the skin following injury or stimulation by the sympathetic nervous system and there they provide a first line of defence against microbes in the environment (Rollins-Smith & Conlon, 2005). AMPs are small (~10-50 amino acid residues), positively charged peptides that bind to negatively charged membrane components on microbial cells (Rinaldi, 2002). Although AMPs are commonly found on anuran skin, their presence has also been characterized in other frog tissues (Groisman *et al.*, 1992; Ohnuma *et al.*, 2010).

Most amphibian species that have been examined to date produce multiple types of AMPs that act alone or in synergy to combat a range of invasive microbes; because of their synergistic effects, the action of a peptide mixture is thought to be more important than any single AMP alone (Barra & Simmaco, 1995; Schadich *et al.*, 2010). Although different genera of frogs express common families of AMPs, the peptides from any given species do not share identical amino acid sequences with other species (Conlon *et al.*,

2004). The brevinin AMPs are found in ranid frogs and share only five invariant residues (Pro³, Ala⁹, Cys¹⁸, Lys²³, Cys²⁴). Uniquely, the skin of the wood frog *R. sylvatica* produces only a single detectable AMP, the novel brevinin-1SY. Brevinin-1SY is a 24 amino acid peptide with tested antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* (Matutte *et al.*, 2000). Skin extracts obtained from wood frog specimens from cold ponds (<7°C) lacked antimicrobial activity, implicating an environmental role in AMP expression (Matutte *et al.*, 2000). Given that *R. sylvatica* may be frozen for weeks at a time, their vulnerability to pathogens during environmental stress was questioned.

Metamorphosis is another key event in the development of the amphibian immune system (Ohnuma *et al.*, 2010). During metamorphosis, amphibians undergo extensive rearrangements of their cells, organs and tissues; this includes substantial changes to their immune system. The high blood corticosteroid activity associated with the climax of metamorphosis is known to suppress the adaptive immune defences and skin peptide secretion has also been shown to be inhibited by (applied) corticosteroids (for review: Rollins-Smith *et al.*, 1997; Simmaco *et al.*, 1998). Tadpoles have small, immature dermal granular glands and this raises the question of the tadpole's ability to protect itself against environmental pathogens using AMPs prior to metamorphosis (Venable, 1964). In fact, the expression of antimicrobial peptide genes have been shown to be synchronized with metamorphosis, with undetectable levels of gene expression before metamorphosis and markedly increased levels observed during the period during which metamorphosis took place (Reilly *et al.*, 1994b; Ohnuma *et al.*, 2006). Therefore, the

expression of AMPs may not only be dependent on environmental impacts, but also the life stage of the frog.

In the present study, we investigate the tissue-specific expression of the brevinin-1SY gene in adult *R. sylvatica* and the impacts that freezing and its associated stresses (anoxia and dehydration) have on mRNA expression levels. We also examine whether the brevinin-1SY gene is expressed in a developmentally-dependent manner in *R. sylvatica*.

MATERIALS AND METHODS

Animals

Male wood frogs (5-9 g) were collected from spring breeding ponds in the Ottawa region in April 2010. Frogs were washed in a tetracycline bath and placed in plastic containers with damp sphagnum moss at 5°C for one week. Control frogs were sampled from this condition, euthanized by pithing, and tissues were quickly excised and flash frozen in liquid N₂. Remaining animals were divided into three groups and given freezing, anoxia, or dehydration exposures; for freezing and anoxia exposures, methods were essentially as in De Croos *et al.* (2004) whereas dehydration exposures were modified from Churchill & Storey (1993). Briefly, for freezing exposure, frogs were enclosed in plastic boxes lined with damp paper towel, and placed in an incubator set to -4.5°C. The frogs were allowed to cool for 45 min and were nucleated by contact with ice forming on the paper towel when body temperature fell below about -0.5°C. Temperature was then adjusted upwards to -3°C and a 24 h freezing exposure was timed from this

point. For anoxia exposure, frogs were placed in sealed plastic containers (previously flushed with nitrogen gas) containing damp paper towel and held on ice. Containers were again flushed with nitrogen gas for 20 min and were then sealed and placed in an incubator set to 5°C for 24 h. For dehydration exposure, frogs were placed in closed plastic buckets in an incubator set to 5°C; animals were weighed at 12 h intervals until 40% of their total body water had been lost. The total body water lost was calculated from the change in mass of the frogs over time:

$$\% \text{ water lost} = [(M_i - M_d) / (M_i - \text{BWC}_i)] \times 100\%$$

where M_i is the initial mass of the frog, M_d is the mass at any given weighing during the experimental dehydration, and BWC_i is the initial body water content of the frog before dehydration which was experimentally determined to be $0.808 \pm 0.012 \text{ g H}_2\text{O g}^{-1} \text{ body mass}$ (Churchill & Storey, 1993). Frogs were sampled from each of the respective experimental conditions, and tissues were sampled as described above for the control animals. All tissue samples were stored at -80°C until use. Conditions for animal care, experimentation, and euthanasia were approved by the Carleton University animal care committee in accordance with guidelines set down by the Canadian Council on Animal Care.

Tadpole eggs were collected from spring breeding ponds in the Ottawa region in April 2010 and raised in an aquarium until the completion of metamorphosis. Their developmental stages were determined using the Gosner 46-stage developmental chronology (Gosner, 1960). Tadpoles were housed in plastic tanks filled with dechlorinated, municipal water; tadpoles were progressively thinned to other containers

to avoid any negative impacts of crowding. The tadpoles were fed boiled endive fragments and goldfish food ad libitum once daily.

Total RNA isolation and quality assessment

Prior to use, all materials and solutions were treated with 0.1% v/v diethylpyrocarbonate (DEPC; BioShop, Burlington, ON) and autoclaved. Total RNA was extracted from tissues (dorsal and ventral skin, lung, stomach, small intestine, and large intestine) using Trizol™ reagent (Invitrogen, Burlington, ON). Briefly, tissue samples (200-400 mg) were homogenized in 1 ml Trizol using a Polytron homogenizer (n=4-5 independent extractions). A 200 µl aliquot of chloroform was added to each sample and subsequently centrifuged at 24,000 g for 15 min at 4°C. Total RNA was removed and precipitated for 10 min in isopropanol (500 µl) at room temperature. The samples were centrifuged at 5,400 g for 15 min at 4°C; the supernatant was discarded and the RNA pellet washed with 1 ml of 70% ethanol by centrifuging the sample at 5,400 g for 5 min and then removing the supernatant. The pellet was air-dried for 10 min before being re-suspended in 20–50 µl of DEPC-treated water. The RNA concentration of the samples was determined on a GeneQuant Pro spectrophotometer (Pharmacia, Baie d'Urfe, QU) at 260 nm. RNA purity was assessed using a ratio of absorbance at 260/280 nm, while RNA quality was examined by observing the integrity of 18S and 28S ribosomal RNA (rRNA) bands on a native agarose gel electrophoresis with ethidium bromide staining.

cDNA synthesis and PCR amplification

For first strand cDNA synthesis, 3 µg of total RNA were diluted using DEPC treated water to achieve a total volume of 10 µl and 1 µl of oligo-dT (200 ng/µl; 5'-

TTTTTTTTTTTTTTTTTTTTT-3'; V = A or G or C) (Sigma Genosys, Oakville, ON) was added to the sample. The samples were incubated at 65°C for 5 min in a PCR machine (Mastercycler Eppendorf, Mississauga, ON) and then chilled on ice for 5 min. Then 4 µl of 5x first strand buffer (Invitrogen, Burlington, ON), 2 µl 100 mM DTT (Invitrogen, Burlington, ON), 1 µl 10 mM dNTPs (Bio Basic, Markham, ON), and 1 µl Superscript II reverse transcriptase (Invitrogen, Burlington, ON) was added to each sample. This mixture was incubated at 42°C for 60 min in a PCR machine and then held at 4°C.

Serial dilutions of the cDNA in DEPC water (10^{-1} to 10^{-3}) were used to amplify brevinin-1SY as well as α -tubulin (used for normalization). The amino acid sequence determined by Matutte *et al.* (2000) was converted into a hypothetical nucleotide sequence and then compared to the nucleotide sequences of other brevinin-1 genes to determine the codon variants most typical from other *Ranidae* frogs. Primers for brevinin-1SY were designed from the consensus amino acid sequence. The primer sequences were as follows:

(1) brevinin-1SY Forward 5'-GAGCCAGATGAABGGATGT-3' (B = G/T/C)

Reverse 5'-TTTGGTTACTGCACAAATCA-3'

(2) α -tubulin Forward 5'-AAGGAAGATGCTGCCAATAA-3'

Reverse 5'-GGTCACATTTACCATCTG-3'.

PCR was performed by combining 5 µl of a cDNA dilution directly with 15 µl of a prepared PCR-master mix. This master mix was comprised of 1.25 µl of primer mixture

(0.03 nmol/ μ l), 15 μ l of DEPC treated water, 0.75 μ l of 10x PCR buffer (Invitrogen, Burlington, ON), 1.5 μ l of 50 mM MgCl₂, 0.5 μ l of 10 mM dNTPs, and 1 μ l of Taq Polymerase (Invitrogen, Burlington, ON). The amplification program consisted of a 7 min denaturation phase at 95°C, a 33–40 cycle amplification phase consisting of 94°C for 1 min, 62°C (brevinin-1SY) or 54°C (α -tubulin) for 1 min, and 72°C for 1.5 min, and a final elongation phase at 72°C for 10 min. The PCR block was cooled to 4 °C before the reaction tubes were removed. To ensure that the amplified products had not reached saturation, initial studies tested serial dilutions of cDNA to identify the dilution (typically 10⁻²) that was non-saturating yet still showed visible bands for quantification purposes.

PCR products were separated on 2.5% (brevinin-1SY) or 1.0% (α -tubulin) agarose gels for 30 min or 20 min at 130 mA, respectively. Agarose was dissolved in 200 ml of 1x TAE buffer (2 M Tris base, 1.1 ml concentrated acetic acid, 1 mM EDTA, pH 8.5) by heating. The gel was cooled slightly before 2 μ l of ethidium bromide (0.3 mg/300 ml) were added and the solution was allowed to solidify in a gel tray. A 3 μ l aliquot of xylene blue loading dye was added to each PCR reaction, 12.5 μ l samples were loaded onto the agarose gels, and electrophoresis was carried out at 130 V in TAE buffer.

Samples of brevinin-1SY and α -tubulin PCR products were excised from the agarose gels using the freeze/squeeze method before sequencing. Briefly, samples were frozen in liquid nitrogen for ~5 min, thawed, and transferred to 0.5 mL Eppendorf tubes that had been punctured in the bottom, plugged with glass wool, and fitted inside 1.5 mL tubes. Samples were centrifuged at 13,845 g for 5 min and eluants were subsequently transferred to new tubes, diluted with 0.1 vol of 3 M sodium acetate and 3 vol of 90% ethanol, and centrifuged at 13,845 g for 15 min. Next, the supernatants were discarded

and 0.5 mL of 70% ethanol was used to wash the DNA precipitate before drying it for 5 min at room temperature. The purified cDNA pellet was diluted in 40 μ l of DEPC water and sent for sequencing at Bio Basic (Markham, ON). Sequences were confirmed as encoding the correct genes by sequence comparison in BLASTN.

Data and statistics

PCR-amplified products were visualized on ethidium bromide stained agarose gels using a Chemi-Genius BioImaging system (Syngene Corp., Frederick, MD) and the band densities quantified using the associated Gene Tools software. Brevinin-1SY PCR products were normalized against the corresponding band intensities of α -tubulin amplified from the same cDNA sample to correct for any minor variations in sample loading. Data are expressed as means $\pm$ SEM derived from a minimum n = 4 independent samples from different animals. Statistical testing of normalized band intensities used one-way ANOVA and a post-hoc test (Student-Newman-Keuls). Protein analysis was completed using the CLC Protein Workbench software (CLC bio, Aarhus N, Denmark).

RESULTS

cDNA cloning of brevinin-1SY from adult wood frogs

Primers for brevinin-1SY were used in RT-PCR to assess the relative mRNA gene expression levels in tissues of adult wood frogs in response to freezing, dehydration, and anoxia exposures. The brevinin-1SY amplicon was 57 nucleotides and was confirmed as encoding a partial sequence of brevinin using BLASTN. The BLAST search showed that

brevinin-1SY transcript shared the closest homology with the *Rana palustris* brevinin-1 PLc sequence (GenBank accession #: AM745088.1) with 80.7% homology to the corresponding sequence fragment (Fig. 2.01).

The brevinin-1SY amplicon encoded 19 amino acids, covering 79% of the full length, 24 amino acid brevinin-1SY peptide (Swiss-Prot #: P82871.1) (Fig. 2.02). The missing amino acids corresponded to the first 3 amino acids at the N terminus and the last 2 amino acids at the C terminus (Fig. 2.02B). A single amino acid substitution occurred between the translated amplicon and the known peptide sequence reported by Mattute *et al.* (2000): an ATG encoding Met, where Ile₁₆ should be, despite the reverse primer encoding the Ile at the correct position. This is likely a sequencing error, since a substitution of the guanine nucleotide in ATG for any other base would encode an Ile. However, three trials of sequencing resulted in this same translation. A BLASTP of the brevinin-1SY peptide (Swiss-Prot #: P82871.1) showed that this peptide had the closest homology with the *Rana catesbeiana* Ranatuerin-4 precursor (GenBank accession #: ACO51651.1) with 79.2% homology to the corresponding sequence fragment (Fig. 2.02).

Since brevinin-1SY was known to be present in *R. sylvatica* skin, this tissue was analyzed first. Control dorsal skin was found to have a 9.78-fold (± 0.66) proportional greater gene expression than ventral skin (± 0.13) when comparing equal amount of RNA. Next, mucosal tissues were tested for the presence of AMPs since these surfaces are also continually exposed to potentially pathogenic organism without high incidence of infectious disease, suggesting the presence of effective defence mechanisms (Bevins, 1994). All other tissues tested were also found to express the brevinin-1SY gene: lung, stomach, small intestine, and large intestine.

The effects of physiological stresses on brevinin-1SY expression were then tested. In response to 24 h freezing, brevinin-1SY mRNA expression levels decreased significantly in both dorsal skin (to 57% of control values) and large intestine (to 50%) (Fig. 2.03). Anoxia exposure for 24 h strongly increased brevinin-1SY mRNA transcript levels in ventral skin by 5.23-fold, but significantly decreased expression to 67 and 69% of control values in small and large intestine, respectively (Fig. 2.03). During 40% dehydration, the gene expression of brevinin-1SY increased significantly in dorsal skin (2.39-fold), ventral skin (3.29-fold), and lungs (1.57-fold) (Fig. 2.03). No significant changes in brevinin-1SY were seen in stomach under any of the experimental conditions.

cDNA cloning of brevinin-1SY during frog development

The PCR primers used to amplify brevinin-1SY in the adult wood frog were used to amplify gene products from embryonic, tadpole and transforming adult stages, specifically over Gosner stages of development 14-45. Tadpoles were divided into Gosner stages based on observable physiological characteristics: Gosner stages 14-20 (tadpoles within eggs), 21-25 (free swimming tadpole), 26-30 (development of the back limb bud), 31-35 (extension of the limb bud into a jointed leg), 36-41 (toe differentiation), 42-43 (development of front legs), and 44-45 (adsorbed tail bud).

Brevinin-1SY was amplified at all stages tested during tadpole growth and metamorphosis. However, gene expression increased strongly in the later stages of development; expression levels significantly increased as compared to stages 14-20 during Gosner stages 36-41 (by 2.87-fold), stages 42-43 (by 4.5-fold), and stages 44-45 (by 6.22-fold). These three Gosner stage brackets were also significantly different from

each other, with Gosner stage 42-43 being 1.57-fold higher than stage 36-41, and Gosner stage 44-45 being 1.38-fold higher than stage 42-43 (Fig. 2.04).

Interestingly, the brevinin-1SY primers amplified a second 282 bp product from tadpole RNA that was not previously observed in adult tissues (Fig. 2.05A). The sequence had little similarity to that of brevinin-1SY; for example, there was only 43.64% identity between the most similar 56 nucleotide region of the two sequences (Fig. 2.05B). Levels of this unidentified gene product were present throughout development but were highest in stages 14-20 and 26-30 and were significantly lower during stages 21-25 (to 31%), 31-35 (to 36%), 36-41 (to 52%), 42-43 (to 48%), and 44-45 (to 57%) as compared with Gosner stages 14-20 (Fig. 2.04). Hence, the expression pattern of this gene product was largely opposite to that of brevinin-1SY. A BLASTN search of this sequence revealed only a short region (maximum 7% query coverage) of similarity to various Ranidae antimicrobial peptide precursors, with sequence 5' - GAGCCAGATGAAACGGATGT-3'. Except for a single base, this sequence was identical to the brevinin-1SY primer, and is therefore is likely of no significance because it could be forced amplification.

The unidentified amplicon translated to a single open reading frame composing 92 amino acids (Fig. 2.05). Analysis of this sequence showed this polypeptide to be mostly hydrophobic (60% of all residues were hydrophobic, 17% were hydrophilic, and 23% were neither) and neutral (80% neutral, 15% negatively charged, 5% positively charged). Glycine (14%) and proline (15%) were the most frequently occurring amino acids. No secondary structure or Pfam domains were identified. One putative glycosylation and one putative phosphorylation for protein kinase C (PKC) site were

identified; they existed adjacent to each other at amino acids 53-56 and 55-57, respectively. Compared with the Kite-Doolittle scale of hydrophobicity, the sites of post-translation modification overlapped one of the most hydrophobic regions of the peptide (amino acids 56-60), making it unlikely that functional posttranslational modifications do occur at the aforementioned sites.

DISCUSSION

The plasticity of *R. sylvatica* that allows this species to survive a number of different environmental stressors provides a natural model to analyze the impacts of environmental effects (freezing, anoxia, dehydration) on the immune system. Environmental impacts on amphibian disease are thought to be contributing to the unprecedented declines of amphibians around the world, yet the relationship dynamic between immunity and environment is not well understood (Fisher, 2007; Bosch *et al.*, 2007).

Environment can change host-pathogen interactions in several ways. First, microbe communities and their viability change with differing environmental conditions, exposing frogs to altered types and patterns of pathogens (Castro *et al.*, 2010; Sheik *et al.*, 2011). For example, the so-called ‘chytrid’ fungus, *Batrachochytrium dendrobatidi*, causes the infectious disease chytridiomycosis (a major cause of death in modern anurans globally). Chytrid infection shows thermal and hydric dependence and therefore shows a significant association with changes in climatic variables (Bosch *et al.*, 2007; Richards-

Zawacki *et al.*, 2007; Bustamante *et al.*, 2010). Hence, the presence of different types and amounts of pathogens may require altered defenses in frogs.

Additionally, being ectothermic, the frog's ability to mount an immune defense may be dependent on environmental temperature (Raffel *et al.*, 2006; Rohr & Raffel, 2010). Both high and low environmental temperature can challenge viability, limit energy availability and trigger metabolic rate depression to support survival during these periods of bioenergetic constraint. Theoretical work maintains that a partially effective immunity can achieve the greatest fitness when balancing the costs of the immune system. Maintaining the immune system is minimal, while mounting an immune response has a significant cost and can draw energy resources from other physiological systems (Derting & Compton, 2003; McDade *et al.*, 2008) – although this may be an unlikely event in the 'budget' of an animal in a hypometabolic state. One might hypothesize that under environmental stress and/or during hypometabolism, frogs would depend more on the competence of the constitutively expressed innate immune system and the maintenance of its non-specific effector molecules (such as AMPs) in an infectious environment rather than more ATP-expensive acquired facets of immune immunity (Bonneaud *et al.*, 2003; McNamara & Buchanan, 2005).

Understanding how climate affects the disease dynamic requires insight into how environment affects host-pathogen interactions via the immune system (Richards-Zawacki *et al.*, 2010). Using *R. sylvatica*, we are able to tease apart whether changes in an immune response are changed universally because of the unifying metabolic rate depression experienced during each freezing, anoxia, and dehydration or whether there are environmentally specific effects that lead to unique patterns of response for each of

the stresses. Additionally, because wood frogs express only a single dermal antimicrobial peptide, expression trends are not confounded by multiple functionalities of differing AMPs.

Previous studies by Matutte *et al.* (2000) revealed that skin from wood frogs collected from cold ponds during the early spring breeding period that occur immediately after winter hibernation lacked detectable concentrations of brevinin-1SY in the skin or any other similar antimicrobial activity. This led to the conclusion that peptide synthesis is dependent on microbial challenge, which was presumably correspondingly weak in the cold winter habitat. This hypothesis was cited in several other papers and backed up by evidence that *R. esculenta* produce very low amounts of AMPs when kept in a sterile environments (Mangoni *et al.*, 2001; Conlon *et al.*, 2004). Indeed, the analysis of gene expression levels of brevinin-1SY in the present study showed a drop in AMP mRNA levels during 24 h freezing in the dorsal skin of *R. sylvatica*, which is in line with the results observed by Matutte *et al.* (2000). Large intestine also showed a freeze-responsive decreased in AMP mRNA expression. However, mRNA levels did not drop in the ventral skin, lungs, stomach, or small intestine of *R. sylvatica* after 24 h of freezing. This is curious, considering that an adaption to match immune response with bacterial challenge in the environment should perhaps occur universally throughout the frog. A hypothesis to rationalize the drop in AMP expression in just dorsal skin and large intestine during freezing might be developed based on the relationship that *R. sylvatica* has with ice-nucleating bacteria over the winter.

Wood frogs survive freezing best when the freezing event is slow and allows lots of time for cryoprotective adjustments such as the synthesis and distribution of the

glucose cryoprotectant or the up-regulation of specific genes (Storey & Storey, 2004a). Slow freezing is ensured if nucleation occurs at a temperature just below the freezing point of body fluids (about -0.5°C) – that is, if supercooling is minimized. To ensure minimal supercooling and ice nucleation close to the freezing point, frogs employ ice nucleators. These include inoculative freezing initiated by environmental ice, the actions of ice-nucleating bacteria, and ice-nucleating proteins in the blood (for review: Storey & Storey, 2004a). In a damp hibernaculum, inoculative freezing due to skin contact with environmental ice is the likely the major mode of nucleation (Constanzo *et al.*, 1999). However, in the event that there is no direct contact with external ice, ice-nucleating bacteria trigger crystallization before supercooling becomes extensive (Layne, 1995). Ice-nucleating active (INA) bacteria in the natural flora of the large intestine (the area with the largest interaction between the host and microorganisms) and dorsal skin of winter wood frogs are thought to be responsible for the characteristic supercooling point of about -2°C of frogs cooled on dry substrate (Lee *et al.*, 1995; Layne, 1995; Mahida *et al.*, 1997). Cold induced down-regulation of AMPs in these areas may allow the increased colonization or viability of INA bacteria during the winter when they are most needed. More investigation of the environmentally inducible nature of INA bacteria is required, since to date they have only been isolated from winter (February) *R. sylvatica*.

One element of natural freeze tolerance in wood frogs is the ability to endure prolonged anoxia and ischemia that allows the animals to survive without oxygen over prolonged periods of freezing. A common response to hypoxia is to alter the perfusion of organs to maintain blood flow to critical organs at the expense of reduced blood flow to the digestive organs (Davies, 1989; Axelsson & Fritsche, 1991; McGaw, 2005). This is

effective in hypoxia tolerant species but in hypoxia sensitive species (especially mammals), the labile cells of the intestinal mucosa are readily injured by periods of hypoxia/reoxygenation and ischaemia/reperfusion (Redan *et al.*, 1990; Xu *et al.*, 1999). Hypoxia often leads to gut damage that allows bacterial translocation: the penetration of gut flora through the intestinal barrier into the sterile tissue beyond (Zhi-Yong *et al.*, 1992; Tazuke *et al.*, 2003). This has drawn focus to the gut as the origin of sepsis and multiple organ dysfunction syndrome (MODS) following hypoxia in humans (Rotstein, 2000). Therefore, one might hypothesize that an increase in gut immune function, such as via up-regulation of AMPs, may benefit this scenario. However, the reverse may be true. Interestingly, gut luminal bacteria and sublethal doses of endotoxins have been found to ameliorate ischaemia/reperfusion injury systematically (potentially by desensitization of the inflammatory response), whereas digestive decontamination in clinical studies has failed to reduce MODS (van der Hoven *et al.*, 2001; Turnage *et al.*, 1994; Neviere *et al.*, 2000; Rotstein, 2000). While primary effects of reperfusion injury are mediated by reactive oxygen species, secondary effects arise from abnormal recruitment of immune effector molecules (Simons *et al.*, 1987; Kong *et al.*, 1998). Likewise, it has been found that the depletion of various intestinal resident immune molecules prevents reperfusion injury in the gut (Chen *et al.*, 2004; Mbachu *et al.*, 2004). Coincident with this scenario, the present results for wood frogs showed that gut (small and large intestine) brevinin-1SY expression decreased over 24 h anoxia. This may reflect a mechanism to limit collateral tissue damage by immune cells during anoxia or may be a preparatory mechanism to combat impending oxidative stress that occurs rapidly following re-oxygenation (for review: Hatfield *et al.*, 2009). Coordinated

changes in antioxidant defenses during anoxia are a commonality between animals that endure variable oxygen environments (for reviews: Hermes-Lima *et al.*, 1998 & 2002). Most of the research has focused on the build-up of antioxidants, but as seen here, alteration of the immune system could perhaps also play a role in the prevention of re-oxygenation injury. Anoxia/hypoxia also occurs during freezing and under high levels of dehydration (Joanisse & Storey, 1996; Hillman, 1978, 1987). Therefore, in line with this hypothesis, the effect of thawing and rehydration would also be predicted to result in decreased AMP levels.

While hypoxic animals often perfuse other organs at the expense of digestive blood flow, selective redistribution of blood flow between cutaneous regions in frogs during changing environmental oxygen content does not occur, and the skin still plays a role in gas exchange during hypoxia in frogs (Boutilier *et al.*, 1986; West & Burggren, 1984; Pinder & Burggren, 1986). Boutilier *et al.* (1986) suggested that the fixed distribution of blood supply to the skin ensures optimal perfusion in spite of environmental changes. Frog lungs, however, have been shown to receive increased blood flow during hypoxia, likely to provide blood in the area where the most oxygen should be available. During anoxia, breathing rate has been shown to progressively decrease until it eventually ceases in *R. pipiens* and since lymph movement is dependent on lung ventilation in frogs, the physiological responses to anoxia may inadvertently lower immunity (Winmill *et al.*, 2004; Hedrick *et al.*, 2007). With a decreased internal immune function, the protection of the skin by AMPs may become increasingly important. This might account for the rise in ventral skin AMP levels observed in *R.*

sylvatica during 24 h anoxia; the heightened AMP mRNA would thus become increasingly comparable to that of the normal levels of the dorsal skin.

The skin of amphibians regulates water uptake and salt balance to meet different physiological conditions (Shoemaker & Nagy, 1977; Greenwald, 1971). Owing to their highly permeable skin, amphibians have a high susceptibility to evaporative water loss. Amphibians tolerate wide variations in water content and ionic strength of their body fluids (Shoemaker, 1992); in the wood frog, endurance of up to 50-60% of total body water loss is a mechanism that aids in freezing when extracellular ice and evaporative water loss cause extensive cellular dehydration (Churchill & Storey, 1993). In fact, freeze tolerance has been suggested to have arisen from a pre-existing mechanism used to defend against water stress (Churchill & Storey, 1994a, 1995). In wood frogs, dehydration alone provokes a hyperglycemia response, comparable to the build-up of cryoprotectants during freezing (Churchill & Storey, 1993, 1994b); similarly, the build-up of urea to protect against colligative water loss is thought to be important in both dehydration and freezing (Costanzo & Lee, 2005; Muir *et al.*, 2007). Parallel molecular responses are also observed between wood frog dehydration and freezing, including a reduced metabolism, activated liver glycogen phosphorylase, and elevated %PKAc and liver second messenger cAMP levels (Pinder *et al.*, 1992; Churchill & Storey, 1994b; Holden & Storey, 1997). Despite these similarities, brevinin-1SY expression patterns differed between dehydration and freezing stresses in wood frogs. Whereas AMP transcript levels were significantly decreased in dorsal skin and small intestine during freezing, dehydration to 40% of total body water loss caused significant increases in AMP mRNA expression levels in lungs and both the ventral and dorsal skin. Similar to

these results, *magainin* mRNA increased during medium and high dehydration and magainin protein levels were elevated during high dehydration in African clawed frogs, *Xenopus laevis* (HA Holden, unpublished data). Frogs rely on both pulmonary and cutaneous gas exchange; therefore, the impacted tissues of lung and skin could be unified by their role in respiration (Gottlieb & Jackson, 1976). Additionally, as the first organ to experience evaporative water loss, the skin is placed to have stress-detecting and signaling functions to trigger survival adaptations (Wu *et al.*, 2008).

Dehydration is accompanied by a markedly reduced blood flow (likely because of decreased blood pressure as a result of lowered blood volume), increased blood viscosity, a decreased number of perfused skin capillaries, and leads to tissue hypoxia because of these difficulties in acquiring and circulating oxygen (Christensen, 1974; Hillman, 1987; Malvin *et al.*, 1992; Churchill & Storey, 1993; Muir *et al.*, 2007; Burggren & Moalli, 1984). A study in *R. pipiens* also showed that during dehydration the skin experiences very high water loss compared with other organs, resulting in significant mass loss due to ecdysis (Smith & Jackson, 1931). These factors greatly impact cutaneous gas permeability. However, despite drastic changes during dehydration, the skin remains important for cutaneous CO₂ excretion (Boutilier *et al.*, 1979; Burggren & Vitalis, 2005). A continued exposure to airborne pathogens, combined with a reduced availability of vasculature or blood perfusion to the infected area could increase the vulnerability of the respiratory organs in dehydrated animals, since many immune effector molecules travel through the blood. Further impairing the internal immune system may be a drastically decreased lymph flow (as observed in *B. marinus* during dehydration) owing to the decreased lung ventilation frequency in amphibians during water loss (Jones *et al.*, 1997).

A compensatory effect might be the observed increase in dermal AMPs to diminish potential infection opportunities in these areas during dehydration. Additionally, skin wound healing, as might be associated with the regeneration of the integument following unnatural ecdysis due to dehydration, has been shown to be associated with an induction of AMPs (Nakazawa *et al.*, 2003; Bardan *et al.*, 2004; Ohnuma *et al.*, 2006).

Development and metamorphosis is associated with massive rearrangement of tissues and systems, including the immune system (for review: Rollins-Smith, 1997). Tadpoles have an immune system that is adequate for their aquatic environment, but is not the same as the mature immune system that these frogs have as adults when they live in both terrestrial and aquatic environments. Previous studies have shown that, in general, AMP mRNA expression is undetectable before the onset of metamorphosis. In *X. laevis*, peptide expression was shown to occur ~2 stages after mRNA expression (Vanable, 1964; Reilly *et al.*, 1994b; Ohnuma *et al.*, 2006). Although small levels of brevinin-1SY transcripts were found in all Gosner stages analyzed, strong increases in transcript levels occurred only at the end stages of metamorphosis (stages 36-45); the timing of these significant increases are consistent the emergence of AMP expression as seen in other species of anurans (Reilly *et al.*, 1994b; Clark *et al.*, 1994; Ohnuma *et al.*, 2006). AMP expression is thought to be induced by thyroid hormones (T_3) associated with metamorphosis, and corresponds with the development of mature mucosal and dermal glands in the adult skin tissue (Ohmura & Wakahara, 1998). The current findings are in accordance with this hypothesis, since significant increases in wood frog brevinin-1SY were synchronized with the localized development of adult epidermal tissue in the transforming tadpole. Significant increases in antimicrobial expression were first

observed at stages 36-41, during which adult epidermis is first found on the limbs of the metamorphic frog and parts of the trunk region. The next significant increase in brevinin-1SY expression (Gosner stages 42-43) was associated with adult skin formation on the remaining regions of the body and parts of the head (except the tail, which does not transition to adult epidermis). Adult skin development is complete at Gosner stage 45 (Suzuki *et al.*, 2009), when the final jump in brevinin-1SY levels was observed in *R. sylvatica*. This relationship between the antimicrobial peptide expression and the development of adult skin suggests that the basal levels of brevinin-1SY gene expression observed before adult skin growth may be associated with the internal organs. Antimicrobial expression has been identified previously, but less commonly, during the tadpole stage, with amplification of basal levels during metamorphosis as observed here (Wabnitz *et al.*, 1998; Ohnuma *et al.*, 2010).

The marked coordination between AMP levels and frog readiness to leave the aquatic environment suggests that AMPs are a requirement for dealing with terrestrial pathogens. The transformation of the larval integument from apical, skein, and basal cells to an adult skin consisting of a stratified squamous epithelium with a developed dermis exposes a vulnerable stratum coreum susceptible to invasion (for review: Yoshizato, 2007; Berger *et al.*, 1999). However, another hypothesis is that AMPs might also play a role in the apoptosis of larval skin cells so that they can be replaced by adult skin cells during metamorphosis; this hypothesis occurred when several antimicrobials were shown to have apoptotic properties (for review: Conlon *et al.*, 2009). Programmed cell death is necessary for the development of the adult amphibian and, like all aspects of metamorphosis, is controlled by thyroid hormones (TH) (for review on apoptosis during

development: Ishizuya-Oka *et al.*, 2010; Hayes, 1997). TH levels peak during the metamorphic climax – this is associated with the apoptotic death of the upper two layers of the epidermis, while the basal layer proliferates to reconstitute the adult dermis (Schreiber & Brown, 2002); peak TH production occurs between stages Gosner stages ~40-44 (Yaoita & Brown, 1990; Regard *et al.*, 1978). In the present study, peak antimicrobial expression of brevinin-1SY occurred between Gosner stages 44-46 in the wood frog, so this dual function cannot be ruled out.

During the amplification of tadpole brevinin-1SY, a second unidentifiable amplicon appeared that was not previously seen in adult tissues. This unidentified amplicon was tadpole specific, down-regulated concurrently with the upregulation of brevinin-1SY but showed little homology with brevinin-1SY. In fact, a BLASTP of the putative protein sequence found no significant similarity to anything. The unidentified amplicon translated to a single open reading frame coding for 92 primarily hydrophobic amino acids and further analysis indicated no likely secondary structure, Pfam domains, or sites of post-translation modification. Since antimicrobial peptides are generally cationic, amphipathic alpha helices with a preponderance of lysine amino acids (Matutte *et al.*, 2000), it would seem unlikely that this polypeptide is a novel AMP.

Analysis of skin AMPs in tadpoles, metamorphs, and adults of Ewing's tree frog also isolated tadpole specific peptides that did not correspond to the known antimicrobials of that species (Schadich *et al.*, 2010). The physiological roles of these peptides are unknown, but were conjectured to include chemical cues to conspecifics or defence against specific pathogens (these peptides were not effective when tested against

E. coli). These same roles, as well as metamorphic roles, could also be predicted for the unidentified amplicon observed in *R. sylvatica* tadpoles.

In conclusion, freeze-responsive genes in wood frogs can also be categorized as responsive to either the anoxia (representing freeze-induced ischemia) or dehydration (representing freeze-induced cell volume reduction) component. The present results show that brevinin-1SY responds to each. The response pattern of brevinin-1SY was different during freezing, anoxia, and dehydration and shows an environmentally regulated, organ-specific expression that addresses the individual needs and physiological changes of each of the tissues so as to promote whole animal survival during that time. The major role of brevinin-1SY in the skin in reacting to the environment and mediating an appropriate immune response is evident because skin was the only tissue that showed a change in AMP expression in response to all three stresses: freezing, anoxia, and dehydration. By contrast, brevinin-1SY expression in the stomach was not affected by changes in the environment. Brevinin-1SY was not only an adult specific AMP, as is common to brevinin forms in other species, but rather was expressed at low basal levels until metamorphosis, when expression was significantly amplified. Interestingly, a tadpole specific amplicon of unknown identity was observed.

(A)

(B)

Brevinin-1SY	100%				
Brevinin-1BLb	77.2%	100%			
Brevinin-1Pa	77.2%	94.7%	100%		
Brevinin-1CHa	77.2%	91.2%	93.0%	100%	
Brevinin-1PLc	80.7%	91.2%	93.0%	93.0%	100%

(C)

```

Brevinin-1SY  GTAGTTGCAGGTCTGGCCGCTAAGGTCTTGCCGTC AATGATTTGTGCAGTAACAAAA
Brevinin-1BLb a-ta-----ca-----t-aa---at-----a---gc---
Brevinin-1Pa  a-ta-----cg-----c---aa---at-----a---gc---
Brevinin-1CHa a-ta-----cg-----aaa-t-at-----a---c---
Brevinin-1PLc --ta-----cg-----t-----aa---at-----a---c---

```

FIG. 2.01. Partial nucleotide sequence of *R. sylvatica* brevinin-1SY and comparison to other frog species.

Partial brevinin-1SY nucleotide sequence from *R. sylvatica* aligned with the brevinin nucleotide sequences from four other species: brevinin-1BLb (*Rana blairi*; Accession no: EU407153.1), brevinin-1Pa (*Rana pipiens*; Accession no: EU407148.1), brevinin-1CHa (*Rana chiricahuensis*; Accession no: DQ923154.1), and brevinin-1PLc (*Rana palustris*; Accession no: AM745088.1) showing (A) homology tree, (B) homology matrix, and (C) regions of conservation (only those nucleotides differing from the *R. sylvatica* brevinin-1SY are indicated) for the five sequences.

FIG. 2.02 (legend on next page)**(A)**

Nucleotide GTAGTTGCAGGTCTGGCCGCTAAGGTCTTGCCGTCAATGATTTGTGCAGTAACAAA
 Protein V V A G L A A K V L P S M I C A V T K

(B)

Translated Amplicon ...VVAGLAAKVLPSMICAVTK..
 |||||:|||||
 Peptide Sequence FLPVVAGLAAKVLPSIICAVTKKC

(C) **(D)**

Brevinin-1SY	100%						
Brevinin-1CHb	79.2%	100%					
Brevinin-1Pb	66.7%	79.2%	100%				
Brevinin-1PLc	75.0%	87.5%	79.2%	100%			
Brevinin-1CG4	70.8%	70.8%	66.7%	75.0%	100%		
Ranatuenn-4	79.2%	66.7%	66.7%	62.5%	58.3%	100%	

(E)

Brevinin-1SY	FLPVVAGLAAKVLPSIICAVTKKC
Brevinin-1CHb	----i-----klf--i----
Brevinin-1Pb	---ii--i----f-k-f--is---
Brevinin-1PLc	----i--v---f--k-f--i----
Brevinin-1CG4	---i-----nf--k-v-ki----
Ranatuenn-4	---fi-r-----f-----s-----

FIG. 2.02. Partial nucleotide and deduced amino acid sequence of *R. sylvatica* brevinin-1SY.

(A) Partial nucleotide and deduced partial amino acid sequence of brevinin-1SY (*R. sylvatica*). (B) A single open reading frame was predicted from the nucleotide sequence and coded for a peptide of 19 amino acids which corresponded to the known amino acid sequence of the brevinin-1SY (Mattute *et al.*, 2000). Brevinin-1SY amino acid sequence (Accession no: P82871.1) was aligned with similar sequences from other species: brevinin-1CHb (*R. chiricahuensis*; Accession no: ABK91541.1), brevinin-1Pb (*R. pipiens*; Accession no: ABB89061.1), brevinin-Plc (*R. palustris*; Accession no: CAN87010.1), brevinin-1CG4 (*Amolops chunganensis*; Accession no: ADM34207.1) and ranatuerin-4 (*Rana catesbeiana*; Accession no: ACO51651.1) showing (C) homology tree, (D) homology matrix, and (E) regions of conservation (only those amino acids differing from the *R. sylvatica* brevinin-1SY are indicated) for the six sequences.

FIG. 2.03. Effects of freezing (24 h), anoxia (24 h), and dehydration (40% total body water lost) on the mRNA expression of brevinin-1SY as determined by RT-PCR. Representative RT-PCR bands of brevinin-1SY (above) and α -tubulin (below) are shown and the corresponding histograms show normalized mean values. Data are means $\pm$ SEM, $n=4-5$ independent trials. ^aSignificantly different from the corresponding control (5°C acclimated) value as determined using a one way ANOVA (SNK), $P<0.05$. ^bSignificantly different from the corresponding freezing value as determined using a one way ANOVA (SNK), $P<0.05$. ^cSignificantly different from the corresponding dehydration value as determined using a one way ANOVA (SNK), $P<0.05$.

FIG. 2.04. Effects of growth and metamorphosis through Gosner Stages 14-20, 21-25, 26-30, 31-35, 36-41, 42-43, and 44-45 on the mRNA expression of brevinin-1SY in *R. sylvatica* as determined by RT-PCR. Representative RT-PCR bands of brevinin-1SY and α -tubulin (internal standard) and corresponding histograms showing normalized mean values. Data are means $\pm$ SEM, $n=4-5$ independent trials. Significantly different from the corresponding Gosner stage ^a 14-20 value, ^b 21-25, ^c 26-30, ^d 31-35, ^e 36-41, or ^f 42-43 value as determined using a one way ANOVA (SNK), $P<0.05$.

(A)

```

1      TTGGAGCCAGATGAAACGGATGTAGGAGCAATGAGTATTCAACCACACGGAGTTCGGTCA
1      L E P D E T D V G A M S I Q P H G V R S

61     CAGTTTCCACAACCGTTGGGGGAGGTGCCACCAGATGAAGATAATGTGGAGGGACTCCCC
21     Q F P Q P L G E V P P D E D N V E G L P

121    TTGGGATTTCATGCGCGGAAGAGCCTTTTGGTATGGGCAACCATACGAAGAGGCCAGTCCCC
41     L G F M A E E P F G M G N H T K R P V P

181    AACGATCCCTCACCCCTGAGAGGAGGATTTTTAATTATCGGTTGGCCAGAGCAAGAAGGG
61     N D P S P L R G G F L I I G W P E Q E G

241    TGTGGAAAATGCCTCGGTACTATGCCAATCGTCAGC
81     C G K C L G T M P I V S

```

(B)

```

Brevinin-1SY TAGTTGCAGGTCTGGCCGCTAAGGTCTTGCCGTCAATGATTGTGCAGTAACAAA
              || |  || ||  ||          || || || | |  || ||  |  ||
UnID mRNA     ATGTGGAGGGACTCCCCTTGGGATTTCATGGCGGAAGAGCCTTTTGGTATGGGCAAC

```

FIG. 2.05. Partial nucleotide and deduced amino acid sequence of *R. sylvatica* unidentified mRNA.

(A) Partial nucleotide sequence of the unidentified mRNA transcript from *R. sylvatica* tadpoles and its deduced partial amino acid sequence. A single open reading frame was predicted from the nucleotide sequence and coded for a polypeptide of 92 amino acids. (B) Alignment of the 56 nucleotide region (shaded in part A) of the unidentified mRNA transcript that showed greatest homology with the corresponding segment of the brevinin-1SY nucleotide sequence.

CHAPTER 3

Hepcidin antimicrobial peptides from the African clawed frog, *Xenopus laevis*

INTRODUCTION

The African clawed frog, *Xenopus laevis*, has been extensively studied since the 1950s as a model of embryonic development, but much less is known about the physiology and biochemistry of the adult animal or its strategies for dealing with environmental stress (Beck & Slack, 2001). Studies have shown that the microbe community changes during desiccation (Amalfitano *et al.*, 2008; Marxsen *et al.*, 2010). Therefore, during estivation in burrows that are drying out or during overland migrations, frogs may be exposed to different types or patterns of pathogens and may need altered defenses. Although many amphibians, including *X. laevis*, can tolerate 30-50% of total body water loss, the effects of dehydration on amphibian immune defenses is lacking and requires investigation (Carey *et al.*, 2001). Because of the high energy cost of mounting an adaptive immune response, we hypothesized that elements of innate immunity might be heightened when frogs enter estivation, potentially triggered as a response to dehydration (Moret *et al.*, 2003). To explore the idea that the innate immune system is heightened during estivation, the regulation of effector molecules of innate immunity were investigated in response to dehydration stress in *X. laevis*.

Antimicrobial peptides (AMPs) are broadly effective defense molecules that exist as part of the innate immune system of all vertebrates. Hepcidin is one of these – a conserved, cationic, amphipathic peptide that is synthesized by the liver and released into the plasma where it functions as both an internal AMP and an iron regulatory hormone (Hu *et al.*, 2008; Park *et al.*, 2001). Hepcidin inhibits efflux of iron from cells by binding to the cellular iron exporter ferroportin causing it to be degraded. Hypoferremia can inhibit the proliferation of some pathogens in the bloodstream and, therefore, the

regulation of iron availability by hepcidin contributes to host defense (Nemeth *et al.*, 2004). Hepcidin expression is regulated by three main signals: transforming growth factor- β (TGF- β), bone morphogenetic protein (BMP), and interleukin 6 (IL6) (Fig. 3.01).

Both BMP and TGF- β ligand binding cause the heteroligmerization of two distantly related type I and type II transmembrane serine/threonine kinase receptors that subsequently activate an evolutionarily conserved transcription factor family called SMADs (Souhelnytskyi *et al.*, 1997; Nakayama *et al.*, 2001; Fig. 3.01). When the BMP ligand is bound by the BMP coreceptor hemojuvelin (HJV), the downstream receptor regulated SMADs (R-SMAD) 1,5, and 8 activate hepcidin transcription (Babitt *et al.*, 2006). By contrast, R-SMADs 2 and 3 are phosphorylated downstream of TGF- β signaling and, when bound by growth differentiation factor 15 (GDF15), are thought to antagonize hepcidin transcription (Tanno *et al.*, 2007; Finkenstedt *et al.*, 2009; Lakhali *et al.*, 2009; Chou & Weiss, 2007; Fig. 3.01). Phosphorylated R-SMADs dissociate from the receptor and oligomerize with the common partner SMAD4 (co-SMAD4); the heteromeric SMAD complex translocates into the nucleus where it regulates hepcidin expression (Nakayama *et al.*, 2001). The inhibitory I-SMADs (SMADs 6 and 7) antagonize TGF- β and BMP signaling by forming a stable complex with the activated type I receptor; this association blocks access to the R-SMADs, preventing their phosphorylation and activation (Hayashi *et al.*, 1997; Hata *et al.*, 1998).

IL6 is a cytokine released in response to an inflammatory stimulus as a part of the acute phase response (APR) of the innate immune system and binds IL6 receptor α and glucoprotein 130 (gp130) complexes (for review: Carbia-Nagashima & Arzt, 2004). This

interaction activates Janus kinases (JAKs) that in turn phosphorylate the signal transducers and activators of transcription (STAT) family, activating them and allowing them to translocate into the nucleus. STAT3, when phosphorylated on tyrosine 705, regulates hepcidin expression directly by binding the promoter region (Wrighting & Andrews, 2006; Fig. 3.01).

The present study examines the effect of dehydration on the expression of hepcidin in *X. laevis* liver including upstream regulation by SMAD and STAT signaling. The data show that hepcidin transcription is upregulated in response to dehydration and STAT3 is implicated in its control.

Fig. 3.01. Molecular events outlining the impact of the SMAD signaling pathway on liver cells. BMP 6 with co-receptor hemojuvelin cause the heteroligmerization of type I and type II SMAD receptors which phosphorylate R-SMAD 5 causing them to bind with SMAD 4, translocate into the nucleus, and activate hepcidin. TGF- β signalling causes the heteroligmerization of type I and type II SMAD receptors which phosphorylate R-SMADs 2 and 3 causing them to bind with SMAD 4, travel into the nucleus, and inhibit hepcidin. SMAD 6 antagonizes BMP signaling by forming a stable complex with the activated type I receptor. IL6 binds its receptor and this interaction activates Janus kinases (JAKs) that in turn phosphorylate the signal transducers and activators of transcription (STAT) family, activating them and allowing them to translocate into the nucleus to activate hepcidin expression. cMYC is a co-regulator of hepcidin expression that is inhibited by SMADs 2 & 3.

MATERIALS AND METHODS

Animals

Female *X. laevis* (31-47 g) were donated from a colony at the University of Toronto in 2010. In our lab, they were rinsed in a tetracycline bath and then held in tanks of dechlorinated water at 22°C for at least 3 weeks before use. Control animals were sampled from these conditions. The remaining animals were divided into two groups and dehydrated to two levels: (1) medium dehydration (24±1 % of total body water lost; n=7) and (2) high dehydration (38 ±1 % of total body water lost; n=6). For dehydration experiments, frogs were placed in closed plastic containers and allowed to air dry over time. Water loss was monitored by weighing the frogs at 12 h intervals. The change in mass of each frog was used to calculate the percentage of total body water lost:

$$\% \text{ water lost} = [(M_i - M_d) / (M_i - \text{BWC}_i)] \times 100\%$$

where M_i is the initial mass of the frog, M_d is the mass at any given weighing during the experimental dehydration, and BWC_i is the initial body water content of the frog before dehydration which was experimentally determined to be $0.74 \pm 0.02 \text{ g H}_2\text{O g}^{-1} \text{ body mass}$ (Malik & Storey, 2009). Frogs were sacrificed by pithing once they reached the predetermined level of water loss and liver was quickly excised, frozen in liquid N_2 and then stored at -80°C .

Preparation of crude tissue extracts

For total protein extracts, frozen liver samples were homogenized 1:5 w:v in homogenization buffer A (20 mM Hepes, 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1

mM Na₃VO₄, 10 mM β-glycerophosphate) with ~1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) added immediately preceding homogenization using a Polytron PT10 homogenizer. Homogenates were centrifuged at 24,000g for 15 min in a Sorval BC-5B refrigerated centrifuge at 4°C and then the supernatant was removed.

For nuclear and cytoplasmic extracts, separate frozen liver samples were homogenized 1:2 w:v in homogenization buffer B (10 mM Hepes, 10 mM KCl, 10 mM EDTA, 10 mM β-glycerol phosphate, 10 mM DTT, 1% v:v Protease Inhibitor Cocktail (Sigma, pH 7.9) using a Dounce homogenizer (20 piston strokes). Homogenates were centrifuged at 24,000g for 10 min at 4°C. The supernatant was removed (cytoplasmic fraction) and the pellet was then resuspended in 150 µl of extraction buffer per gram of starting tissue. Extraction buffer contained 10 mM Hepes, 0.2 M NaCl, 0.5 mM EDTA, 5% v:v glycerol, 10 mM β-glycerophosphate, 10 mM DTT, and 1% v:v Protease Inhibitor Cocktail. Following 1 h rocking on ice, the resuspension was centrifuged at 24,000g for 10 min at 4°C. The supernatant was removed (nuclear fraction).

Soluble protein concentration in all extracts was quantified by the Coomassie blue dye-binding method using the Bio-Rad prepared reagent (BioRad Laboratories, Hercules, CA) with bovine serum albumin as the standard; absorbance at 595 nm was measured on a MR5000 microplate reader. Samples were normalized to a constant protein concentration by the addition of small amounts of homogenization buffer A or B (as required) and then aliquots were mixed 1:1 v:v with 2x loading buffer (100 mM Tris-base, 4% w:v SDS, 20% v:v glycerol, 0.2 w:v bromophenol blue, 10% w:v 2-

mercaptoethanol), boiled for 5 min, cooled to room temperature, and transferred to long-term storage at -20°C.

Western blotting

Equal amounts of protein were loaded into lanes of SDS polyacrylamide gels and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on a BioRad mini-gel apparatus at room temperature in running buffer (3.02 g Tris Base, 18.8 g glycine and 1 g SDS per litre, pH 8.3) at 180 V for 45 min. Proteins were transferred to PVDF membrane at 160 mA for 1.5 h using a wet-transfer method in transfer buffer (25 mM Tris, 192 mM glycine, 10% v:v methanol, pH 8.5). Membranes were blocked with either milk (3%; 20 min) or polyvinylalcohol (PVA; 1 min) and washed three times with Tris-Buffered Saline with 0.05% v:v Tween (TBST), before being incubated with primary antibody overnight at 4°C. Following incubation, the membranes were washed as above in TBST, and incubated with HRP-linked anti-rabbit IgG secondary antibody (BioShop, Burlington, ON) diluted 1:4000 v:v in TBST for 1 h at room temperature, and then rewashed in TBST as above. Immunoreactive bands were visualized with enhanced chemiluminescence using the ChemiGenius Bioimaging System (Syngene, Frederick, MD). Subsequently, membranes were stained using Coomassie blue (0.25% w:v Coomassie brilliant blue, 7.5% v:v acetic acid, 50% v:v methanol). Antibodies recognizing SMAD 2 (Cat. No. 5339), P-SMAD 2, (Ser465/467; Cat. No. 3108), SMAD 4 (Cat. No. 9515), SMAD 5 (Cat. No. 9517), P-SMAD 1/5 (Ser463/465; Cat. No. 9516), SMAD 6 (Cat. No. 9519), and P-STAT 3 (Tyr705; Cat. No. 9131) were purchased from Cell Signalling (Danvers, MA). SMAD 3 (Cat. No. A01224) and P-SMAD 3 (Ser425; Cat. No. A01215) was purchased from GenScript (Piscataway, NJ) whereas cMYC (Cat.

No. SC-764) and P-MYC (Thr 58/Ser 62; Cat. No. SC-8000-R) were from Santa Cruz (Santa Cruz, CA). All primary antibodies were rabbit polyclonal IgG diluted 1:1000.

Electrophoretic mobility shift assay

DNA oligonucleotides were designed based on the consensus DNA binding element of SMAD 3 and produced by Sigma Genosys (Oakville, ON). The biotinylated probe (SMAD 3 5'-Biotin-AAGCTTGTCTCCAAGGCAGACAATGT-3') and the complement probe (SMAD 3 5'-c-ACATTGTCTGCCTTGGAGACAAGCTT -3') were diluted in water to 500 pmol/ μ l and combined 1:1 v:v for a total volume of 20 μ l. The probe mixture was incubated for 10 min at 94°C in a thermocycler, and then allowed to anneal by slowly cooling to room temperature.

Each sample was prepared by combining 10 ng of double stranded probe, 7 μ g of nuclear extract (prepared as described above), 1 μ g of poly d(I-C) (Sigma-Aldrich, Oakville, ON), 2 μ l of EMSA binding buffer (10 mM Tris, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 2.5 μ M DTT, pH 7.8) and diluting to a final volume of 10 μ l with water. The samples were incubated for 30 min at 15°C in a thermocycler, combined with 1 μ l of 6x DNA loading dye (BioShop, Burlington, ON) and loaded into the lanes of a non-denaturing polyacrylamide gel alongside a turtle liver-E2F positive control. The gel had previously been subjected to electrophoresis on a BioRad mini-gel apparatus in TBE buffer (90 mM Tris, 90 mM boric acid, 20 mM EDTA) at 120 V for 10 min at 4°C before loading. Following loading, the gel was further electrophoresed for at 120 V for 50 min in TBE buffer at 4°C.

Proteins were transferred to Pall Biodyne B nylon membrane (that had been pre-soaked in TBE buffer) using a wet-transfer method using TBE buffer as transfer buffer. The membrane was fixed in an oven for 1 h at 80°C, blocked in 1x blocking buffer (Affymetrix, Santa Clara, CA) for 30 min at room temperature, probed with streptavidin-HRP (BioShop, Burlington, ON) diluted 1:1000 in 1x blocking buffer for 30 min at room temperature, and washed with 1x wash buffer (Affymetrix, Santa Clara, CA). Biotin reactive bands were visualized with enhanced chemiluminescence using the ChemiGenius Bioimaging System.

Total RNA isolation and quality assessment

RNA isolation and assessment were conducted as described previously in Chapter 2.

cDNA synthesis and PCR amplification

cDNA synthesis was performed as described previously in Chapter 2.

Primers for the target genes were designed using the Primer Designer Program v3.0 (Scientific and Educational Software) based on *X. laevis* or *X. tropicalis* sequences.

The primer sequences were as follows:

(1) SMAD 2	Forward	5'-AGTCATCATGAACTGAAAGC-3'
	Reverse	5'-GGTTCCGAATAGGTGACAGG-3'
(2) SMAD 4	Forward	5'-ACGGACATCTTCAGCATCAC-3'
	Reverse	5'-TTGTGCGGTTGCGGCTTGTT-3'
(3) SMAD 5	Forward	5'-CTTCCACACCGAGCCAGAAC-3'

	Reverse	5'-ACTGACAGCAGGAGCGATGC-3'
(4) cMYC	Forward	5'-CCAGAGTCAACCGCCACAGA-3'
	Reverse	5'-GACACCGCTTCAGAACTAAC-3'
(5) Hepcidin I	Forward	5'-GATCTGTAGCTCTATTAGG-3'
	Reverse	5'-GCATTTGCCACAGCCCTTGAA-3'
(6) Hepcidin II	Forward	5'-CTGCCTGCTGCTCCTTCTCT-3'
	Reverse	5'-GTCTCTTGGTCCGGAACAGAG-3'
(7) α -tubulin	Forward	5'-AAGGAAGATGCTGCCAATAA-3'
	Reverse	5'-GGTCACATTTACCATCTG-3'

PCR was performed by mixing 5 μ l of a cDNA dilution with 1.25 μ l of primer mixture (0.03 nmol/ μ l), 15 μ l of DEPC treated water, 0.75 μ l of 10x PCR buffer (Invitrogen, Burlington, ON), 1.5 μ l of 50 mM MgCl₂, 0.5 μ l of 10 mM dNTPs, and 1 μ l of Taq Polymerase (Invitrogen, Burlington, ON). The amplification program consisted of 7 min at 95°C; 30–40 cycles of 94°C for 1 min, annealing at 51–62°C for 1 min, and 72°C for 1.5 min, and 72°C for 10 min. Annealing temperatures were 57.5°C for SMAD 2 and 4, 62°C for SMAD 5, 61.7°C for cMYC, 51.5°C for Hepcidin 1 and 2, and 54°C for α -tubulin. To ensure that the amplified products had not reached saturation, initial studies tested serial dilutions of cDNA to identify the dilution (typically 10⁻²) that was non-saturating yet still showed visible bands for quantification purposes. PCR products were separated on 1% agarose gels (or 2% for hepcidin) for 20 min at 130 mA. Gels were prepared by adding agarose to 200 ml of TAE buffer (2 M Tris base, 1.1 ml concentrated acetic acid, 1 mM EDTA, pH 8.5). The agarose was dissolved by heating and then 2 μ l of

ethidium bromide (0.3 mg/300 ml) were added, before the solution was allowed to solidify in a gel tray. A 3 μ l aliquot of xylene blue loading dye were added to each PCR reaction and 12.5 μ l samples were loaded onto the gels; electrophoresis was carried out at 130 V in TAE buffer.

Samples of amplification products were excised from the agarose gels using the freeze/squeeze method as described previously in Chapter 2.

Data quantification and statistics

Chemiluminescent blots and ethidium bromide stained agarose gels were visualized using a Chemi-Genius BioImaging system (Syngene Corp., Frederick, MD) and band densities were quantified using the associated Gene Tools software.

Immunoreactive band intensities were normalized against the summed intensity of a group of Coomassie-stained protein bands in the same lane that did not change between control and experimental states; the summed intensities of these bands were confirmed as unchanging by comparing them to the immunoreactive bands of glyceraldehyde-3-phosphate dehydrogenase, a constitutive housekeeping gene and accepted reference protein. The normalization step allowed for the correction of any minor variations in sample loading. PCR products were normalized against the corresponding band intensity of α -tubulin amplified from the same cDNA sample. Data are expressed as means $\pm$ SEM, n = 3-5 independent samples from different animals. Statistical testing of normalized band intensities used one-way ANOVA and a post-hoc test (Student-Newman-Keuls) or the Student's *t*-test.

RESULTS

The antibodies used in the following study cross-reacted with only a single protein band at the expected molecular masses after electrophoresis of *X. laevis* tissue extracts. These immunoreactive bands were compared to, and corresponded to, the respective immunoreactive bands in 13-lined ground squirrel liver positive controls that were assessed on the same blots. The proteins analyzed are as follows with their identified molecular weight in brackets: SMAD 2 (60 kDa), SMAD 3 (52 kDa), SMAD 4 (70 kDa), SMAD 5 (60 kDa), SMAD 6 (62 kDa), cMYC (70 kDa), and STAT 3 (85 kDa).

Dehydration effects on hepcidin

Using gene-specific primers designed from *X. tropicalis* hepcidin I a 108 base pair (bp) amplicon of hepcidin I encoding 35 amino acids was amplified from *X. laevis* liver (Fig. 3.02A). This gene fragment showed 94.3% similarity to the respective region of the *X. tropicalis* homolog (Fig. 3.02B), and spanned amino acids 47-81 of the *X. tropicalis* sequence, representing ~43% of the hepcidin I protein. Although similar to the hepcidin I sequence from *X. tropicalis*, the *X. laevis* hepcidin I protein sequence did not show considerable identity to hepcidin from other animals (Fig. 3.02B,C).

Analysis of the hepcidin I sequence showed this peptide to be fairly equally hydrophobic and hydrophilic (34% of all residues were hydrophobic, 37% were hydrophilic, and 29% were neither) and neutral (80% of all residues were neutral, 3% were negative, 17% were positive). Cysteine was the most frequently occurring amino acid, composing 23% of the residues. A Kyte-Doolittle plot showed a large hydrophobic

stretch from amino acids 15 to 25 and a hydrophilic site from amino acids 6 to 12. A beta strand secondary structure was identified from amino acids 14-20, overlapping the hydrophobic stretch (surface probability estimated to be 0). A putative phosphorylation site (PKC) was identified at amino acids 8-10, within the hydrophilic stretch, and where the surface probability of the peptide was estimated to be highest. No Pfam domains were identified.

Using gene-specific primers designed from *X. tropicalis* hepcidin II a 112 bp amplicon of hepcidin I encoding 37 amino acids was amplified from *X. laevis* liver (Fig. 3.03A). This gene fragment showed 89.2% similarity to the respective region of the *X. tropicalis* homolog (Fig. 3.03B), and spanned amino acids 22-58 of the *X. tropicalis* sequence, representing ~46% of the hepcidin II protein. Although similar to the hepcidin II sequence from *X. tropicalis*, the *X. laevis* hepcidin II protein sequence did not show considerable identity to hepcidin from other animals (Fig. 3.03B,C).

Analysis of the hepcidin II sequence showed this peptide to be mostly hydrophobic (49% of all residues were hydrophobic, 24% were hydrophilic, and 27% were neither) and neutral (76% of residues were neutral, 14% were negatively charged, 11% were positively charged). Serine was the most frequently occurring amino acid, composing 14% of the amino acids. A Kyte-Doolittle plot showed a hydrophilic stretch from amino acids 19-24. One putative phosphorylation site (PKC) was identified at amino acids 33-35, overlapping a hydrophilic region with the highest surface probability of the fragment. No secondary structure or Pfam domains were identified.

The mRNA expression levels of hepcidin I increased significantly by 3.58-fold during medium dehydration and then decreased to 53% of the medium dehydration value under high dehydration conditions, a level that was 1.89-fold higher than the control (Fig. 3.04). Hepcidin II mRNA transcripts increased significantly only during high dehydration by 2.07-fold as compared with controls (Fig. 3.04).

Dehydration effects on the TGF- β activated SMAD pathway

The expression levels of R-SMAD 2 were measured by RT-PCR and immunoblotting in muscle from control, medium dehydrated, and high dehydrated African clawed frogs. RT-PCR amplified a 362 base pair (bp) product encoding 120 amino acids that matched amino acids 151-270 of the *X. laevis* SMAD 2 (GenBank ID: AAB39329.1) protein when using a BLAST search, representing ~24% of the entire protein (Fig. 3.05A). This fragment showed the closest similarity to SMAD 2 from Western clawed frog (Fig. 3.05B), although considerable identity (98.15% identity) was observed between all SMADs in this region (Fig. 3.06)

SMAD 2 mRNA transcript levels were reduced during dehydration to 73% and 74% of the control levels under medium and high dehydration conditions, respectively (Fig. 3.07A). Correspondingly, total protein levels of SMAD 2 decreased significantly to 64% and 61% of control levels in response to medium and high dehydration (Fig. 3.07B). Transcription factors must move into the nucleus in order to exert their effect on gene expression and, therefore, changes in the distribution of SMADs between nuclear and cytoplasmic fractions is a useful indicator of changes in SMAD transcriptional activity. The relative amounts of SMAD 2 in cytoplasmic and nuclear fractions of *X. laevis* liver

were evaluated under control versus high dehydration situations. Under high dehydration conditions, a significant decrease to 79% in the cytoplasmic content of SMAD 2 was seen, reflecting the decreases in total SMAD protein levels reported above; interestingly, a strong 2.06-fold increase is observed in SMAD 2 nuclear levels (Fig. 3.07C). Phosphorylated (activated) SMAD 2 (Ser 465/467) decreased significantly during high dehydration, falling to 68% of the control values (Fig. 3.07D). This was reflected by a decrease in relative levels of both the cytoplasmic and nuclear P-SMAD 2 content to 65% and 69% of their control values, respectively (Fig. 3.07E).

R-SMAD 3 protein exhibited an expression pattern similar to SMAD 2 in response to dehydration. Total SMAD 3 protein levels decreased significantly to 52% and 71% during medium and high dehydration, respectively (Fig. 3.08A). This was reflected by a decrease in the cytoplasmic SMAD 3 content to 69% during high dehydration. Under high dehydration the nuclear content of SMAD 3 increased by 1.45 – fold (Fig. 3.08B), similar to SMAD 2. Phosphorylated (activated) SMAD 3 (Ser 425) decreased significantly during high dehydration, falling to 73% of its control values respectively (Fig. 3.08C), with a resulting 79% decrease in nuclear levels, but no change in cytoplasmic levels (Fig. 3.08D). P-SMAD 3 (but not P-SMAD 2) binds directly to DNA in the nucleus and hence P-SMAD 3 binding to DNA was assessed using an electrophoretic mobility shift assay (EMSA) – a technique to analyze protein interactions with DNA. P-SMAD 3 binding to DNA was reduced significantly to 84% of control level during high dehydration, mirroring the drop in P-SMAD 3 levels localized to the nucleus during high dehydration (Fig. 3.08E).

Dehydration effects on co-SMAD 4

Co-SMAD 4 expression levels in liver were measured by RT-PCR and immunoblotting from control, medium dehydrated, and high dehydrated African clawed frogs. RT-PCR amplified a 166 bp product encoding 54 amino acids that matched amino acids 298-351 of the *X. laevis* SMAD 4 (GenBank ID: AA16968.1) protein when using a BLAST search, representing ~10% of the entire protein (Fig. 3.09A). SMAD 4 showed close percent similarity to a number of organisms (Fig. 3.09B), with a 98.15% identity observed between all analyzed SMAD 4s in the region corresponding to the amplified fragment (Fig. 3.10). SMAD 4 mRNA transcript levels decreased significantly to 79% and 74% of control values during medium and high dehydration (Fig. 3.11A), respectively, while total protein levels mirrored this trend and decreased by 71% and 67%, respectively (Fig. 3.11B). Nuclear levels did not change, but cytoplasmic levels in liver from high dehydration frogs decreased to 60% of control cytoplasmic levels (Fig. 3.11C).

Dehydration effects on the BMP activated SMAD pathway

R-SMAD 5 expression levels were measured by RT-PCR and immunoblotting from the liver of control, medium dehydrated, and high dehydrated African clawed frogs. RT-PCR amplified a 240 bp product encoding 79 amino acids that matched amino acids 386-464 of the *X. laevis* SMAD 5 (GenBank ID: AA170467) protein when using a BLAST search, representing ~13% of the entire protein (Fig. 3.12A). This region of SMAD 5 showed low percent similarity to a number of organisms (Fig. 3.12B); however, an 88.14% identity was observed between all analyzed SMAD 5s in the region

corresponding to the amplified fragment (Fig. 3.13). SMAD 5 mRNA levels increased by 3.45 and 2.07 fold during medium and high dehydration, respectively (Fig. 3.14A). SMAD 5 total protein levels reflected this pattern, increasing by 1.73 and 1.56 - fold during medium and high dehydration, respectively (Fig. 3.14B). The observed increase in total SMAD 5 levels was reflected by a significant increase by 3.07-fold during high dehydration in cytoplasmic values while nuclear values did not change (Fig. 3.14C). P-SMAD 5 (Ser 463/465) levels showed the reverse pattern to SMAD 5 total protein, decreasing to 58% and 56% of control levels during medium and high dehydration, respectively (Fig. 3.14D). The observed drop in P-SMAD 5 levels during high dehydration were reflected by a significant decrease in relative cytoplasmic levels to 65% of control values (Fig. 3.14E).

I-SMAD 6 expression levels were also measured by immunoblotting in liver from control, medium dehydrated, and high dehydrated African clawed frogs. However, total protein and nuclear versus cytoplasmic distribution showed no changes in response to either levels of dehydration (Fig. 3.15A,B).

Dehydration effects on P-STAT3

P-STAT3 3 (Tyr 705) expression levels were measured by immunoblotting for control, medium dehydrated, and high dehydrated African clawed frog liver. The relative amount of phosphorylated STAT 3 significantly increased by 1.77-fold under high dehydration conditions (Fig. 3.16A) and a significant 1.83-fold increase in nuclear-localized phospho-protein was also seen during high dehydration (Fig. 3.16B).

Dehydration effects on cMYC

cMYC expression levels were measured by RT-PCR and immunoblotting in the liver of control, medium dehydrated, and high dehydrated African clawed frogs. RT-PCR amplified a 369 bp product encoding 123 amino acids that matched amino acids 219-281 of the *X. laevis* cMYC (GenBank ID: AAH70712.1) protein when using a BLAST search, representing ~15% of the entire protein (Fig. 3.17A). This region of cMYC was not very similar to a number of organisms (Fig. 3.17B), with a 55.77% identity observed between all analyzed cMYC proteins in the region corresponding to the amplified fragment (Fig. 3.18). Transcript levels of cMYC increased by 2.37 and 2.70 – fold during medium and high dehydration (Fig. 3.19A), respectively, and total protein levels mirrored this trend and increased by 1.47 and 1.36 – fold, respectively (Fig. 3.19B). This increase was reflected by an increase in cMYC nuclear levels to 1.29 – fold (Fig. 3.19C). P-cMYC levels increased significantly under medium dehydration conditions by 1.38 – fold over control levels, but then decreased significantly during high dehydration to 65% of medium dehydration levels, returning the relative phosphorylation levels to that of the control (Fig. 3.19D). Correspondingly, no changes were seen in the nuclear or cytoplasmic content during high dehydration (Fig. 3.19E).

DISCUSSION

Adaptations for surviving extensive dehydration offer opportunities to examine the mechanisms by which protein regulation confers an ability of animals to survive extreme environmental conditions. *X. laevis* can experience seasonally dry conditions that

result in evaporation of their aquatic habitat, leaving them to face desiccation stress. To survive, the frogs can enter a hypometabolic state of estivation. Entry into hypometabolism has been shown to affect the immune system of frogs including marked lymphocyte depletion, decreased primary and secondary lymphoid organs, and reduced complement levels in *Rana pipiens* during cold-induced hibernation and a decreased dermal expression of antimicrobial peptides in *R. sylvatica* while frozen over the winter (Cooper *et al.*, 1992; Maniero & Carey, 1996; Matutte *et al.*, 2000). Further studies have shown how cold temperatures alone generally diminish the immune system of ectothermic vertebrates including non-hibernating frogs, lizards, and fish (Maniero & Carey, 1996). Environmental change has also been blamed for the mass declines and extinctions of anuran species by placing stress on their immune systems in such a way that they can no longer adequately control pathogens (Raffel *et al.*, 2006). Despite studies analyzing the impact of the cold on immune function, the impact of hypometabolism at warm temperatures, such as that experienced seasonally by *X. laevis* has not previously been investigated.

Hepcidin is an evolutionarily conserved, iron-regulatory and antimicrobial liver-expressed peptide. While only one form of hepcidin exists in humans, multiple forms have been identified in various fish species and two forms (hepcidin I and hepcidin II) have been found in *X. tropicalis* (Hirono *et al.*, 2005; Kim *et al.*, 2005; Huang *et al.*, 2007; Yang *et al.*, 2007; Hu *et al.*, 2008). In the present study, two differentially regulated hepcidin cDNA sequences were amplified from *X. laevis* liver whereas previously, only one form was thought to exist based on the EST tag CB199190 (Hu *et al.*, 2006). *X. laevis* hepcidin I and hepcidin II cDNA sequences showed considerable

identity to the *X. tropicalis* hepcidin I and hepcidin II amino acid sequences. This gene conservation between frog species is consistent with the high degree of structural conservation previously found between all hepcidin forms. In fact, in contrast to other antimicrobial peptides, hepcidin is highly conserved throughout vertebrate evolution (Shi and Camus, 2006; Padhi and Verghese, 2007; Hu *et al.*, 2008).

The N-terminal amino acids are responsible for hepcidin's interaction with ferroportin, potentially because of the presence of a metal binding motif in this area (Clark *et al.*, 2011; Tselepis *et al.*, 2010).; however, only the C' terminal region of hepcidin I and the central region of hepcidin II were amplified from *X. laevis*. Hepcidin is generally known to be cysteine rich and form two beta sheets (for review: Diamond *et al.*, 2009). Consistent with its role as an AMP, hepcidin is amphipathic; the human hepcidin form has a hairpin structure stabilized by 4 disulfide bonds (Nemeth *et al.*, 2005). In line with this structure, the amplified hepcidin I peptide was found also to be cysteine rich and was shown to have a region that theoretically forms a beta strand structure. These characteristics were not observed in the peptide fragment of hepcidin II. Despite the demonstration of two theoretical PKC phosphorylation sites in both hepcidin sequences, previous studies seemingly have not shown any type of post-translation modification.

Hepcidin has been shown to be mitigated by hypoxia in mammals and fish (Shi & Camus, 2006). The urea accumulation occurring in *X. laevis* during estivation can also cause an increase in the haemoglobin-oxygen binding properties of the *Xenopus*' blood, such that there is an inhibited oxygen release (Jokumsen & Weber, 1980). This effect, combined with an increased blood viscosity and reduced blood volume due to dehydration, decreases the blood's ability to fully oxygenate tissues and *X. laevis* can

experience hypoxia as a general consequence of estivation (Malik & Storey, 2009). Therefore, it was interesting to find that transcript levels of both hepcidin I and II were increased during dehydration despite the probable hypoxia experienced by dehydrated frogs. A similar scenario was observed in sea bass liver, where hepcidin transcription levels increased in spite of observed anemia; in this case, a response to infection apparently overrode the need for iron, demonstrating the dual functionality of hepcidin (Rodrigues *et al.*, 2006). Thus, in contrast to the immunodeficiency observed during states of metabolic suppression in the cold, the increased hepcidin levels reflect a heightened immune status to protect aestivating *X. laevis* from infection during dehydration.

To investigate the transcription factor(s) inducing the hepcidin expression, this study explored the possibility of hepcidin control by SMADs and STAT-3 by analyzing the protein and mRNA changes in these signalling pathways in response to dehydration. SMAD signalling through TGF- β and BMP have previously been established to have opposing effects on the transcription of hepcidin. The ability of SMADs to either activate or repress transcription is conferred by the transcriptional partners with which they associate (Chen *et al.*, 2002).

BMPs are activated in response to rising circulating iron levels and signal through BMP receptors and, in mammalian cells (but not in zebrafish), also signal through the BMP co-receptor hemojuvelin (HJV) to positively regulate hepcidin expression (Babitt *et al.*, 2006; Gibert *et al.*, 2011). BMP activated SMADs have also been shown to partially mediate the response by hepcidin to inflammation – although its role is thought to be minor compared to that of STAT 3 (Verga Falzacappa *et al.*, 2008). Although BMP

activated R-SMAD 5 levels were upregulated in this study at both the mRNA and protein levels in *X. laevis* liver, the total amount of activated P-SMAD 5 localized to the nucleus did not change. This was consistent with the fact that the protein levels of I-SMAD 6, whose role it is to inhibit the entry of SMAD 5 into the nucleus, did not change during dehydration. The maintenance of P-SMAD 5 in the nucleus occurred despite an overall suppression in P-SMAD 5 levels during high dehydration. These results indicate that dehydration had no effect on the activity of the BMP activated SMAD signalling pathway, therefore, the BMP activated iron responsive SMAD pathway cannot account for the elevated levels of hepcidin during high dehydration in *X. laevis*.

Unlike BMP signalling, TGF- β signalling has been shown to be a negative regulator of hepcidin. Since TGF- β signalling via SMADs has been shown to down-regulate the IL6-induced phosphorylation of STAT3 in BLAST cells and epithelial cells, this might be the method of hepcidin mitigation (Walia *et al.*, 2003; Wierenga *et al.*, 2002). The present analysis of the TGF- β signalling SMAD pathway showed a reduced protein expression of both SMAD 2 (supported by decreased SMAD 2 mRNA levels) and SMAD 3 in response to medium and high dehydration, combined with a decreased presence of the activated (phosphorylated) forms of these proteins in the nucleus in response to high dehydration. Once inside the nucleus, P-SMAD 3, but not P-SMAD 2, binds directly to the nuclear consensus sequence CAGA (by contrast, SMAD 2 participates in DNA-bound complexes via SMAD 4) (Zawel *et al.*, 1998; Massagué & Wotton, 2000). This study shows that P-SMAD 3 binding to its DNA element decreases during dehydration, which is consistent with a decreased amount of P-SMAD 3 in the nucleus during dehydration. Taken together, these data indicate a decrease in the activity

of the TGF- β mediated SMAD signalling pathway under high dehydration conditions. A release from this antagonizing pathway during high dehydration could account for some of the increases seen in the *X. laevis* hepcidin liver levels.

STAT 3 stimulates hepcidin expression in response to pro-inflammatory cytokines (notably IL6), and is responsible for controlling the anemia of disease (Sakamori *et al.*, 2010). Dehydration has been shown to promote inflammation in a number of mammalian pathologies including cystic fibrosis and keratoconjunctivitis (Boucher, 2007; Niederkorn *et al.*, 2006; Zheng *et al.*, 2010; Barbet *et al.*, 1988). In *X. laevis*, a significant increase in phosphorylated STAT 3 levels and its proportion in the nucleus occurred in response to high dehydration, indicating an overall elevation in the activity of this transcription factor. This indicates that dehydration may promote an inflammation pathway during high dehydration, which could account for some of the increases seen in the *X. laevis* hepcidin liver levels under high dehydration conditions.

Interestingly, both the iron regulated (SMAD) and pro-inflammatory pathways (via IL-6 and STAT3) required SMAD 4 for signalling to hepcidin in mouse cells (Wang *et al.*, 2005). In the SMAD pathways, SMAD 4 combines with phospho-R-SMADs before they can translocate into the nucleus (Nakayama *et al.*, 2001); STAT3 requires SMAD 4 in order to affect the transcription of hepcidin (Wang *et al.*, 2005). SMAD 4 mRNA transcript and protein levels were reduced in response to medium and high dehydration, minimizing their availability for either of the pathways to function. Despite this, the proportion of SMAD 4 in the nucleus remained unchanged demonstrating that its ability to accompany transcription factors to the nucleus is not affected by dehydration.

Taken together, the analysis of the SMAD and STAT pathways for trends that reflect the impact of dehydration on hepcidin revealed that active transcription factors from these pathways were only modified in response to high dehydration (i.e. an increase in P-STAT 3, and a decrease in P-SMADs 2 and 3). While this trend is mirrored in the elevation of hepcidin II mRNA in response to high dehydration, hepcidin I showed increased transcript levels as early as medium dehydration. Therefore, while the increase in the hepcidin activating P-STAT 3 protein and the decrease in the hepcidin antagonizing SMAD 2 and 3 proteins could be suggested to mediate enhanced hepcidin II (and potentially hepcidin I) transcription during high dehydration, another transcription factor would need to be implicated in the augmentation of hepcidin II mRNA under medium dehydration.

In addition to having been identified as a hepcidin co-regulator in HepG2 and BHK cells, cMYC has also been shown to control cellular iron levels by coordinating the expression of iron-regulatory protein 2 and ferritin heavy chain (Bayele *et al.*, 2006; Wu *et al.*, 1999). cMYC is also a key protein controlling cellular proliferation as it induces both cell cycle progression and apoptosis (Amati *et al.*, 1993). Iron levels and cell division are intricately entwined, with iron depletion leading to cell cycle G1/S arrest by changing the expression levels of cyclins and cdks (Le & Richardson, 2002). Animals that enter into hypometabolic states are thought to exploit cell cycle arrest to conserve energy (for reviews: Biggar & Storey, 2009; Storey & Storey, 2007). The TGF- β mediated SMAD signalling pathway has been shown to repress cMYC and, correspondingly, the present results show that the down regulation of TGF- β mediated SMAD proteins during dehydration is accompanied by increased cMYC mRNA and

protein in response to both medium and high dehydration (Bayele *et al.*, 2006; Yagi *et al.*, 2002; Chen *et al.*, 2002). Notably, P-cMYC increased only during medium dehydration, with no change in nuclear levels during high dehydration. The phospho-form of cMYC is stability regulated; phosphorylation at residue Ser-62 alone stabilizes cMYC, while the subsequent, dependent phosphorylation of Thr 58 targets cMYC for ubiquitin-mediated proteasome degradation; both phospho-forms play roles in the transactivation of genes (Gupta *et al.*, 1993; Sears *et al.*, 2000). From the current data, it is not possible to discern whether the stabilizing or destabilizing phosphorylated form is upregulated during medium dehydration. However, it has been previously demonstrated that cMYC phosphorylation is mediated by the Ras effector pathways; while the Raf/Mek/Erk branch of the Ras pathway is cMYC stabilizing via Ser62 phosphorylation, the phosphatidylinositol-3-OH kinase (PI3K)–Akt branch inhibits glycogen synthase kinase-3 β (GSK-3 β), preventing GSK-3 β from phosphorylating cMYC on Thr 58 (Yeh *et al.*, 2004). Cessation of the Ras signal results in a loss of PI3K-Akt activity, allowing GSK-3 β to be released from inhibition and phosphorylate cMYC on the destabilizing Thr 58 residue. Previous work by Malik & Storey (2009) showed that the Erk cascade had a strong conserved activation during dehydration in *X. laevis* tissues; this included significant increases in the phosphorylated (activated) forms of c-Raf, Mek1/2, and Erk1/2 during medium dehydration in liver, with a return to control levels by Mek1/2, and Erk1/2 under high dehydration. This is the same phosphorylation pattern observed for cMYC during dehydration, and the activation of the Ras pathway, as evidenced by the upregulated MAPK cascade, provides compelling evidence for cMYC phosphorylation to be a result of enhanced stabilization of Ser 62 phosphorylation.

Taken together, these trends in cMYC protein expression reflect the pattern induced in hepcidin I in response to dehydration, suggesting that, akin to other animals, cMYC may act upstream of the hepcidin peptide in *X. laevis*.

The differential regulation of hepcidin I and II in response to dehydration suggests the possibility of a functional divergence between the hepcidin forms in *X. laevis*. This idea has been presented before in light of the multiple copies of hepcidin found in fish species as well as *X. tropicalis* (Cho *et al.*, 2009; Wang *et al.*, 2009; Hu *et al.*, 2008; Padhi & Verghese, 2007). In *X. tropicalis*, hepcidin II was suggested to be iron-regulatory whereas hepcidin I was thought to function as an antimicrobial peptide (Hu *et al.*, 2008). The hepcidin form(s) affected by iron loading in *X. laevis* cannot be hypothesized here (since the upstream pathway showed no change during dehydration), but the expression of hepcidin II mRNA corresponded with the protein expression trends of STAT 3, suggesting that hepcidin II might function in innate immunity – although the increased expression levels of hepcidin I during high dehydration could also be a result of STAT 3 influence. Alternatively, this may be another mechanism to establish a diminished iron status to ward off disease. The correlation between cMYC and hepcidin I expression may provide insight into the control of cell-cycle progression in estivation by regulating iron pools through hepcidin I. Therefore, this data further supports differing roles of hepcidin I and II in lower vertebrates and may be a response to the dual environment that the African clawed frog can face seasonally. This was reasoning was hypothesized in teleost fish as well, where teleost hepcidin diversity was posited to be a reflection of the complexity of the environmental challenges these fish cope with during their lives (Xu *et al.*, 2008).

In summary, Hecpudin I and II mRNA transcripts were identified in *X. laevis* and both were elevated in response to dehydration, reflecting a heightened AMP response and a diminished availability of iron throughout the body. We report the suppression of activated proteins in the TGF- β activated SMAD pathway but not the BMP activated pathway in response to dehydration and an upregulation of STAT 3 and cMYC, implicating these transcription factors as playing important roles during dehydration. Previous studies show that cMYC and STAT 3 are involved in hepcidin regulation. The expression patterns of these players in *X. laevis* liver suggest that they may also control hepcidin I and II expression in African clawed frogs. This is the first study to explore these transcription factor pathways and immune/iron regulatory proteins with respect to dehydration.

FIG. 3.02 (legend on next page)**(A)**

```

1      ATGCTCTGGAGCCTCTACTTAGGAGCAAGAGACATTTCCACCTCTCCATCTGCGTCCACT
1      A L E P L L R S K R H S H L S I C V H

61     GCTGCAACTGCTGCAAATTCAAGGGCTGTGGCAAATGCTGTCTGACCT
20     C C N C C K F K G C G K C C L T

```

(B) **Homology matrix of 5 sequences**

```

African clawed frog 100%
Western clawed frog 94.3% 100%
Zebrafish           54.3% 32.1% 100%
Human               40.0% 26.6% 28.6% 100%
Chicken             28.6% 21.0% 13.6% 19.7% 100%

```

(C)

```

African clawed frog ALEPLLRSKRHSHLSICVHCCNCKFKGCGKCCLT 35
Western clawed frog ALEPLLRSKRqSHLSICVHCCNCKyKGCGKCCLT 35
Zebrafish           dplvLfRtKRqSHLSlCrfCCkCCrnKGCGyCCkf 35
Human               mpmfqrRrrRdtHfpICifCCgCChrskCGmCCkT 35
Chicken             sLrPvgaScRdnsecItmlCrknr.....CfLr 28
Consensus           r c c

```

FIG. 3.02. Partial nucleotide and deduced amino acid sequence of *X. laevis* hepcidin I.

(A) Partial cDNA sequence of *hepcidin I* from the African clawed frog, with the corresponding translated amino acid sequence. Nucleotides and amino acids are numbered on the left. The nucleotide sequence was 108 bases and encoded 35 amino acids. The sequence covered amino acids 47-81 of the Western clawed frog sequence (Accession no. AAI56007.1), representing ~43% of the total hepcidin I protein.

(B) Homology tree and homology matrix produced from an alignment of the partial African clawed frog hepcidin I protein sequence with hepcidin from Western clawed frog (*X. tropicalis*; Accession no. AAI56007.1), zebrafish (*Danio rerio*; Accession no. AAI63916.1), chicken (*Gallus gallus*; Accession no. AAS99322.1) and human (*Homo sapiens*; Accession no. ABF47098.1). The percentage values correspond to shared identity between corresponding species.

(C) Comparison of the partial African clawed frog (*X. laevis*) hepcidin I protein sequence with the corresponding region in hepcidin I from Western clawed frog (*X. tropicalis*; Accession no. AAI56007.1), zebrafish (*Danio rerio*; Accession no. AAI63916.1), chicken (*Gallus gallus*; Accession no. AAS99322.1) and human (*Homo sapiens*; Accession no. ABF47098.1).

FIG. 3.03 (legend on next page)**(A)**

```

1      CAGCGCATCCCTTGGTGGGAATGAAATAAAGGCTCCCGAACACCCGATCTCTGAGTCTCA
1      S A S L G G N E I K A P E H P I S E S Q

61     GTGGGGGGAATCTGATGCTCTGGGACCTCTGTTCCGGACCAAGAGACAACCTC
21     W G E S D A L G P L F R T K R Q L

```

(B) **Homology matrix of 5 sequences**

```

African clawed frog 100%
Western clawed frog 89.2% 100%
Human                20.0% 28.2% 100%
Zebrafish            18.9% 20.0% 29.3% 100%
Chicken              15.2% 14.7% 21.1% 11.6% 100%

```

(C)

```

African clawed frog SASLGGNEIKAPEHPISES.....QWGESDALGPLFRT 33
Western clawed frog SASLsGNEIKAPEHPISES.....eqGESDALGPLFRT 33
Human               SvfpqqtgqlAelqPqd.....raGaraswmPmFqr 31
Zebrafish           vpfiqqvqdehhveseelqenqhlteaehrladplvLFRT 40
Chicken             SllLsqvycaslhqPqp.....llrlkrmtPFwr 29
Consensus                               f

African clawed frog KRQL 37
Western clawed frog KRhL 37
Human               rRrr 35
Zebrafish           KRQs 44
Chicken             gvsL 33
Consensus

```

FIG. 3.03. Partial nucleotide and deduced amino acid sequence of *X. laevis* hepcidin II.

(A) Partial cDNA sequence of *hepcidin II* from the African clawed frog, with the corresponding translated amino acid sequence. Nucleotides and amino acids are numbered on the left. The nucleotide sequence was 112 bases and encoded 37 amino acids. The sequence covered amino acids 22-58 of the Western clawed frog sequence (Accession no. ABL75284.1), representing ~46% of the total hepcidin II protein.

(B) Homology tree and homology matrix produced from an alignment of the partial African clawed frog hepcidin II protein sequence with hepcidin from Western clawed frog (*X. tropicalis*; Accession no. ABL75284.1), zebrafish (*Danio rerio*; Accession no. NP_001018873.1), chicken (*Gallus gallus*; Accession no. AAS99322.1) and human (*Homo sapiens*; Accession no. ABF47098.1). The percentage values correspond to shared identity between corresponding species.

(C) Comparison of the partial African clawed frog (*X. laevis*) hepcidin II protein sequence with the corresponding region in hepcidin II from Western clawed frog (*X. tropicalis*; Accession no. ABL75284.1), zebrafish (*Danio rerio*; Accession no. NP_001018873.1), chicken (*Gallus gallus*; Accession no. AAS99322.1) and human (*Homo sapiens*; Accession no. ABF47098.1).

FIG. 3.04. Effects of medium and high dehydration on the mRNA expression of hepcidin I and hepcidin II in *X. laevis* liver. Effects of dehydration on hepcidin mRNA levels as determined by RT-PCR. Representative hepcidin amplicons are shown with corresponding α -tubulin bands below. Histograms show relative changes in normalized hepcidin mRNA. Data are means $\pm$ SEM, $n=3-5$ independent trials. Significantly different from ^acontrol or ^bmedium dehydration values using a one way ANOVA and post hoc SNK test.

FIG. 3.05 (legend on next page)**(A)**

```

1      TATGCTTTTAACCTTAAAAAAGATGAAGTATGTGTCAATCCTTACCATTATCAGAGGGTG
1      Y  A  F  N  L  K  K  D  E  V  C  V  N  P  Y  H  Y  Q  R  V

61     GAGACACCAGTTTTACCACCTGTATTAGTTCCACGGCACACAGAAATCTTGACAGAGCTG
21     E  T  P  V  L  P  P  V  L  V  P  R  H  T  E  I  L  T  E  L

121    CCACCTCTTGATGACTACACGCATTCCATTCCAGAAAACACTAATTTTCCTGCAGGGATT
41     P  P  L  D  D  Y  T  H  S  I  P  E  N  T  N  F  P  A  G  I

181    GAACCTCAGAGCAATTATATTCCAGAAACACCACCTCCTGGATATATTAGTGAAGATGGA
61     E  P  Q  S  N  Y  I  P  E  T  P  P  P  G  Y  I  S  E  D  G

241    GAACTAGCGATCAGCAACTTAACCAAAGCATGGACACAGGGTCACCAGCTGAGCTGTCT
81     E  T  S  D  Q  Q  L  N  Q  S  M  D  T  G  S  P  A  E  L  S

301    CCGAGTACACTTTCTCCAGTCAACCACAATCTCGATTTGCAACCTGTCACCTATTCGGAA
101    P  S  T  L  S  P  V  N  H  N  L  D  L  Q  P  V  T  Y  S  E

361    CC

```

(B) **Homology matrix of 5 sequences**

African clawed frog	100%				
Western clawed frog	100.0%	100%			
Chicken	99.2%	88.1%	100%		
Human	98.3%	93.4%	88.6%	100%	
Zebrafish	93.3%	89.5%	85.6%	80.1%	100%

FIG. 3.05. Partial nucleotide and deduced amino acid sequence of *X. laevis* SMAD 2.

(A) Partial cDNA sequence of *SMAD 2* from the African clawed frog, with the corresponding translated amino acid sequence. Nucleotides and amino acids are numbered on the left. The nucleotide sequence was 362 bases and encoded 120 amino acids. The sequence covered amino acids 151-270 of the African clawed frog sequence (Accession no. AAB39329.1), representing ~24% of the total SMAD 2 protein. Redundant deviations from the Genbank sequence for this gene (Accession no. AAB39329.1) are underlined.

(B) Homology tree and homology matrix produced from an alignment of the partial African clawed frog SMAD 2 protein sequence with SMAD 2 from Western clawed frog (*X. tropicalis*; Accession no. NP_001011168.1), zebrafish (*Danio rerio*; Accession no. AAF06737.1), chicken (*Gallus gallus*; Accession no. NP_989892.1) and human (*Homo sapiens*; Accession no. AAC39657.1). The percentage values correspond to shared identity between corresponding species.

African clawed frog	YAFNLKKDEVCVNPHYHYQRVETPVLPPVLVPRHTEILTEL	40
Western clawed frog	YAFNLKKDEVCVNPHYHYQRVETPVLPPVLVPRHTEILTEL	40
Chicken	YAFNLKKDEVCVNPHYHYQRVETPVLPPVLVPRHTEILTEL	40
Human	YAFNLKKDEVCVNPHYHYQRVETPVLPPVLVPRHTEILTEL	40
Zebrafish	YAFNLKKDEVCVNPHYHYQRVETPVLPPVLVPRHTEILTEL	40
Consensus	yafnlkkdevcvnpyhyqrvetpvlppvlvprhteiltel	
African clawed frog	PPLDDYTHSIPENTNFPAGIEPQSNIYIPETPPPGYISEDG	80
Western clawed frog	PPLDDYTHSIPENTNFPAGIEPQSNIYIPETPPPGYISEDG	80
Chicken	PPLDDYTHSIPENTNFPAGIEPQSNIYIPETPPPGYISEDG	80
Human	PPLDDYTHSIPENTNFPAGIEPQSNIYIPETPPPGYISEDG	80
Zebrafish	PPLDDYTnSIPENTNFPtGIEPpnNYIPETPPPGYISEDG	80
Consensus	pplddyT sipentnfp gieP nyipetpppgyisedg	
African clawed frog	ETSDQQLNQSMGTGSPAELSPSTLSPVNHNLDLQPVTYS	119
Western clawed frog	ETSDQQLNQSMGTGSPAELSPSTLSPVNHNLDLQPVTYS	119
Chicken	ETSDQQLNQSMGTGSPAELSPSTLSPVNHsLDLQPVTYS	119
Human	ETSDQQLNQSMGTGSPAELSPtTLSPVNHsLDLQPVTYS	119
Zebrafish	EaSDQQmNQSMGTGSPAELSPSTLSPVNHgmDLQPVTYS	119
Consensus	e sdqq nqsmgtgspaelsp tLspvnh dlqpvtys	

FIG. 3.06. Comparison of the partial African clawed frog (*X. laevis*) SMAD 2 protein sequence with the corresponding region in SMAD 2 from Western clawed frog *X. tropicalis*; Accession no. NP_001011168.1), zebrafish (*Danio rerio*; Accession no. AAF06737.1), chicken (*Gallus gallus*; Accession no. NP_989892.1) and human (*Homo sapiens*; Accession no. AAC39657.1).

FIG. 3.07. Effects of medium and high dehydration on the mRNA and protein expression of SMAD 2 in *X. laevis* muscle. (A) Effects of dehydration on the mRNA expression of *SMAD 2* as determined by RT-PCR. Representative *SMAD 2* amplicons are shown with corresponding α -tubulin bands below. Effects of dehydration on protein levels as determined by Western blotting after normalization to total protein: (B) total SMAD 2, (C) cytoplasmic/nuclear SMAD 2, (D) P-SMAD 2 (Ser 465/467), and (E) cytoplasmic/nuclear P-SMAD 2 (Ser 465/467) as determined by Western blotting. Representative Western bands show relative SMAD 2 protein levels. Histograms show relative changes in normalized SMAD 2 transcripts or protein. Data are means $\pm$ SEM, $n = 3-5$ independent trials. Significantly different from the ^acontrol or ^bmedium dehydration values using a one way ANOVA and post hoc SNK test or Students *t*-test, $P < 0.05$.

FIG. 3.08. Effects of medium and high dehydration on the mRNA and protein expression of SMAD 3 in *X. laevis* liver. Effects of dehydration on protein content as determined by Western immunoblotting after normalization to total protein: (A) total SMAD 3 levels, (B) cytoplasmic/nuclear SMAD 3 levels, (C) relative P-SMAD 3 (Ser 425) levels, and (D) cytoplasmic/nuclear P-SMAD 3 (Ser 425) levels. Representative Western bands show SMAD 3 protein levels. (E) Effects of dehydration on the relative binding by SMAD 3 in nuclear extracts as determined by an EMSA. Representative EMSA shows relative SMAD 3 binding levels. Histograms show relative changes in normalized SMAD 3 protein. Data are means $\pm$ SEM, $n = 3-5$ independent trials. Significantly different ^acontrol or ^bmedium dehydration values using a one way ANOVA and post hoc SNK test or Students *t*-test, $P < 0.05$.

FIG. 3.09 (legend on next page)**(A)**

```

1      ATTACTGGCCTGTCCATAATGAGCTTGCATTTAGCCACCAATCTCAAATCACCCGGCTC
1      Y  W  P  V  H  N  E  L  A  F  Q  P  P  I  S  N  H  P  A

61     CTGATTATTGGTGTCTCCATTGCCTACTTTGAAATGGATGTTCAAGTGGGAGAGACCTTTA
20     P  D  Y  W  C  S  I  A  Y  F  E  M  D  V  Q  V  G  E  T  F

121    AAGTTCCTCAAGCTGTCCCATCGTCACTGTTGATGGATATGTGGA
40     K  V  P  S  S  C  P  I  V  T  V  D  G  Y  V

```

(B) **Homology matrix of 4 sequences**

```

African clawed frog 100%
Western clawed frog 98.1% 100%
Human                98.1% 90.2% 100%
Zebrafish            96.3% 84.5% 85.2% 100%

```

FIG. 3.09. Partial nucleotide and deduced amino acid sequence of *X. laevis* SMAD 4.

(A) Partial cDNA sequence of *SMAD 4* from the African clawed frog, with the corresponding translated amino acid sequence. Nucleotides and amino acids are numbered on the left. The nucleotide sequence was 166 bases and encoded 54 amino acids. The sequence covered amino acids 298-351 of the African clawed frog sequence (Accession no. AA16968.1), representing ~10% of the total SMAD 4 protein.

(B) Homology tree and homology matrix produced from an alignment of the partial African clawed frog SMAD 4 protein sequence with SMAD4 from Western clawed frog (*X. tropicalis*; Accession no. AAI35846.1), zebrafish (*Danio rerio*; Accession no. ACA58502.1), and human (*Homo sapiens*; Accession no. BAB40977.1). The percentage values correspond to shared identity between corresponding species. No sequence was annotated for Chicken (*Gallus gallus*) SMAD 4.

African clawed frog	YWPVHNELAFQPPISNHPAPDYWCSIAYFEMDVQVGETFK	40
Western clawed frog	YWPVHNELAFQPPISNHPAPeYWCSIAYFEMDVQVGETFK	40
Human	YWPVHNELAFQPPISNHPAPeYWCSIAYFEMDVQVGETFK	40
Zebrafish	YWPVHNEiAFQPPISNHPAPeYWCSIAYFEMDVQVGETFK	40
Consensus	ywpvhne afqppisnhpap ywcsiayfemdvqvgetfk	
African clawed frog	VPSSCPIVTVDGYV	54
Western clawed frog	VPSSCPIVTVDGYV	54
Human	VPSSCPIVTVDGYV	54
Zebrafish	VPSSCPIVTVDGYV	54
Consensus	vpsscpivtvdgyv	

FIG. 3.10. Comparison of the partial African clawed frog (*X. laevis*) SMAD 4 protein sequence with the corresponding region in SMAD 4 from Western clawed frog (*X. tropicalis*; Accession no. AAI35846.1), zebrafish (*Danio rerio*; Accession no. ACA58502.1), and human (*Homo sapiens*; Accession no. BAB40977.1). No sequence was annotated for Chicken (*Gallus gallus*) SMAD 4.

FIG. 3.11. Effects of medium and high dehydration on the mRNA and protein expression of SMAD 4 in *X. laevis* liver. (A) Effects of dehydration on the mRNA expression of *SMAD 4* as determined by RT-PCR. Representative *SMAD 4* amplicons are shown with corresponding α -tubulin bands below. Effects of dehydration on protein levels as determined by Western blotting after normalization to total protein: (B) SMAD 4 levels and (C) cytoplasmic/nuclear SMAD 4 levels. Representative Western bands show relative SMAD 4 protein levels. Histograms show relative changes in normalized SMAD 4 transcripts or protein. Data are means $\pm$ SEM, $n=3-5$ independent trials. Significantly different from ^acontrol or ^bmedium dehydration values using a one way ANOVA and post hoc SNK test or Students *t*- test, $P < 0.05$.

FIG. 3.12 (legend on next page)**(A)**

```

1      CATCTAACTGTCCCGTGGTCACAGTGGATGGATATGTGGACCCCTCTGGTGGAGATCGGT
1      S  N  C  P  V  V  T  V  D  G  Y  V  D  P  S  G  G  D  R

61     TTTGCCTTGGTCAGCTTTCTAACGTGCATCGCACAGACACTAGTGAGCGTGCAAGGCTTC
20     F  C  L  G  Q  L  S  N  V  H  R  T  D  T  S  E  R  A  R  L

121    ACATCGGGAAGGGAGTGCAGCTTGAGTGTCTGGGGCGAGGGAGACGTATGGATGAGGTGCC
40     H  I  G  K  G  V  Q  L  E  C  R  G  E  G  D  V  W  M  R  C

181    TCAGTGATCACGCCGTGTTTGTCCAGAGTTATTACTTGGACAGGGAAGCAGGACGAGCGC
60     L  S  D  H  A  V  F  V  Q  S  Y  Y  L  D  R  E  A  G  R  A

```

(B) **Homology matrix of 5 sequences**

African clawed frog	100%				
Western clawed frog	47.4%	100%			
Chicken	50.0%	84.0%	100%		
Human	50.0%	82.9%	96.8%	100%	
Zebrafish	50.0%	82.2%	93.1%	91.8%	100%

FIG. 3.12. Partial nucleotide and deduced amino acid sequence of *X. laevis* SMAD 5.

(A) Partial cDNA sequence of *SMAD 5* from the African clawed frog, with the corresponding translated amino acid sequence. Nucleotides and amino acids are numbered on the left. The nucleotide sequence was 240 bases and encoded 79 amino acids. The sequence covered amino acids 386-464 of the African clawed frog sequence (Accession no. AA170467), representing ~13% of the total SMAD5 protein. Redundant deviations from the Genbank sequence for this gene (Accession no. AA170467) are underlined.

(B) Homology tree and homology matrix produced from an alignment of the partial African clawed frog SMAD 5 protein sequence with SMAD 5 from Western clawed frog (*X. tropicalis*; Accession no. AAH75389.1), zebrafish (*Danio rerio*; Accession no. AAI65695.1), chicken (*Gallus gallus*; Accession no. AAX56945.1) and human (*Homo sapiens*; Accession no. AAB66353.1). The percentage values correspond to shared identity between corresponding species.

African clawed frog	VTVDGYVDPSSGGD.RFCLGQLSNVHRTDTSERARLHIGKG	39
Western clawed frog	VlIDGftDPSnnknRFCLGLLSNVnRnsTiEntRrHIGKG	40
Chicken	VlVDGftDPSnnknRFCLGLLSNVnRnsTiEntRrHIGKG	40
Human	VlVDGftDPSnnksRFCLGLLSNVnRnsTiEntRrHIGKG	40
Zebrafish	VlVDGftDPSnnknRFCLGLLSNVnRnsTiEntRrHIGKG	40
Consensus	v dg dps rfclg lsnv r t e r higkg	
African clawed frog	VQLECRGEGDVWMRCLSDHAVFVQSYLDREAG	72
Western clawed frog	VhLyyvG.GeVyaeCvSDssiFVQSrncnyqhG	72
Chicken	VhLyyvG.GeVyaeCLSDssiFVQSrncnyhhG	72
Human	VhLyyvG.GeVyaeCLSDssiFVQSrncnfhhG	72
Zebrafish	VhLyyvG.GeVyaeCLSDtsiFVQSrncnyhhG	72
Consensus	v l g g v c sd fvqs g	

FIG. 3.13. Comparison of the partial African clawed frog (*X. laevis*) SMAD 5 protein sequence with the corresponding region in SMAD 5 from Western clawed frog (*X. tropicalis*; Accession no. AAH75389.1), zebrafish (*Danio rerio*; Accession no. AAI65695.1), chicken (*Gallus gallus*; Accession no. AAX56945.1) and human (*Homo sapiens*; Accession no. AAB66353.1).

FIG. 3.14. Effects of medium and high dehydration on the mRNA and protein expression of SMAD 5 in *X. laevis* liver. (A) Effects of dehydration on *SMAD 5* transcript levels as determined by RT-PCR. Representative *SMAD 5* amplicons are shown with corresponding α -tubulin bands below. Effects of dehydration on relative protein levels as determined by Western blotting after normalization to total protein: (B) SMAD 5, (C) cytoplasmic/nuclear SMAD 5, (D) P-SMAD 5 (Ser463/465), and (E) cytoplasmic/nuclear SMAD 5 (Ser463/465). Representative Western bands are shown as well as histograms showing relative changes in normalized SMAD 5 transcripts or protein. Data are means $\pm$ SEM, $n=3-5$ independent trials. Significantly different from ^acontrol or ^bmedium dehydration values using a one way ANOVA and post hoc SNK test or Students *t*-test, $P < 0.05$.

FIG. 3.15. Effects of medium and high dehydration on the protein expression of SMAD 6 in *X. laevis* liver. Effects of dehydration on protein (A) SMAD 6 levels and (B) cytoplasmic/nuclear SMAD 6 levels, as determined by Western blotting. Representative Western bands show SMAD 6 protein levels. Histograms show relative changes in SMAD 6 protein normalized to total protein. Data are means $\pm$ SEM, $n = 3-5$ independent trials. Significantly different from ^acontrol or ^bmedium dehydration values using a one way ANOVA and post hoc SNK test or Students *t*- test, $P < 0.05$.

FIG. 3.16. Effects of medium and high dehydration on the protein expression of STAT 3 in *X. laevis* liver. Effects of dehydration on protein (A) STAT 3 levels and (B) cytoplasmic/nuclear STAT 3 levels, as determined by Western blotting. Representative Western bands show STAT 3 protein levels. Histograms show relative changes in STAT 3 protein normalized to total protein. Data are means $\pm$ SEM, $n = 3-4$ independent trials. Significantly different from ^acontrol or ^bmedium dehydration values using a one way ANOVA and post hoc SNK test or Students *t*- test, $P < 0.05$.

FIG. 3.17 (legend on next page)**(A)**

```

1      CCTCGTGTGATGGGAGCTTAAATCTCGGAGGGACCAACAGGAGCAGCCATGAATTCCTCC
1      S C D G S L N L G G T N R S S H E F L

61     AGGACCCCAGCTCGGATTGTGTTGACCCTTCAGTGGTCTTCCCATACCCACTGAACGACA
20     Q D P S S D C V D P S V V F P Y P L N D

121    GCATTTCCAACGCCAGCTCACCTTGCCAAGATCTCATGTTGGAAACACCACCCATCAGCA
40     S I S N A S S P C Q D L M L E T P P I S

181    GCAACAGCAGCAGTAGTGAATCAGAAGACGAACAGGAAGACGATGACGACGATGAAGACT
60     S N S S S S E S E D E Q E D D D D D E D

241    GCGACGAGGAAGAGGAGATTGACGTTGTACGGTAGAAAAAAGGCAGACGGCGTCCAGGC
80     C D E E E E I D V V T V E K R Q T A S R

301    GGATGGAATCCGGTTCTCATTCGCAGTCCTCCCGACCCACCACAGCCCGTTAGTTCTGA
100    R M E S G S H S Q S S R P H H S P L V L

361    AGCGGTGTC
120    K R C

```

(B) **Homology matrix of 4 sequences**

African clawed frog	100%			
Zebrafish	67.3%	100%		
Chicken	62.0%	71.6%	100%	
Human	65.4%	69.0%	76.3%	100%

FIG. 3.17. Partial nucleotide and deduced amino acid sequence of *X. laevis* cMYC.

(A) Partial cDNA sequence of *cMYC* from the African clawed frog, with the corresponding translated amino acid sequence. Nucleotides and amino acids are numbered on the left. The nucleotide sequence was 369 bases and encoded 123 amino acids. The sequence covered amino acids 219-281 of the African clawed frog sequence (Accession no. AAH70712.1), representing ~15% of the total cMYC protein.

(B) Homology tree and homology matrix produced from an alignment of the partial African clawed frog cMYC protein sequence with cMYC from zebrafish (*Danio rerio*; Accession no. NP_571487.2), chicken (*Gallus gallus*; Accession no. NP_001026123.1), and human (*Homo sapiens*; Accession no. NP_002458.2). The percentage values correspond to shared identity between corresponding species. No sequence was annotated for Western clawed frog (*X. tropicalis*) cMYC.

Zebrafish	PNSSSSSGSDSEDEEEEEDEEEEEEEEEEEEEEEEEIDVV	40
Chicken	-.tt--...---.-q-ed-.....-----	20
Human	-.tt--...---.-q-----.....-----	20
African clawed frog	s-----..e--.d-q--dddd-dcd-.....-----	30
Zebrafish	TV.EKRQ.K.R.HE...TDASE..SRYP..SPLVLKRC	67
Chicken	-la-anesess.t-s.s-e---ehck.-hh-----	55
Human	s-.----apgkrs-sgsp-s-gg.h-k.-ph-----	55
African clawed frog	--.----tas-rm-sgshsq-s....r-hh-----	63

FIG. 3.18. Comparison of the partial African clawed frog (*X. laevis*) cMYC protein sequence with the corresponding region in cMYC from zebrafish (*Danio rerio*; Accession no. NP_571487.2), chicken (*Gallus gallus*; Accession no. NP_001026123.1), and human (*Homo sapiens*; Accession no. NP_002458.2). No sequence was annotated for Western clawed frog (*X. tropicalis*) cMYC.

FIG. 3.19. Effects of medium and high dehydration on the mRNA and protein expression of cMYC in *X. laevis* liver. (A) Effects of dehydration on the expression of *cMYC* transcripts as determined by RT-PCR. Representative *cMYC* amplicons are shown with corresponding α -tubulin bands below. Effects of dehydration on protein levels as determined by Western blotting after normalization to total protein: (B) total cMYC, (C) cytoplasmic/nuclear cMYC, (D) P-cMYC (Thr 58/Ser 62), and (E) cytoplasmic/nuclear P-cMYC (Thr 58/Ser 62). Representative Western bands are shown together with histograms showing relative changes in normalized cMYC transcripts or protein. Data are means $\pm$ SEM, $n = 3-5$ independent trials. Significantly different from ^acontrol or ^bmedium dehydration values using a one way ANOVA and post hoc SNK test or Student's *t*-test, $P < 0.05$.

CHAPTER 4

Expression of SMAD transcription factors in the skeletal muscle of the African clawed frog, *Xenopus laevis*

INTRODUCTION

This chapter transitions the thesis from looking at the immune system and analyzing the SMAD pathway with respect to its involvement in AMP (hepcidin) regulation in liver to the possible involvement of SMADs in the skeletal muscle response during dehydration. As the mechanisms behind SMAD signaling have previously been described in detail in Chapters 1 and 3, this introduction only provides an overview of this pathway's relevance in skeletal muscle.

Skeletal muscle is a highly plastic tissue that has a remarkable morphogenic ability to respond to environmental stimuli. Muscle mass is maintained or augmented by usage. By contrast, the plastic response to periods of inactivity, disuse, or unmet energy demands typically leads to degenerative changes that culminate in the atrophy of muscle fibres and decreased mechanical performance (for reviews: Fitts *et al.*, 2000; Powers *et al.*, 2007).

Few studies have investigated the effects of muscle disuse or hypocaloric atrophy in animals that experience natural bouts of dormancy (Mantle *et al.*, 2009). Those that have found that atrophy in these animals occurs to a much lesser extent than would occur in humans or many other vertebrates under similar conditions (for reviews: Carey *et al.*, 2003; Shavlakadze & Grounds, 2006). The protection against muscle atrophy during estivation is thought to be an adaptive mechanism to maintain locomotor performance during the small temporal windows that estivator species have for fitness dependent behaviours such as feeding and breeding (for review: James *et al.*, 2010). The molecular mechanisms behind this atrophy protective phenomenon remain to be elucidated. The African clawed frog, *Xenopus laevis* provides a fascinating model to investigate the

molecular responses of growth regulators in muscles to estivation as they can maintain this period of dormancy for up to 8 months without food or water (Tinsley & Kobel, 1996).

The processes that control the synthesis and degradation of muscle are tightly regulated by a variety of growth factors including transforming growth factor- β (TGF- β) and insulin signalling (for review: Jackman & Kandarian, 2004; Glass *et al.*, 2010). Together these molecular controls affect muscle stem cell proliferation and myotube differentiation, primarily through the SMAD signal transduction pathway (Zhu *et al.*, 2000). This interplay imparts the conventional plasticity of skeletal muscle, with atrophy being characterized by decreases in protein synthesis and increases in protein degradation.

Myostatin (growth-differentiation factor 8, GDF8) is a member of the TGF- β superfamily that is expressed and secreted predominantly by skeletal muscle and has the conserved function of negatively regulating skeletal muscle size (for review: Tobin & Celeste, 2005). Myostatin mutations result in gross and widespread hypertrophy and increases in skeletal muscle mass/hyperplasia (McPherron & Lee, 1997; Mosher *et al.*, 2007). Conversely, myostatin overexpression results in muscle atrophy (Reisz-Porszasz *et al.*, 2003). Myostatin mRNA and protein are naturally elevated during muscle atrophy (Reardon *et al.*, 2001; Shao *et al.*, 2007; Wójcik *et al.*, 2008).

Myostatin functions by binding activin receptors and triggering phosphorylation of receptor-regulated SMADs (R-SMADs) 2 and 3 (Zhu *et al.*, 2004; Fig. 4.01). Interestingly, one of the downstream targets of SMAD 2/3 is myostatin, suggesting that

myostatin autoregulates its own expression through activation of SMADs (Allen & Unterman, 2007).

Insulin signaling is another major pathway that controls muscle growth (Sartori *et al.*, 2009; O'Neill *et al.*, 2010; Fig. 4.01). Binding of insulin-like growth factor 1 (IGF-1) triggers the activation of phosphatidylinositol-3-kinase (P13K). P13K in turn facilitates the translocation of Akt (protein kinase B) to the membrane where it is phosphorylated (activated) (Stitt *et al.*, 2004). Once activated, Akt drives many anabolic processes in muscle. For example, Akt targets the phosphorylation and inactivation of the atrophy inducing forkhead box O (FoxO) transcription factors (Kandarian & Jackman, 2006) and the inhibitors of the growth enhancing mammalian target of rapamycin (mTOR) pathway (Inoki *et al.*, 2002; McMullen & Hallenbeck, 2010). Akt is also the mediator between energy metabolism and insulin sensitivity; Akt stimulates glucose transport by insulin by stimulating the translocation of glucose transporter 4 (GLUT4) to the plasma membrane, where it facilitates the uptake of plasma glucose (Mîinea *et al.*, 2005; Anhê *et al.*, 2010). Therefore, the absence of insulin signaling also contributes to reduced muscle mass and functions. R-SMAD 5 (but not SMAD 1 or 8) is specifically linked to the improvement of insulin uptake in skeletal muscles by enhancing Akt expression and phosphorylation (Anhê *et al.*, 2010).

In the present chapter, the molecular responses of the SMAD pathway are characterized in response to dehydration in *X. laevis* and interpreted to provide insights into the regulation of muscle mass and glucose transport during dehydration and estivation.

FIG. 4.01. Molecular events outlining the impact of the SMAD signalling pathway on skeletal muscle cells. BMP 9 causes the heteroligmerization of type I and type II SMAD receptors which phosphorylate R-SMAD 5 causing it to bind with SMAD 4, translocate into the nucleus, and activate Akt. Once phosphorylated through the insulin signalling pathway, Akt stimulated the translocation of GLUT4 to the plasma membrane. SMAD 6 antagonizes BMP signaling by forming a stable complex with the activated type I receptor. TGF- β signalling causes the heteroligmerization of type I and type II SMAD receptors which phosphorylate R-SMADs 2 and 3 causing them to bind with SMAD 4, translocate into the nucleus, and activate myostatin but inhibit cMYC.

MATERIALS AND METHODS

Animals

X. laevis were treated, sacrificed, and tissues collected as described previously in Chapter 3.

Protein extracts and Western blotting

Total protein and subcellular nuclear and cytoplasmic extracts were isolated, normalized, and samples prepared as described previously in Chapter 3. Protein samples for SMAD and cMYC protein analysis were separated using SDS-PAGE, transferred onto PVDF membrane, and immunoreactive bands visualized as described previously in Chapter 3. Myostatin and GLUT4 were separated on 12% SDS-PAGE gels for 45 min at 180 V, and then transferred and visualized as described previously in Chapter 3.

Antibodies recognizing SMAD and cMYC proteins and their dilutions are described previously in Chapter 3. Rabbit polyclonal IgG antibodies recognizing GLUT4 (1:4000; Cat. No. sc-7938) and myostatin (1:2000; Cat. No. AB3239) were purchased from Santa Cruz (Santa Cruz, CA) and Chemicon Millipore (Billerica, MA), respectively, and were used after blocking with milk (2.5%; 30 min).

Electrophoretic mobility shift assay

DNA oligonucleotide probes, sample preparation, and separation are described previously in Chapter 3.

Total RNA isolation, cDNA synthesis and PCR amplification

RNA isolation and assessment were conducted as described previously in Chapter 2. cDNA synthesis, primer design, and gene sequencing were also completed as

described in Chapter 3. Primers for SMAD and cMYC genes were those used in Chapter 3 whereas primer sequences for myostatin were:

Myostatin	Forward	5'-GGAGCACGGTCCACTGGAAT-3'
	Reverse	5'-CACTGTCAATGGATAACGGC -3'

RT-PCR was performed as previously described using an annealing temperature of 61.7 °C and 35 amplification cycles. To ensure that the amplified products had not reached saturation, initial studies tested serial dilutions of cDNA to identify the dilution (typically 10^{-2}) that was non-saturating yet still showed visible bands for quantification purposes. PCR products were separated on 1% agarose gels for 20 min at 130 mA.

Data quantification and statistics

Data quantification and statistics were conducted as described in Chapter 3.

RESULTS

The antibodies used were each shown to cross-react with only a single protein band at the expected molecular mass after electrophoresis of *X. laevis* tissue extracts. These immunoreactive bands were compared to, and corresponded to, the respective bands in 13-lined ground squirrel liver positive controls that were analyzed on the same blots. The proteins analyzed were as follows with their identified molecular weight in brackets: SMAD 2 (60 kDa), SMAD 3 (52 kDa), SMAD 4 (70 kDa), SMAD 5 (60 kDa), SMAD 6 (62 kDa), cMYC (70 kDa), Myostatin (13 kDa), and GLUT4 (46 kDa).

Dehydration effects on the TGF- β activated SMAD pathway

The expression levels of R-SMAD 2 were measured by RT-PCR and immunoblotting in muscle from control, medium dehydrated, and high dehydrated African clawed frogs. RT-PCR amplified a 362 base pair (bp) product encoding 120 amino acids that matched the *X. laevis* SMAD 2 (GenBank ID: AAB39329.1) protein when using a BLAST search (previously described in Chapter 3). *SMAD 2* mRNA and protein levels showed no significant changes over the course of dehydration (Fig. 4.02A,B). Because transcription factors must translocate into the nucleus to potentiate their effect on gene expression, changes in the distribution of SMADs between nuclear and cytoplasmic fractions are a useful indicator of changes in SMAD transcriptional activity. Accordingly, the cytoplasmic versus nuclear distribution of SMAD 2 was analyzed but no significant changes occurred between control and high dehydration states in either nuclear or cytoplasmic levels (Fig. 4.02C). However, the relative amount of phosphorylated (activated) SMAD 2 (Ser 465/467) increased significantly by 1.95-fold during high dehydration, indicating a phosphorylation activation event (Fig. 4.02D). This was reflected by a 1.56-fold increase in the nuclear P-SMAD 2 content in high dehydration (Fig. 4.02E).

R-SMAD 3 protein levels were significantly increased by 1.58-fold and 1.69-fold during medium and high dehydration, respectively (Fig. 4.03A). This was accompanied by a 1.78-fold significant increase in the nuclear-located content of SMAD 3 (Fig. 4.03B). Phosphorylated SMAD 3 (Ser 425) content increased under high dehydration by 1.35-fold (Fig. 4.03C), with a resulting 2.00-fold increase in P-SMAD 3 in the nucleus during that time (Fig. 4.03D). P-SMAD 3 (unlike P-SMAD 2) binds directly to nuclear

DNA and this binding ability was assessed using an electrophoretic mobility shift assay (EMSA). A 1.58-fold increase in P-SMAD 3 bound to DNA occurred under high dehydration conditions and reflected the elevated protein levels in the nuclear fractions reported above (Fig. 4.03E).

Dehydration effects on myostatin

Using gene specific primers designed from *X. tropicalis* a 248 base pair (bp) amplicon of *myostatin* encoding 82 amino acids was amplified from *X. laevis* muscle (Fig. 4.04A). This gene fragment showed 98.8% similarity to the respective region of the *X. tropicalis* homolog (Fig. 4.04B), and spanned amino acids 201-282 of the *X. tropicalis* sequence, representing ~21% of the myostatin protein. This fragment was found to have a conserved TGF- β propeptide domain (amino acids 1-54), consistent with the role of this protein in signalling through the SMAD pathway. The *X. laevis* myostatin protein sequence also showed considerable identity to myostatin from other animals (Fig. 4.05). In particular, there was only one amino acids difference between the *X. laevis* and *X. tropicalis* sequences over the 81 amino acid segment. The mRNA expression levels of myostatin decreased significantly to 38 and 39% of control values during medium and high dehydration, respectively (Fig. 4.06A). This corresponded to a significant decrease in myostatin protein to 79% of control under high dehydration conditions (Fig. 4.06B).

Dehydration effects on cMYC

cMYC expression levels were measured by RT-PCR and immunoblotting in the muscle of control, medium dehydrated, and high dehydrated African clawed frogs. The *cMYC* product was 369 nucleotides, which translated to a 123 amino acid fragment that

matched the *X. laevis* cMYC protein (GenBank ID: AAH70712.1) when using a BLAST search (described in Chapter 3). Transcript levels of *cMYC* increased significantly under medium dehydration by 4.15-fold and by 3.52-fold in high dehydration (Fig. 4.07A). This enhanced expression was translated to the protein level. cMYC increased significantly by 1.75-fold in medium dehydration, and remained elevated during high dehydration (Fig. 4.07B). This increase in protein correlated to an augmentation of nuclear cMYC levels to 1.84-fold over controls during high dehydration; cytoplasmic levels of cMYC did not change (Fig. 4.07C). Relative levels of phosphorylated cMYC (Thr 58/Ser 62) stayed constant under medium and high dehydration, with no change in the nuclear or cytoplasmic distribution of P-cMYC during high dehydration (Fig. 4.07D,E).

Dehydration effects on the BMP activated SMAD pathway

R-SMAD 5 expression levels were measured by RT-PCR and immunoblotting in muscle of control, medium dehydrated, and high dehydrated African clawed frogs. RT-PCR amplified a 240 nucleotide product encoding 79 amino acids that matched the SMAD 5 protein (GenBank ID: AAI70467) in a BLAST search (described in Chapter 3). *SMAD 5* mRNA increased significantly by 1.39-fold during medium dehydration, but decreased significantly to 42% of control values under high dehydration conditions (Fig. 4.08A). SMAD 5 protein levels did not reflect the changes in transcript levels, suggesting translational regulation; SMAD 5 protein levels fell significantly during medium dehydration to 67% of controls, but rose again during high dehydration, returning to control values (Fig. 4.08B). As expected, no significant changes were observed in the nuclear or cytoplasmic distribution of SMAD 5 during high dehydration

(Fig. 4.08C). Analysis of P-SMAD 5 (Ser463/465) content showed a decrease to 83% of control values during medium dehydration, followed by a return to control levels during high dehydration (Fig. 4.08D). Nuclear and cytoplasmic contents of P-SMAD 5 remained unchanged between control and high dehydration (Fig. 4.08E).

The expression levels of I-SMAD 6 opposed those of P-SMAD 5, with protein levels increasing during medium dehydration by 1.75-fold before returning to control levels during high dehydration (Fig. 4.09A). No changes in the nuclear or cytoplasmic levels of SMAD 6 occurred between control and high dehydration (Fig. 4.09B).

Dehydration effects on GLUT4

GLUT4 protein levels increased significantly by 1.23-fold between medium and high dehydration in muscle of African clawed frogs as determined by immunoblotting (Fig. 4.10).

Dehydration effects on co-SMAD 4

Co-SMAD 4 is required for the accumulation of R-SMADs in the nucleus. SMAD 4 expression levels in muscle were analyzed by RT-PCR and immunoblotting from control, medium dehydrated, and high dehydrated African clawed frogs. RT-PCR amplified a 166 nucleotide product encoding 54 amino acids that matched the *X. laevis* SMAD 4 protein (GenBank ID: AA16968.1) using a BLAST search (described in Chapter 3). SMAD 4 mRNA transcript levels were elevated significantly by 3.14-fold and 3.08-fold in medium and high dehydration, respectively (Fig. 4.11A). This corresponded to a significant increase in SMAD 4 total protein content by 1.80-fold during high dehydration (Fig. 4.11B). This result was reflected by an increase in nuclear

SMAD 4 protein by 1.55-fold during high dehydration without significant changes to cytoplasmic SMAD 4 levels (Fig. 4.11C).

DISCUSSION

The muscles of animals that endure natural bouts of dormancy, such as estivating frogs, have been shown to be significantly more resistant to muscle wasting as compared to humans or other animals that do not naturally experience long periods of inactivity (for review: Hudson & Franklin, 2002b). However, the molecular underpinnings of this phenomenon have not been fully elucidated. Here, the molecular responses of muscles to estivation in *X. laevis* were analyzed in response to dehydration with a focus on the SMAD pathway. The R-SMADs play an important role in governing the downstream impacts of myostatin and Akt, the negative and positive regulators of skeletal muscle growth, respectively (Lee & McPherron, 2001; Anhê *et al.*, 2010).

Studies have shown an upregulation in myostatin expression in rodents in situations of muscle disuse such as during exposure to microgravity or hind limb suspension (Lalani *et al.*, 2000; Wehling *et al.*, 2000); as well, myostatin has been linked to FoxO transcription factors in the mediation of atrophy-related protein degradation, highlighting the role of myostatin in atrophic pathways (McFarlane *et al.*, 2006; Gilson *et al.*, 2007). In contrast to these experiments, myostatin mRNA levels decreased significantly in response to both medium and high dehydration in *X. laevis* skeletal muscle. Furthermore, myostatin protein showed a time-delayed translational response to these changes in mRNA, with protein levels falling only during high dehydration. It has

been previously shown that the inhibition of myostatin signaling results in the hypertrophy of mature skeletal muscle fibres. This has also been corroborated in *X. laevis*, where the *ex vivo* treatment of skeletal muscles fibres with a myostatin inhibitor promoted muscle hypertrophy (Watt *et al.*, 2010). Along with the lowered levels of myostatin, a coincident significant increase in cMYC mRNA and protein was observed in *X. laevis* muscle under both medium and high dehydration. cMYC is a potent promoter of cell cycle progression and growth, and an increase in cMYC levels (as seen here) is a known early event in the response of skeletal muscle to hypertrophic stimuli (Whitelaw & Hesketh, 1992). These data suggest that, in response to dehydration, signals promoting muscle hypertrophy occur despite the energy limitations imparted by dormancy. These molecular adaptations may be used as mechanisms to prevent muscle atrophy in *X. laevis* under these stress conditions.

Myostatin potentiates its effects by downstream signaling through R-SMADs 2 and 3. Interestingly, the levels of phosphorylated (activated) SMADs 2 and 3 and nuclear co-SMAD 4 were significantly increased under high dehydration conditions, suggesting enhanced SMAD signaling despite lowered levels of myostatin. One of the downstream targets of myostatin/SMAD signaling is the *myostatin* gene itself; therefore, myostatin is thought to autoregulate in a positive feedback manner. Another target of TGF- β signaling is the *cMYC* gene; SMAD 3 exerts its anti-proliferative effects in part by inhibiting the synthesis of cMYC (Yagi *et al.*, 2002; Kowalik, 2002). As previously mentioned, myostatin mRNA levels were diminished in medium and high dehydration, whereas cMYC mRNA was enhanced. These results seemingly imply an uncoupling

between myostatin and cMYC transcription and the SMAD pathway in *X. laevis* skeletal muscle during dehydration.

Concomitant with the downregulation of myostatin and the upregulation of cMYC during medium and high dehydration, an upregulation of SMAD 3 total protein was observed. This was accompanied by increased nuclear levels of SMAD 3 protein which is likely a reflection of the fact that unstimulated SMADs 2/3/4 monomers shuttle between the cytoplasm and nucleus (Nicolás *et al.*, 2004). Indeed, SMAD 3 (but not SMAD 2) is translocated into the nucleus just as readily as P-SMAD 3, and shows a similar pattern of localization once inside the nucleus as P-SMAD 3 or P-SMAD 3/4 complexes (Chen *et al.*, 2005). However, unphosphorylated SMAD 2/3 does not preferentially travel into the nucleus because of their association with cytoplasmic retention partners (for review: Reguly & Wrana, 2003). SMAD 3 alone (but not SMADs 1/2/4) is able to bind directly and stimulate the promoter region of I-SMAD 7, and synergy with SMAD 4 greatly increases binding stability and stimulation (Nagarajan *et al.*, 1999; Denissova *et al.*, 2000). This is consistent with the fact that overexpression of SMAD 3 has been shown to induce downstream effects (Chen *et al.*, 1996). I-SMADs play a role in a negative feedback loop that modulates the signaling by TGF- β , activin, and BMP. Transcription of I-SMADs 6 and 7 is rapidly and directly induced by TGF- β signalling, providing a mechanism of negative feedback (Nakao *et al.*, 1997; Afrakhte *et al.*, 1998; Takase *et al.*, 1998). Whereas I-SMAD 6 only inhibits BMP-mediated SMADs, I-SMAD 7 inhibits all R-SMADs. Recent articles have suggested that that myostatin gene expression is controlled by a balance between R-SMAD and I-SMAD levels, whereby myostatin signaling exerts positive feedback, but the induction of SMAD

7 exerts negative feedback (Zhu *et al.*, 2004; Forbes *et al.*, 2006). cMYC transcription would also be affected because of the antagonizing effect of SMAD 7 on SMADs 2/3. Therefore, the apparent mismatch in SMAD signaling and downstream target induction seen in the present work may be a result of I-SMAD elevation by exogenous SMAD 3 during medium and high dehydration. Coincident with this theory, an induction in I-SMAD 6 protein was observed in medium dehydration and there were no changes in SMAD 3 phosphorylation despite elevated levels of SMAD protein. However, increases in the phosphorylation of both SMAD 2/3 during high dehydration, suggest against inhibition at this time point. Therefore, during high dehydration SMAD signaling is seemingly not the main pathway controlling myostatin expression or synthesis. Since SMADs are known to regulate a large number of processes in the body beyond growth, including apoptosis, immunity cell fate and migration, to name a few, the elevation of activated SMADs 2/3 may be in response to other signaling molecules besides myostatin (for review: Ross & Hill, 2008).

Myostatin transcription is controlled by several SMAD independent pathways, with the FoxO1 transcription factor being thought to play a role in the atrophy mediated gene expression of myostatin (Allen & Unterman, 2007). Interestingly, nuclear FoxO1 expression is down regulated under high dehydration conditions in *X. laevis* skeletal muscle tissue, suggesting that this transcription factor may be part of the missing link (Malik & Storey, 2011).

Dehydration in frogs is associated with the accumulation of osmolytes in their plasma and tissues to provide colligative resistance to water loss. *X. laevis* mainly rely on increased plasma urea concentrations during dehydration, but glucose, another

common colligative osmolyte, is also elevated in the plasma in some species during dehydration in levels high enough to have colligative effects (Balinsky *et al.*, 1967; Churchill & Storey, 1993,1994; Malik & Storey, 2009). Glucose serves as a main source of energy for skeletal muscle, but during estivation the main fuel source is typically stored lipids (Seymour, 1973; Hermes-Lima *et al.*, 2001). Therefore, the regulation of glucose uptake is evidently changed during dehydration. Because glucose uptake in muscles is intertwined with factors regulating muscle growth, the effects of dehydration on glucose transportation in muscle cells were also examined in this study.

GLUT4 is the primary glucose transporter isoform in skeletal muscle cells and is responsible for glucose uptake upon insulin stimulation (for Review: He *et al.*, 2007). Akt is an insulin-responsive protein kinase necessary for stimulating the translocation of GLUT4 to the plasma membrane and thus the activation of Akt is a rate limiting step for glucose uptake (van Dam *et al.*, 2005; Anhê *et al.*, 2010). SMAD 5 has been shown to promote glucose uptake through GLUT4 by promoting upstream Akt expression and phosphorylation (Anhê *et al.*, 2010). Myostatin also regulates glucose uptake and metabolism. In contrast to SMAD 5, myostatin causes the dephosphorylation of Akt and the inhibition of myostatin has been shown to increase insulin sensitivity and glucose uptake in muscles by upregulating GLUT4, whereas increases in myostatin have the opposite effect (Amirouche *et al.*, 2009; Guo *et al.*, 2009; Antony *et al.*, 2007; Chen *et al.*, 2010).

Analysis of SMAD 5 during dehydration in *X. laevis* showed a significant decrease in SMAD 5 phosphorylation during medium dehydration. This likely occurs because of a combination of lowered levels of SMAD 5 protein and increased levels of I-

SMAD 6 at the same time. SMAD 6 forms stable interactions with BMP type I receptors, preventing the phosphorylation of downstream BMP mediated SMADs, such as SMAD 5 (Goto *et al.*, 2007). During high dehydration, total and P-SMAD 5 levels increase and SMAD 6 levels decrease, returning them to control values. While the drop in SMAD 5 levels correlated with no effect on GLUT4, the elevation in P-SMAD 5 signaling during high dehydration combined with the lowered inhibition by myostatin may enhance Akt signaling since GLUT4 protein was elevated between medium and high dehydration (Anhê *et al.*, 2010; Antony *et al.*, 2007).

Glut4 is an exercise responsive gene and its upregulation is triggered in part by the hypoxia that can develop in exercising muscles when oxygen demand by working muscle outstrips oxygen delivery capacity (Chiu *et al.*, 2004; Garcia-Roves *et al.*, 2005). Studies have shown that hypoxia stress alone also increases glucose uptake in a GLUT4 dependent manner in skeletal muscles (Zierath *et al.*, 1998). Hypoxia can develop in *X. laevis* during high dehydration as a consequence of an increased blood viscosity and decreased blood volume (Hillman, 1978). Therefore, the enhancement of GLUT4 in *X. laevis* may be a response to the imposed hypoxia. In frogs, hypoxia stress triggers the mobilization of liver glycogen to produce plasma glucose and this was also found to be true in *X. laevis* (Malik & Storey, 2009). Increased levels of GLUT4 in *X. laevis* skeletal muscle could enhance the uptake of the available plasma glucose into the cells for use as a substrate in anaerobic glycolysis, since a hypoxic environment is not conducive to aerobic lipid oxidation. Indeed, hypoxia-mediated increases in blood glucose are well-known to contribute to anaerobic energy generation (D'cona *et al.*, 1978; Donohoe & Boutilier, 1998). Interestingly, GLUT4 mRNA and protein were also elevated in the

skeletal muscle of the 13-lined ground squirrel, *Spermophilus tridecemlineatus*, during torpor (Tessier & Storey, 2010). Like estivators, hibernating ground squirrels show very little muscle atrophy despite weeks of inactivity and rely on stored lipids for energy (for review: Carey *et al.*, 2003). However, unlike the aforementioned frogs, squirrels do not require the colligative protection of endogenous osmolytes or undergo hypoxia, suggesting an additional role of GLUT4 in the muscles of animals resistant to atrophy that requires further investigation.

In conclusion, this study provides a molecular view of the proceedings of key proteins involved in muscle metabolism during dehydration-induced estivation. These data suggest that the characteristic protection of muscles against muscle wasting in estivating frogs may be mediated by lowered levels of myostatin and increased levels of cMYC. Changes to SMADs in both the TGF- β /activin and BMP pathway implicate their importance in the adaptation of muscles to dehydration stress (and/or estivation); however, the relationship between activated SMADs 2/3 and downstream targets *myostatin* and *cMYC* is seemingly decoupled. Elevated levels of GLUT4 perhaps in response increased P-SMAD 5 and decreased myostatin may increase plasma glucose transport into muscle cells, providing added substrate under hypoxic conditions created by high dehydration. Since myostatin inhibits activation of the Akt/mTOR pathway, and here we see a down-regulation of myostatin, an investigation into the positive growth factors of the P13K/Akt/mTOR pathway will provide an intriguing complement to this study and provide further insight into how estivators protect their muscles from atrophy (Trendelenburg *et al.*, 2009; Watt *et al.*, 2010).

FIG. 4.02. Effects of medium and high dehydration on the mRNA and protein expression of SMAD 2 in *X. laevis* muscle. (A) Effects of dehydration on the mRNA expression of *SMAD 2* as determined by RT-PCR. Representative *SMAD 2* amplicons are shown with corresponding α -tubulin bands below. Effects of dehydration on protein levels as determined by Western blotting after normalization to total protein: (B) total SMAD 2 levels, (C) cytoplasmic/nuclear SMAD 2 levels, (D) P-SMAD 2 (Ser 465/467) levels, and (E) cytoplasmic/nuclear P-SMAD 2 (Ser 465/467) levels as determined by Western blotting. Representative Western bands show SMAD 2 protein levels. Histograms show relative changes in normalized SMAD 2 transcripts or protein. Data are means $\pm$ SEM, $n = 3-5$ independent trials. Significantly different from the ^acontrol or ^bmedium dehydration values using a one way ANOVA and post hoc SNK test or Student's *t*-test, $P < 0.05$.

FIG. 4.03. Effects of medium and high dehydration on the mRNA and protein expression of SMAD 3 in *X. laevis* muscle. Effects of dehydration on protein content as determined by Western immunoblotting after normalization to total protein: (A) total SMAD 3 levels, (B) cytoplasmic/nuclear SMAD 3 levels, (C) relative P-SMAD 3 (Ser 425) levels, and (D) cytoplasmic/nuclear P-SMAD 3 (Ser 425) levels. Representative Western bands show SMAD 2 protein levels. (E) Effects of dehydration on the relative binding by SMAD 3 in nuclear extracts as determined by an EMSA. Representative EMSA shows relative SMAD 3 binding levels. Histograms show relative changes in normalized SMAD 3 protein. Data are means $\pm$ SEM, $n = 3-5$ independent trials. Significantly different ^acontrol or ^bmedium dehydration values using a one way ANOVA and post hoc SNK test or Students *t*-test, $P < 0.05$.

FIG. 4.04 (legend on next page)

(A)

1

1

61

20

121

40

181

60

241

80

TTGATGTGAAAACAGCACTACAAAACCTGGCTAAAGCAACCTGCATCTACTCTAGGAATTG

D V K T A L Q N W L K Q P A S T L G I

AAATTAAAGCTTGTGATGAAAATGGTCGTGATCTACCAATTGCATTCGTGGTTCAAATG

E I K A C D E N G R D L P I A F R G S N

AAGATGGACTGAACCCATTTATTGAAGTTAAAGTTATGGATACGCTAAAAAGATCGAGAA

E D G L N P F I E V K V M D T L K R S R

GGGATTTTCGGACTTGACTGTGATGAACATTCAACAGAATCGAGGTGTTGCCGTTATCCAT

R D F G L D C D E H S T E S R C C R Y P

TGACAGTG

L T V

(B)

Homology matrix of 5 sequences

African clawed frog	100%				
Western clawed frog	98.8%	100%			
Chicken	81.5%	77.5%	100%		
Human	82.7%	76.9%	91.7%	100%	
Zebrafish	60.5%	63.1%	69.9%	69.8%	100%

FIG. 4.04. Partial nucleotide and deduced amino acid sequence of *X. laevis* myostatin.

(A) Partial cDNA sequence of *myostatin* from the African clawed frog, with the corresponding translated amino acid sequence. Nucleotides and amino acids are numbered on the left. The nucleotide sequence was 248 bases and encoded 82 amino acids. The sequence covered amino acids 201-282 of the Western clawed frog sequence (Accession no. XP_002931568.1), representing ~21.68% of the total myostatin protein.

(B) Homology tree and homology matrix produced from an alignment of the partial African clawed frog myostatin protein sequence with myostatin from Western clawed frog (*X. tropicalis*; Accession no. XP_002931568.1), zebrafish (*Danio rerio*; Accession no. AAQ11222), and human (*Homo sapiens*; Accession no. ABI48514). The percentage values correspond to shared identity between corresponding species.

African clawed frog	DVKTALQNWLKQPASTLGIEIKACDENGRLPIAFRGSNE	40
Western clawed frog	-----	40
Chicken	----v-----e-n-----f--t----avt-p-pg-	40
Human	----v-----e-n-----l----h--avt-p-pg-	40
Zebrafish	---qv-tv-----etnr----n-y-ak-n--avtstetg-	40
African clawed frog	DGLNPFIEVKVMDTLKRSRRDFGLDCDEHSTESRCCRYPLT	81
Western clawed frog	-----p-----	81
Chicken	-----l--r-t--p-----	81
Human	-----l---t--p-----	81
Zebrafish	---l--m---isegp--i---s-----n-s-----	81

FIG. 4.05. Comparison of the partial African clawed frog (*X. laevis*) myostatin protein sequence with the corresponding region in myostatin from Western clawed frog (*X. tropicalis*; Accession no. XP_002931568.1), zebrafish (*Danio rerio*; Accession no. AAQ11222), chicken (*Gallus gallus*; Accession no. AAK18000), and human (*Homo sapiens*; Accession no. ABI48514).

FIG. 4.06. Effects of medium and high dehydration on the mRNA and protein expression of myostatin in *X. laevis* muscle. (A) Effects of dehydration on the mRNA expression of *myostatin* as determined by RT-PCR. Representative *myostatin* amplicons are shown with corresponding α -tubulin (normalization control) bands below. (B) Effects of dehydration on myostatin protein levels as determined by Western blotting after normalization to total protein. Representative Western bands show myostatin protein levels. Histograms show relative changes in normalized myostatin transcripts or protein. Data are means $\pm$ SEM, $n = 3-5$ independent trials. Significantly different from corresponding respective ^acontrol or ^bmedium dehydration values using a one way ANOVA and post hoc SNK test, $P < 0.05$

FIG. 4.07. Effects of medium and high dehydration on the mRNA and protein expression of cMYC in *X. laevis* muscle. (A) Effects of dehydration on the expression of cMYC transcripts as determined by RT-PCR. Representative cMYC amplicons are shown with corresponding α -tubulin bands below. Effects of dehydration on protein levels as determined by Western blotting after normalization to total protein: (B) total cMYC, (C) cytoplasmic/nuclear cMYC, (D) P-cMYC (Thr 58/Ser 62), and (E) cytoplasmic/nuclear P-cMYC (Thr 58/Ser 62). Representative Western bands are shown together with histograms showing relative changes in normalized cMYC transcripts or protein. Data are means $\pm$ SEM, $n = 3-5$ independent trials. Significantly different from ^acontrol or ^bmedium dehydration values using a one way ANOVA and post hoc SNK test or Student's *t*-test, $P < 0.05$.

FIG. 4.08. Effects of medium and high dehydration on the mRNA and protein expression of SMAD 5 in *X. laevis* muscle. (A) Effects of dehydration on *SMAD 5* mRNA levels as determined by RT-PCR. Representative *SMAD 5* amplicons are shown with corresponding α -tubulin bands below. Effects of dehydration on relative protein levels as determined by Western blotting after normalization to total protein: (B) SMAD 5, (C) cytoplasmic/nuclear SMAD 5, (D) P-SMAD 5 (Ser463/465), and (E) cytoplasmic/nuclear SMAD 5 (Ser463/465). Representative Western bands are shown as well as histograms showing relative changes in normalized SMAD 5 transcripts or protein. Data are means $\pm$ SEM, $n=3-5$ independent trials. Significantly different from ^acontrol or ^bmedium dehydration values using a one way ANOVA and post hoc SNK test or Students *t*-test, $P < 0.05$.

FIG. 4.09. Effects of medium and high dehydration on the protein expression of SMAD 6 in *X. laevis* muscle. Effects of dehydration on protein (A) SMAD 6 levels and (B) cytoplasmic/nuclear SMAD 6 levels, as determined by Western blotting after normalization to total protein. Representative Western bands show relative SMAD 6 protein levels. Histograms show relative changes in normalized SMAD 6 protein. Data are means $\pm$ SEM, $n=3-5$ independent trials. Significantly different from ^acontrol or ^bmedium dehydration values using a one way ANOVA and post hoc SNK test or Students *t*-test, $P < 0.05$

FIG. 4.10. Effects of medium and high dehydration on the protein expression of GLUT4 protein in *X. laevis* muscle as determined by Western blotting after normalization to total protein. Representative Western bands show relative GLUT 4 protein levels. Histograms show relative changes in normalized GLUT 4 protein. Data are means $\pm$ SEM, $n=3-4$ independent trials. ^aSignificantly different from control values using a one way ANOVA and post hoc SNK test or Students *t*-test, $P < 0.05$.

FIG. 4.11. Effects of medium and high dehydration on the mRNA and protein expression of SMAD 4 in *X. laevis* muscle. (A) Effects of dehydration on the mRNA expression of SMAD 4 as determined by RT-PCR. Representative SMAD 4 amplicons are shown with corresponding α -tubulin bands below. Effects of dehydration on protein levels as determined by Western blotting after normalization to total protein: (B) SMAD 4 levels and (C) cytoplasmic/nuclear SMAD 4 levels. Representative Western bands show relative SMAD 4 protein levels. Histograms show relative changes in normalized SMAD 4 transcripts or protein. Data are means $\pm$ SEM, $n=3-5$ independent trials. Significantly different from ^acontrol or ^bmedium dehydration values using a one way ANOVA and post hoc SNK test or Students *t*-test, $P < 0.05$.

CHAPTER 5

General Discussion

Amphibians inhabit all continents except Antarctica and have evolved a number of adaptations to survive in highly variable environments. The two examples examined in this thesis were the North American wood frog, *Rana sylvatica*, which is freeze tolerant, and the African clawed frog, *Xenopus laevis*, which is dehydration tolerant to ~35% of total body water lost. Investigations into the physiological and biochemical changes that occur under these stresses have allowed the mechanisms of survivability to be elucidated. Metabolic rate depression (MRD) to reduce total energy expenditure and the use of colligative osmolytes to protect against dehydration/freezing are two general principles that have been identified in frogs adapting to environmental stress. However, despite global MRD that includes strong suppression of transcription and translation, certain suites of genes and proteins are upregulated during cellular stress, and these have been identified in being conducive to survivorship.

Despite the adverse conditions that amphibians must have faced throughout the many years that they have inhabited the Earth (an estimated 350 million years), in the last 40 years amphibians have faced an unprecedented decline, now making them the most threatened vertebrate class on the planet with as many as one-third of amphibian species at risk of extinction (Sodhi *et al.*, 2008; Carey *et al.*, 1999; Stuart *et al.*, 2004). This has prompted research on the normal fitness of frogs, analyzing what keeps these animals healthy, so as to better identify and understand deviations that could signal compromised health. How the environment impacts the immune system and the muscle fitness of the frogs *R. sylvatica* and *X. laevis* has been the focus of my thesis, with the emphasis being on the gene and protein regulation of antimicrobial peptides (AMPs) and the SMAD family of transcription factors (with respect to AMP regulation and muscle mass).

Dormancy and the Immune System

Environmental stress impacts host-parasite relationships among amphibians (Pounds *et al.*, 1999; Woodhams *et al.*, 2007), both directly and indirectly affecting the immune system (for review: Blaustein *et al.*, 2010). How environment impacts amphibian disease is a major question in ecology, which has become increasingly important as scientists try to understand amphibian population declines (Raffel *et al.*, 2006; Kielgast *et al.*, 2010). This is because environmental change is often blamed for the increases in infectious disease that are contributing to the global declines of amphibian populations. In order to understand the emergence of infectious disease, the impact of environmental factors on the disease dynamic needs to be understood (Fisher, 2007).

Previous studies analyzing the immune system of amphibians in response to physical environmental changes have shown that prolonged cold exposure, as occurs during hibernation, results in signs of substantial adaptive immunodeficiency. These include: an atrophied thymus during the winter, progressive losses of hemapoietic populations, decreased proliferation of T-lymphocytes, marked lymphocyte depletion, prolonged transplantation graft survival time, and an absence of immunological responsiveness to immunization (Miodoński *et al.*, 1996; Jozkowicz & Plytycz, 1998; Maniero & Carey, 1997; Cooper *et al.*, 1992). Despite these severe changes, analysis of innate immune components shows signs of selective activity. These include significant increases in neutrophils (but decreases in eosinophils) and a maintained low temperature response by the complement system (but diminished overall levels of complement

molecules) (Maniero & Carey, 1997; Cooper *et al.*, 1992; Ruben *et al.*, 1977; Day *et al.*, 1970).

Metamorphosis poses another period of adaptive immune incompetency. During the water-breathing juvenile (tadpole) stage, lymphocyte populations expand into an immune-competent system. During metamorphosis, amphibians undergo extensive rearrangements of their cells, organs and tissues and many metabolic processes, including the immune system. Amphibian metamorphosis is under neuroendocrine control and these hormones cause amphibians to dismantle their premetamorphic immune system by inducing apoptosis in susceptible lymphocytes and inhibiting lymphocyte proliferation. This substantially decreases lymphocyte populations in the thymus, liver, and spleen by at least 40% (for review: Rollins-Smith, 1998). Experiments have shown that during this time, tolerance is easily induced (Pasquier & Haimovich, 1976). Therefore, the dismantlement of the juvenile immune system may serve to eliminate unnecessary lymphocytes that may not be able to tolerate newly emerging adult-specific antigens and would consequently react adversely with adult tissues, potentially causing negative auto-immune reactions (Rollins-Smith, 1998; Pasquier & Haimovich, 1976). Following metamorphosis, lymphocyte populations expand to generate a renewed immune system. Antibodies of the mature adult immune system have a higher affinity and greater diversity than those created by the juvenile immune system and are tolerant of the adult-specific molecules. Normally, metamorphs pass through the period of immune incompetency without danger, suggesting that molecules of the innate immune system must be keeping them healthy in the interim. An elevated innate immune system has been demonstrated previously at the onset of metamorphosis in sea squirts and insects,

however being invertebrates, these animals lack a functional adaptive immune system (Davidson & Swalla, 2002; Altincicek & Vilcinskas, 2006).

Chapters 2 and 3 explored the impacts of cellular stress on AMP expression. AMPs are effector molecules of the innate immune system that are commonly found on frog skin and mucosal tissues, thereby providing the first line of defense against environmental microbes. AMPs were thought to be good candidates for protecting frogs while under the energy restriction of dormancy. In Chapter 2, the skin and mucosal tissues of *R. sylvatica* were scanned for expression of its singular dermal AMP, brevinin-1SY. The expression of brevinin-1SY was confirmed in all tissues tested, and the study went on to characterize the response of this AMP to freezing, anoxia, dehydration, and metamorphosis using RT-PCR. Although changes in expression were generally limited, each tissue showed a stress specific response by its AMP. Freezing was associated with a decrease in AMP expression in both dorsal skin and large intestine, perhaps to promote the winter growth of the ice nucleating bacteria found in these areas. Anoxia was associated with a decrease in AMP expression in the small and large intestine, and an elevation in AMP expression in the ventral skin. The blood is preferentially shunted away from the gut during anoxia, and a lowered level of AMPs may help mitigate a destructive immune response upon reperfusion. With lowered internal immune defenses, the external barrier becomes increasingly important, potentially accounting for the increased levels of skin AMPs during anoxia. Finally, dehydration was associated with increased AMP mRNA in dorsal and ventral skin and lung. This suggests the preferential protection of the respiratory tissues during dehydration. Interestingly, the AMP expression of the stomach did not change with respect to any of the stresses, reflecting

the fact that the stomach is not used during dormancy and thus would not be exposed to food borne pathogens. The tissue specific expression of brevinin-1SY in response to each stress suggests a shared defense function that protects the dormant animal either immunologically its presence or in other ways by its absence (for example, in the case of freezing, AMP down regulation may allow ice nucleating bacteria to grow). Although the regulation of AMPs during stress implicates their importance, the few tissue-specific selective elevations in AMPs dismiss the idea of a global protective response by AMPs. However, the regulation of brevinin-1SY in the skin as a response to freezing, anoxia, or dehydration preserves their importance in the protection of the skin from pathogens during environmental stress. Indeed, this is the most likely organ to face environmental pathogens while the animal is in a hypometabolic state. The selective enhancement of innate immune AMPs in certain tissues suggests that, in addition to regulating which parts of the immune system remain active during dormancy, where these immune effectors are active is also regulated. During metamorphosis, brevinin-1SY expression is strongly correlated with metamorphosis and the development of adult skin, suggesting the enhanced protection of metamorphs by AMPs. These results remain consistent with theoretical work that suggests that a partially effective immune system can achieve the greatest fitness when balancing the costs of the immunity and are reflective of the studies (above) that show selective regulation of aspects of the innate immune system (Behnke *et al.*, 1992).

In Chapter 3, the effect of dehydration (potentially leading to an estivating state) on the expression and regulation of another AMP, hepcidin, was analyzed in *X. laevis* liver. While studies have focused on the impacts of cold-induced dormancy on the

immune system, seemingly few have looked at hot, dry conditions that induce dormancy. This is surprising considering that bacterial growth is temperature dependent and could be expected to be accelerated at high environmental temperatures. Hecpcidin is an AMP synthesized in the liver that also plays a role as an iron regulatory hormone. The data provided in this thesis is the first report of multiple hepcidin forms in *X. laevis* and the differential regulation of these peptides in response to dehydration. Transcript levels of hepcidin I increased significantly in liver in response to medium and high dehydration, whereas hepcidin II transcripts rose only under high dehydration. Protein levels of the transcription factor STAT 3, an inducer of hepcidin expression, rose during high dehydration and cMYC, an iron and cell-proliferation regulator, rose during medium and high dehydration suggesting their control over hepcidin expression. At the same time, members of TGF- β mediated SMAD pathway, which antagonizes hepcidin expression, were suppressed under high dehydration further allowing an environment conducive to hepcidin expression. Interestingly, active members of the BMP-mediated SMAD pathway that promotes hepcidin expression in response to iron loading, were also suppressed during dehydration (but with no change in active nuclear levels), suggesting that enhancement of hepcidin under dehydration stress is not due to its iron regulatory role but rather to its immune function. Accordingly, STAT 3 has been shown to be the pathway through which inflammation triggers hepcidin (Huang *et al.*, 2009). These data further supports a tissue selective role of AMPs in response to environmental stresses, and the limited data presented here in both the wood frog and African clawed frog suggests that desiccation resistance benefits most from the enhancement of these innate immune molecules. Therefore, these data suggests that while studies have shown that the

adaptive immune system is severely impaired in frogs during dormancy, the elementary effectors of the innate immune system are still providing some protection of these animals against environmental pathogens. The tissue selective nature of their expressions, however, means researchers must be wary of making generalizations claiming the presence or absence of innate molecules for fear that important tissue-specific information becomes overlooked.

Estivation and Skeletal Muscle Mass

Prolonged disuse or energy insufficiency typically leads to muscle atrophy in mammals. However, animals that undergo natural bouts of MRD, such as estivators, show limited muscle wasting despite being inactive for many months (sometimes even years). Frog skeletal muscle changes during estivation have been extensively characterized in the green-striped burrowing frog, *Cyclorana albogultata*. These have documented preserved muscle mass, muscle cross-sectional area, water content and myofibre number in the powerful jumping muscles (crurais & gastronemius) following nine months of estivation (Mantle *et al.*, 2009). Following three months of estivation, these frogs have the immediate capability to resume muscle function as determined by the maintenance of *in vitro* force production and swimming performance (Hudson & Franklin, 2002a). The molecular changes underlining this capacity in muscle of estivating frogs have not yet been fully elucidated.

With the fitness advantages of preserving muscle mass being clear, especially considering the limited time that many estivating species are actually active during the year, an important aspect of dehydration tolerance in *X. laevis* might involve the

induction of muscle preserving pathways. In Chapter 4, the myostatin/SMAD signaling pathway was examined in heterogeneous hind leg skeletal muscle of *X. laevis* to examine its potential contribution to atrophy protection. Myostatin is a negative regulator of muscle mass that has been shown to be upregulated in atrophied muscles and downregulated in hypertrophied muscles. In order to preserve muscle mass, the myostatin pathway may need to be diminished. Indeed, a downregulation of both myostatin mRNA and protein were found in *X. laevis* skeletal muscle during dehydration. A similar study in the 13-lined ground squirrels showed that while myostatin levels were maintained during hibernation, the protein levels of its inhibitor were enhanced, suggesting the control of myostatin at the signaling level (Brooks *et al.*, 2011). Furthermore, cMYC, a proliferation enhancing transcription factor, was upregulated during dehydration. Thus, the signaling and expression of myostatin was seemingly decoupled from its signaling pathway since active SMADs 2/3 were upregulated in response to dehydration. Finally, levels of GLUT4 were enhanced under high dehydration conditions, with possible control by enhanced SMAD 5 and decreased myostatin levels as compared to medium dehydration. These studies suggest that instead of molecular signals inhibiting muscle growth, as would occur during normal bouts of inactivity, signals initiating muscle growth are put in place during estivation.

SMAD Family of Transcription Factors

SMAD transcription factors are a network of signaling molecules that propagate signals from the TGF- β superfamily of ligands. My thesis work adds this signaling pathway to a growing number of transcription factors that are differentially regulated during dehydration in the African clawed frog (Malik & Storey, 2009; Malik & Storey,

2011). A comparison of the SMAD activation in the liver and muscle shows that these two organs are selectively regulated. In fact, the expression patterns of P-SMADs 2-4 directly oppose each other in the two tissues. This is in alignment with the different functional outcomes for which SMADs signal in the muscle and liver and the inherent differences in the muscle and liver tissues themselves. In general, most of the P-SMADs showed no regulation during medium dehydration, the exceptions being P-SMAD 5 in liver and muscle, and SMAD 6 in muscle. Relative phosphorylation levels of the R-SMADs were all suppressed in liver during high dehydration, suggesting that this transcription factor pathway does not play a significant role in gene upregulation during estivation in this tissue. Alternatively, the phosphorylated R-SMADs of the TGF- β /activin mediated pathway and co-SMAD 4 were all selectively upregulated in muscle, suggesting that this pathway has a significant role to play in muscle viability during dehydration. Increases to the phosphorylation levels in R-SMADs were all reflected in the nuclear fractions.

All studied targets showed evidence for transcriptional regulation. The regulatory patterns of P-SMADs observed in the liver during dehydration correlated well with the changes found in the assessed downstream targets: *hepcidin 1*, *hepcidin 2*, and *cMYC*. However, this was not true of the relationship between P-SMADs and the assessed target genes (*myostatin*, *cMYC*) in the muscle. *cMYC* was analyzed in both liver and muscle tissue, and was found to be upregulated in both tissues during medium and high dehydration at both the transcript and protein level. The products of these genes, as discussed in the individual chapters, could provide mechanisms that sustain the African

clawed frog under desiccating conditions in estivation by providing protection against pathogens and protective measures against muscle atrophy.

Future Studies

This thesis touched on a few of the processes and molecular mechanisms that facilitate the survival of frogs under environmental stress conditions, yet much remains to be explored.

The data presented in Chapters 2 and 3 hinted at a tissue selective regulation and, in some cases, activation of antimicrobial peptides in response to imposed stress (freezing, anoxia, dehydration). However, further work is needed to validate that protein expression reflects the trends observed for these AMP transcripts. mRNA and protein expression are not always correlated, as is evidenced in this thesis by SMAD 5 in muscle, and since peptides hold the effector function, supplemental work needs to be conducted in order for conclusions to be drawn on their protective function. Furthermore, a number of other antimicrobials have been identified in the African clawed frog. In addition to high levels of expression in the skin, two of these AMPs, magainin and PGLa, are also known to be distributed throughout the stomach and gut (Moore *et al.*, 1991; Reilly *et al.*, 1994a). Magainin has previously been found to be upregulated at both the transcript and protein level in the of skin *X. laevis* during dehydration, similar to the effects of dehydration on brevinin-1SY expression in the skin of *R. sylvatica* (HA Holden, unpublished data). Further characterization of magainin and PGLa in the stomach and gut, and potentially the lungs, during dehydration in *X. laevis* will provide a comparison

to the work completed in *R. sylvatica* and allow trends to be visualized and potential principles to be formulated.

The activation of STAT 3 in liver, as observed in Chapter 3, suggests other immune targets that may be responsive during dehydration. STAT 3 is known to play a hepatoprotective role against persistent inflammatory stress (Wang *et al.*, 2011a). STAT 3 is activated in liver resident macrophages called Kupffer cells by pro-inflammatory cytokines. Through the integration of these signals, STAT 3 either potentiates the proinflammatory response or inhibits it through the activation of the suppressor of cytokine signaling 3 (SOCS3) (for review: Wang *et al.*, 2011b). Since inflammation is the trigger leading to an adaptive immune response, and evidence suggests that the adaptive response may be absent in hypometabolic states, an analysis of STAT 3 induced SOCS3 would provide an interesting assessment of whether crosstalk still occurs between the innate and adaptive immune system during dormancy.

The data in Chapter 4 suggests that major biochemical reorganization occurs in the skeletal muscle of *X. laevis*. Here it was shown that myostatin levels were downregulated while cMYC and GLUT4 were upregulated in response to dehydration. The negative growth signals of the myostatin signaling pathway are typically balanced by the positive growth signals from the Akt/mTOR signaling pathway. The mTOR signaling pathway is a key component of the insulin receptor network and the primary cellular process responsible for regulating protein synthesis. To expand the picture of the processes that interplay in skeletal muscle to prevent muscular atrophy over prolonged dormancy, a study of the mTOR pathway would provide data complementary to that presented here.

The TGF- β /activin mediated P-SMADs (SMADs 2/3) and co-SMAD 4 in *X. laevis* were the only SMADs that were upregulated during dehydration in muscle and liver. Interestingly, their signaling capacity was not correlated with myostatin signaling, suggesting that an alternative signal crucial to muscles in dehydration is using the pathway to propagate its signal. Besides myostatin, TGF- β 1 is the other member of the TGF- β superfamily that is of particular importance in skeletal muscle (for review: Burks & Cohn, 2011). TGF- β 1 is an anti-inflammatory cytokine expressed during myogenesis and is physiologically upregulated in the regeneration of skeletal muscle following injury (Serrano & Muñoz-Cánoves, 2010; Gosselin & McCormick, 2004). TGF- β 1 signals through both the canonical TGF- β pathway (i.e. SMADs 2/3/4) and the non-canonical mitogen-activated protein kinase (MAPK) pathway (for review: Burks & Cohn, 2011). Evidence of the potential role of TGF- β 1 signaling is the activation of MAPK family members from both the extracellular signal-regulated kinases (ERK) pathway and the c-Jun N-terminal kinase (JNK) pathway in *X. laevis* skeletal muscle during dehydration (Malik & Storey, 2009; Malik, 2009). This prompts future work looking at the expression of myogenesis targets in the myocyte enhancer factor-2 (MEF2) family, for which SMADs are a co-modulator of transcription (Quinn *et al.*, 2001). MEF2A and MEF2C protein and phosphorylation levels were previously shown to be upregulated in the muscle tissue of hibernating 13-lined ground squirrels, implicating their value in preserving muscle during dormancy (Tessier & Storey, 2010). Interestingly, GLUT4 is a downstream target of MEFs, and this protein was also found to be significantly upregulated during high dehydration in *X. laevis*.

Conclusions

The data presented in this thesis concludes antimicrobial peptides, belonging to innate immunity, are an important component of the stress response in anurans. Brevinin-1SY is subject to activation in response to environmental stresses in a tissue selective as well as a developmentally dependent manner in wood frogs. Comparably enhanced transcription of hepcidin occurs in liver of African clawed frogs. Dehydration also diminishes the negative growth regulator myostatin in skeletal muscle of *X. laevis*. Furthermore, SMAD transcription factors were subject to activation in the muscle of African clawed frogs, but not the liver, during high dehydration. These data reflect the importance of suppressing metabolic rate in the liver, with the selective activation of key functions that contribute to liver preservation. With the new knowledge presented, a plethora of exciting leads and questions are generated that will help to unravel the mysteries behind how wood frogs and African clawed frogs protect their tissues to endure long term environmental stress.

APPENDICES

APPENDIX A:
List of Publications

Published:

Holden HA, Storey KB. 2011. Reversible phosphorylation regulation of NADPH-linked polyol dehydrogenase in the freeze-avoiding gall moth, *Epiblema scudderiana*: role in glycerol metabolism. Arch Insect Biochem Physiol. 77: 32-44.

In preparation:

Holden HA, Storey KB. Developmental and environmental impacts on brevinin-1SY expression in the wood frog, *Rana sylvatica*.

Holden HA, Storey KB. Hepcidin antimicrobial peptides from the African clawed frog, *Xenopus laevis*.

Holden HA, Storey KB. Expression of SMAD transcription factors in the skeletal muscle of the African clawed frog, *Xenopus laevis*.

Holden HA, Storey KB. Reversible phosphorylation regulation of NADPH-linked polyol dehydrogenase in the freeze-tolerant gall moth, *Eurosta solidaginis*.

Holden HA, Storey KB. Regulation of NADP-Isocitrate Dehydrogenase by State-Dependent Reversible Phosphorylation in Anoxia Tolerant Red-Eared Slider Turtles, *Trachemys scripta elegans*.

APPENDIX B: Communications at Scientific Meetings

Oral presentations:

Holden HA, Storey KB. Surviving Freezing: Impacts on Innate Immunity in the Wood Frog, *Rana sylvatica*. Canadian Society of Zoology Annual Meeting. Ottawa, ON. May 16-20, 2011. **(Selected Talk; Hoar Award Nominee)**

Holden HA, Tessier SM. Conquering Adversity. Celebration of Women in Science and Engineering. Ottawa, ON. April 5, 2011. **(Selected Talk)**

Holden HA, Storey KB. Surviving Freezing: Impacts on the Immune System. Infection and Immunity Research Forum. London, ON. November 5, 2010. **(Selected Talk, 1st Prize)**.

Holden HA, Storey KB. Sub-Zero Survival: Animal Responses to Freezing Temperatures. Celebration of Women in Science and Engineering. Ottawa, ON. April 8, 2010.

Poster presentations:

Holden HA, Storey KB. Impact of Dehydration on the Immune System in the African Clawed Frog, *Xenopus Laevis*. Ottawa-Carleton Institute of Biology Symposium. Ottawa, ON. April 28-29, 2011. **(2nd Prize)**

Holden HA, Storey KB. Immune responses to environmental stresses: roles of the antimicrobial peptide brevinin-1SY during hypometabolism. Canadian Society for Life Science Research Meeting. Montreal, ON. August 13-14, 2010.

Holden HA, Storey KB. Regulation of NADP-Isocitrate Dehydrogenase in Anoxia Tolerant *Trachemys scripta elegans* by State-Dependent Phosphorylation. Ottawa-Carleton Institute of Biology Symposium. Ottawa, ON. April 13, 2010.

Holden HA, Storey KB. Regulation of NADP-Isocitrate Dehydrogenase in Anoxia Tolerant *Trachemys scripta elegans* by State-Dependent Phosphorylation. Chemistry and Biochemistry Graduate Research Conference. Montreal, QU. November 20-21, 2009.

REFERENCES

- Afrakhte M, Moren A, Jossan S, Itoh S, Sampath K, Westermarck B, Heldin CH, Heldin NE, Ten Dijke P. 1998. Induction of inhibitory Smad6 and Smad7 mRNA by TGF-beta family members. *Biochem Biophys Res Commun.* 249: 505-511.
- Alexander SS, Bellerby CW. 1938. Experimental studies on the sexual cycle of the South African clawed toad (*Xenopus laevis*). *J Exp Biol.* 15: 74-81.
- Allen DL, Unterman TG. 2007. Regulation of myostatin expression and myoBLAST differentiation by FoxO and SMAD transcription factors. *Am J Physiol Cell Physiol.* 292: C188-C199.
- Altincicek B, Vilcinskas A. 2006. Metamorphosis and collagen-IV-fragments stimulate innate immune response in the greater wax moth, *Galleria mellonella*. *Dev Comp Immunol.* 30: 1108-1118.
- Amalfitano S, Fazi S, Zoppini A, Barra Caracciolo A, Grenni P, Puddu A. 2008. Responses of benthic bacteria to experimental drying in sediments from Mediterranean temporary rivers. *Microb Ecol.* 55: 270-279.
- Amati B, Littlewood TD, Evan GI, Land H. 1993. The c-Myc protein induces cell cycle progression and apoptosis through dimerization with Max. *EMBO J.* 12: 5083-5087.
- Amiche M, Seon AA, Pierre TN, Nicolas P. 1999. The dermaseptin precursors: a protein family with a common preproregion and a variable C-terminal antimicrobial domain. *FEBS Lett.* 456: 352-356.
- Amirouche A, Durieux AC, Banzet S, Koulmann N, Bonnefoy R, Mouret C, Bigard X, Peinnequin A, Freyssen D. 2009. Down-regulation of Akt/mammalian target of rapamycin signaling pathway in response to myostatin overexpression in skeletal muscle. *Endocrinology.* 150: 286-294.
- Andreu D, Rivas L. 1998. Animal antimicrobial peptides: an overview. *Biopolymers.* 47: 415-433.
- Anhê FF, Lellis-Santos C, Leite AR, Hirabara SM, Boschero AC, Curi R, Anhê GF, Bordin S. 2010. Smad5 regulates Akt2 expression and insulin-induced glucose uptake in L6 myotubes. *Mol Cell Endocrinol.* 319: 30-38.
- Antony N, Bass JJ, McMahon CD, Mitchell MD. 2007. Myostatin regulates glucose uptake in BeWo cells. *Am J Physiol Endocrinol Metab.* 293: E1296-1302.
- Axelsson M, Fritsche R. 1991. Effects of exercise, hypoxia and feeding on the gastrointestinal blood flow in the Atlantic cod *Gadus morhua*. *J Exp Biol.* 158: 181-198.
- Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, Campagna JA, Chung RT, Schneyer AL, Woolf CJ, Andrews NC, Lin HY. 2006. Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. *Nat Genet.* 38: 531-539.

- Balinsky JB, Choritz EL, Coe CGL, Van Der Schans GS. 1967. Amino acid metabolism and urea synthesis in naturally aestivating *Xenopus laevis*. *Comp Biochem Physiol*. 22: 59-68.
- Barbet JP, Chauveau M, Labbé S, Lockhart A. 1988. Breathing dry air causes acute epithelial damage and inflammation of the guinea pig trachea. *J Appl Physiol*. 64: 1851-1857.
- Bardan A, Nizet V, Gallo RL. 2004. Antimicrobial peptides and the skin. *Expert Opin Biol Th*. 4: 543-549.
- Barra D, Simmaco M. 1995. Amphibian skin: a promising resource for antimicrobial peptides. *Trends Biotechnol*. 13: 205-209.
- Bayele HK, McArdle H, Srai SK. 2006. Cis and trans regulation of hepcidin expression by upstream stimulatory factor. *Blood*. 108: 4237-4245.
- Beck CW, Slack MW. 2001. An amphibian with ambition: a new role for *Xenopus* in the 21st century. *Genome Biol*. 2: REVIEWS1029.
- Behler JL, King FW. 1979. The Audubon Society Field Guide to North American Reptiles and Amphibians. Alfred A. Knopf, New York.
- Behnke JM, Barnard CJ, Wakelin D. 1992. Understanding chronic nematode infections: evolutionary considerations, current hypotheses and the way forward. *Int J Parasitol*. 22: 861-907.
- Bentley PJ, Yorio T. 1979. Do frogs drink? *J Exp Biol*. 79: 41-44.
- Berger L, Spear R, Kent A. 1999. Diagnosis of chytridiomycosis in amphibians by histological examination. *Zoos Print J*. 15: 184-190.
- Bevins CL. 1994. Antimicrobial peptides as agents of mucosal immunity. *Ciba Found Symp*. 186: 250-269.
- Biggar KK, Storey KB. 2009. Perspectives in cell cycle regulation: lessons from an anoxic vertebrate. *Curr Genomics*. 2009. 10: 573-584.
- Blaustein AR, Walls SC, Bancroft BA, Lawler JJ, Searle CL, Gervasi SS. 2010. Direct and indirect effects of climate change on amphibian populations. *Diversity*. 2: 281-313.
- Bonneaud C, Mazuc J, Gonzalez G, Haussy C, Chastel O, Faivre B, Sorci G. 2003. Assessing the cost of mounting an immune response. *Am Nat*. 161: 367-379.
- Bosch J, Carrascal LM, Durán L, Walker S, Fisher MC. 2007. Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? *Proc Biol Sci*. 274: 253-260.
- Boucher RC. 2007. Evidence for airway surface dehydration as the initiating event in CF airway disease. *J Intern Med*. 261: 5-16.

- Bouma HR, Carey HV, Kroese FG. 2010. Hibernation: the immune system at rest? *J Leukoc Biol.* 88: 619-624.
- Boutilier RG, Glass ML, Heisler N. 1986. The relative distribution of pulmocutaneous blood flow in *Rana catesbeiana*: effects of pulmonary or cutaneous hypoxia. *J Exp Biol.* 126: 33-39.
- Boutilier RG, Randall DJ, Shelton G, Toews DP. 1979. Acid-base relationships in the blood of the toad, *Bufo marinus*. II. The effects of dehydration. *J Exp Biol.* 82: 345-355.
- Brooks NE, Myburgh KH, Storey KB. 2011. Myostatin levels in skeletal muscle of hibernating ground squirrels. *J Exp Biol.* 214: 2522-2527.
- Burggren WW, Moalli R. 1984. Active regulation of cutaneous gas exchange by capillary recruitment in amphibians: experimental evidence and a revised model for skin respiration. *Resp Physiol.* 55: 379-392.
- Burggren WW, Vitalis TZ. 2005. The interplay of cutaneous water loss, gas exchange and blood flow in the toad, *Bufo woodhousei*: adaptations in a terrestrially adapted amphibian. *J Exp Biol.* 208: 105-112.
- Burks T, Cohn RD. 2011. Role of TGF- β signaling in inherited and acquired myopathies. *Skeletal Muscle.* 1: 19.
- Bustamante HM, Livo LJ, Carey C. 2010. Effects of temperature and hydric environment on survival of the Panamanian golden frog infected with a pathogenic chytrid fungus. *Integr Zool.* 5: 143-153.
- Buttgereit F, Burmester GR, Brand MD. 2000. Bioenergetics of immune functions: fundamental and therapeutic aspects. *Immunol Today.* 21: 192-199.
- Cai Q, Storey KB. 1997. Up-regulation of a novel gene by freezing exposure in the freeze tolerant wood frog (*Rana sylvatica*). *Gene* 198: 305-312.
- Calamita G, Gounon P, Gobin R, Bourguet J. 1994. Antidiuretic response in the urinary bladder of *Xenopus laevis*: presence of typical aggregophores and apical aggregates. *Biol Cell.* 80: 35-42.
- Carbia-Nagashima A, Arzt E. 2004. Intracellular proteins and mechanisms involved in the control of gp130/JAK/STAT cytokine signaling. *Life.* 56: 83-88.
- Carey HV, Andrews MT, Martin SL. 2003. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. *Physiol Rev.* 83: 1153-1181.
- Carey C, Gohen N, Rollins-Smith L. 1999. Amphibian declines: an immunological perspective. *Dev Comp Immunol.* 23: 459-472.
- Carey C, Heyer WR, Wilkinson J, Alford RA, Arntzen JW, Halliday T, Hungerford L,

- Castro HF, Classen AT, Austin EE, Norby RJ, Schadt CW. 2010. Soil microbial community responses to multiple experimental climate change drivers. *Appl Environ Microbiol.* 76: 999-1007.
- Chen CR, Kang Y, Siegel PM, Massagué J. 2002. E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression. *Cell.* 110: 19-32.
- Chen HB, Rud JG, Lin K, Xu L. 2005. Nuclear targeting of transforming growth factor-beta-activated Smad complexes. *J Biol Chem.* 280: 21329-21336.
- Chen Y, Lebrun JJ, Vale W. 1996. Regulation of transforming growth factor beta- and activin-induced transcription by mammalian Mad proteins. *Proc Natl Acad Sci U S A.* 93: 12992-12997.
- Chen Y, Lui VC, Rooijen NV, Tam PK. 2004. Depletion of intestinal resident macrophages prevents ischaemia reperfusion injury in gut. *Gut.* 53: 1772-1780.
- Chen Y, Ye J, Cao L, Zhang Y, Xia W, Zhu D. 2010. Myostatin regulates glucose metabolism via the AMP-activated protein kinase pathway in skeletal muscle cells. *Int J Biochem Cell Biol.* 42: 2072-2081.
- Chiu LL, Chou SW, Cho YM, Ho HY, Ivy JL, Hunt D, Wang PS, Kuo CH. 2004. Effect of prolonged intermittent hypoxia and exercise training on glucose tolerance and muscle GLUT4 protein expression in rats. *J Biomed Sci.* 11: 838-846.
- Cho YS, Lee SY, Kim KH, Kim SK, Kim DS, Nam YK. 2009. Gene structure and differential modulation of multiple rockbream (*Oplegnathus fasciatus*) hepcidin isoforms resulting from different biological stimulations. *Dev Comp Immunol.* 33: 46-58.
- Chou ST, Weiss MJ. 2007. Diseased red blood cells topple iron balance. *Nat Med.* 13: 1020-1021.
- Christensen CU. 1974. Adaptations in the water economy of some anuran amphibian. *Comp. Biochem Physiol A.* 47: 1035-1049.
- Churchill TA, Storey KB. 1993. Dehydration tolerance in wood frogs: a new perspective on the development of amphibian freeze tolerance. *Am J Physiol.* 265: R1324-R1332.
- Churchill TA, Storey KB. 1994a. Effects of dehydration on organ metabolism in the frog *Pseudacris crucifer*: hyperglycemic responses to dehydration mimic freezing-induced cryoprotectant production. *J Comp Physiol B.* 164: 492-498.
- Churchill TA, Storey KB. 1994b. Metabolic responses to dehydration by liver of the wood frog *Rana sylvatica*. *Can J Zool.* 72: 1420-1425.
- Churchill TA, Storey KB. 1995. Metabolic effects of dehydration on an aquatic frog, *Rana pipiens*. *J Exp Biol.* 198: 147-154.
- Clarke BT. 1997. The natural history of amphibian skin secretions, their normal functioning and potential medical applications. *Biol Rev Camb Philos Soc.* 72: 365-379.

- Clark DP, Durell S, Maloy WL, Zasloff M. 1994. Ranalexin. A novel antimicrobial peptide from bullfrog (*Rana catesbeiana*) skin, structurally related to the bacterial antibiotic, polymyxin. *J Biol Chem.* 269: 10849-10855.
- Clark RJ, Tan CC, Preza GC, Nemeth E, Ganz T, Craik DJ. 2011. Understanding the structure/activity relationships of the iron regulatory peptide hepcidin. *Chem Biol.* 18: 336-343.
- Collins JP, Lewis MA. 1979. Overwintering tadpoles and breeding season variation in the *Rana pipiens* complex in Arizona. *Southwest Nat.* 14: 371-373.
- Conlon JM, Iwamuro S, King JD. 2009. Dermal cytolytic peptides and the system of innate immunity in anurans. *Ann N Y Acad Sci.* 1163: 75-82.
- Conlon JM, Kolodziejek J, Nowotny N. 2004. Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. *Biochim Biophys Acta.* 1696: 1-14.
- Cooper EL, Wright RK, Klempau AE, Smith CT. 1992. Hibernation alters the frog's immune system. *Cryobiology.* 29: 616-631.
- Costanzo JP, Bayuk JM, Lee RE Jr. 1999. Inoculative freezing by environmental ice nuclei in the freeze-tolerant wood frog, *Rana sylvatica*. *J Exp Zool.* 284: 7-14.
- Costanzo JP, Lee RE Jr. 2005. Cryoprotection by urea in a terrestrially hibernating frog. *J Exp Biol.* 208: 4079-4089.
- Davidson B, Swalla BJ. 2002. A molecular analysis of ascidian metamorphosis reveals activation of an innate immune response. *Development.* 129: 4739-4751.
- Davies DG. 1989. Distribution of systemic blood flow during anoxia in the turtle, *Chrysemys scripta*. *Respir. Physiol.* 78: 383-390.
- Day NK, Good RA, Finstad J, Johannsen R, Pickering RJ, Gewurz H. 1970. Interactions between endotoxic lipopolysaccharides and the complement system in the sera of lower vertebrates. *Proc Soc Exp Biol Med.* 133: 1397-1401.
- De Croos JNA, McNally JD, Palmieri F, Storey KB. 2004. Up-regulation of the mitochondrial phosphate carrier during freezing in the wood frog *Rana sylvatica*: potential roles of transporters in freeze tolerance. *J Bioenerg Biomemb.* 36: 229-239.
- Denissova NG, Pouponnot C, Long J, He D, Liu F. 2000. Transforming growth factor beta-inducible independent binding of SMAD to the Smad7 promoter. *Proc Natl Acad Sci U S A.* 97: 6397-6402.
- D'eona ME, Boutilier RG, Toews DP. 1978. Anaerobic contributions during progressive hypoxia in the toad *Bufo marinus*. 60: 7-10. *Comp Biochem Physiol A.*

- Derting TL, Compton S. 2003. Immune response, not immune maintenance, is energetically costly in wild white-footed mice (*Peromyscus leucopus*). *Physiol Biochem Zool.* 76: 744-752.
- Derynck R, Zhang YE. 1996. Intracellular signalling: the MAD way to do it. *Curr Biol.* 6: 1226-1229.
- Derynck R, Zhang YE. 2003. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature.* 425: 577-584.
- Diamond G, Beckloff N, Weinberg A, Kisich KO. 2009. The roles of antimicrobial peptides in innate host defense. *Curr Pharm Des.* 15: 2377-2392.
- DiAngelo JR, Bland ML, Bambina S, Cherry S, Birnbaum MJ. 2009. The immune response attenuates growth and nutrient storage in *Drosophila* by reducing insulin signaling. *Proc Natl Acad Sci U S A.* 106: 20853-20858.
- Donohoe PH, Boutilier RG. 1998. The protective effects of metabolic rate depression in hypoxic cold submerged frogs. *Respir Physiol.* 111: 325-336.
- Duellman WE, Trueb L. 1986. *Biology of Amphibians*. McGraw-Hill Publishing Co., New York.
- Feder ME, Burggren WW. 1992. *Environmental physiology of amphibians*. University of Chicago press, Ltd., Chicago.
- Finkenstedt A, Bianchi P, Theurl I, Vogel W, Witcher DR, Wroblewski VJ, Murphy AT, Zanella A, Zoller H. 2009. Regulation of iron metabolism through GDF15 and hepcidin in pyruvate kinase deficiency. *Br J Haematol.* 144: 789-793.
- Fisher MC. 2007. Potential interactions between amphibian immunity, infectious disease and climate change. *Anim Conserv.* 10: 420-421.
- Fitts RH, Riley DR, Widrick JJ. 2000. Physiology of a microgravity environment: Microgravity and skeletal muscle. *J Appl Physiol.* 89: 823-839.
- Forbes D, Jackman M, Bishop A, Thomas M, Kambadur R, Sharma M. 2006. Myostatin auto-regulates its expression by feedback loop through Smad7 dependent mechanism. *J Cell Physiol.* 206: 264-272.
- Funkhouser D, Goldstein L. 1973. Urea response to pure osmotic stress in the aquatic toad *Xenopus laevis*. *Am J Physiol.* 224: 524-529.
- Garcia-Roves PM, Jones TE, Otani K, Han DH, Holloszy JO. 2005. Calcineurin does not mediate exercise-induced increase in muscle GLUT4. *Diabetes.* 54: 624-628.
- Gibert Y, Lattanzi VJ, Zhen AW, Vedder L, Brunet F, Faasse SA, Babitt JL, Lin HY, Hammerschmidt M, Fraenkel PG. 2011. BMP signaling modulates hepcidin expression in zebrafish embryos independent of hemojuvelin. *PLoS One.* 6: e14553.

Gilson H, Schakman O, Combaret L, Lause P, Grobet L, Attaix D, Ketelslegers JM, Thissen JP. 2006. Myostatin gene deletion prevents glucocorticoid-induced muscle atrophy. *Endocrinology*. 148: 452-60.

Glass D, Roubenoff R. 2010. Recent advances in the biology and therapy of muscle wasting. *Ann N Y Acad Sci*. 1211: 25-36.

Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*. 16: 183-190.

Gosselin LE, McCormick KM. 2004. Targeting the immune system to improve ventilatory function in muscular dystrophy. *Med Sci Sports Exerc*. 36: 44-51.

Goto K, Kamiya Y, Imamura T, Miyazono K, Miyazawa K. 2007. Selective inhibitory effects of Smad6 on bone morphogenetic protein type I receptors. *J Biol Chem*. 282: 20603-20611.

Gottlieb G, Jackson DC. 1976. Importance of pulmonary ventilation in respiratory control in the bullfrog. *Am J Physiol*. 230: 608-613.

Greenwald L. 1971. Sodium balance in the leopard frog (*Rana pipiens*). *Physiol. Zool*. 44: 149-161.

Groisman EA, Parra-Lopez C, Salcedo M, Lipps CJ, Heffron F. 1992. Resistance to host antimicrobial peptides is necessary for *Salmonella* virulence. *Proc Natl Acad Sci U S A*. 89: 11939-11943.

Grundy JE, Storey KB. 1998. Antioxidant defences and lipid peroxidation damage in estivating toads, *Scaphiopus couchii*. *J Comp Physiol B*. 169: 132-142.

Guo T, Jou W, Chanturiya T, Portas J, Gavrilova O, McPherron AC. 2009. Myostatin inhibition in muscle, but not adipose tissue, decreases fat mass and improves insulin sensitivity. *PLoS One*. 4: e4937.

Gupta S, Seth A, Davis RJ. 1993. Transactivation of gene expression by Myc is inhibited by mutation at the phosphorylation sites Thr-58 and Ser-62. *Proc Natl Acad Sci U S A*. 90: 3216-3220.

Hata A, Lagna G, Massagué J, Hemmati-Brivanlou A. 1998. Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes Dev*. 12: 186-197.

Hatfield S, Belikoff B, Lukashev D, Sitkovsky M, Ohta A. 2009. The antihypoxia-adenosinergic pathogenesis as a result of collateral damage by overactive immune cells. *J Leukoc Biol*. 86: 545-548.

Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA Jr, Wrana JL, Falb D. 1997. The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell*. 89: 1165-1173.

Hayes TB. 1997. Steroids as potential modulators of thyroid hormone activity in anuran metamorphosis. *Amer Zool*. 37: 184-194.

He A, Liu X, Liu L, Chang Y, Fang F. 2007. How many signals impinge on GLUT4 activation by insulin? *Cell Signal*. 19: 1-7.

Hedger MP, Winnall WR, Phillips DJ, de Kretser DM. 2011. The regulation and functions of activin and follistatin in inflammation and immunity. *Vitam Horm*. 85: 255-297.

Hedrick MS, Drewes RC, Hillman SS, Withers PC. 2007. Lung ventilation contributes to vertical lymph movement in anurans. *J Exp Biol*. 210: 3940-3945.

Hermes-Lima M, Storey JM, Storey KB. 1998. Antioxidant defenses and metabolic depression. The hypothesis of preparation for oxidative stress in land snails. *Comp Biochem Physiol B Biochem Mol Biol*. 120: 437-48.

Hermes-Lima M, Storey JM, Storey KB. 2001. Antioxidant defences and animal adaptation to oxygen availability during environmental stress. In: Storey KB, Storey JM (eds) *Cell and molecular responses to stress*, vol 2. Elsevier Science. Amsterdam, pp 263-287.

Hermes-Lima M, Zenteno-Savín T. 2002. Animal response to drastic changes in oxygen availability and physiological oxidative stress. *Comp Biochem Physiol C Toxicol Pharmacol*. 133: 537-56.

Hillman S. 1978. The roles of oxygen delivery and electrolyte levels in the dehydrational death of *Xenopus laevis*. *J Comp Physiol*. 128: 169-175.

Hillman S. 1980. Physiological correlates of differential dehydration tolerance in anuran amphibians. *Copeia* 1980: 125-129.

Hillman S. 1987. Dehydrational effects on cardiovascular and metabolic capacities in anuran amphibians. *Physiol Zool*. 60:608-613.

Hirono I, Hwang JY, Ono Y, Kurobe T, Ohira T, Nozaki R, Aoki T. 2005. Two different types of hepcidins from the Japanese flounder *Paralichthys olivaceus*. *FEBS J*. 272: 5257-5264.

Holden CP, Storey KB. 1997. Second messenger and cAMP-dependent protein kinase responses to dehydration and anoxia stresses in frogs. *J Comp Physiol B*. 167: 305-312.

Hu X, Camus AC, Aono S, Morrison EE, Dennis J, Nusbaum KE, Judd RL, Shi J. 2006. Channel catfish hepcidin expression in infection and anemia. *Comp Immunol Microbiol Infect Dis*. 30: 55-69.

- Hu X, Ward C, Aono S, Lan L, Dykstra C, Kemppainen RJ, Morrison EE, Shi J. 2008. Comparative analysis of *Xenopus tropicalis* hepcidin I and hepcidin II genes. *Gene*. 426: 91-96.
- Huang H, Constante M, Layoun A, Santos MM. 2009. Contribution of STAT3 and SMAD4 pathways to the regulation of hepcidin by opposing stimuli. *Blood*. 113: 3593-3599.
- Huang PH, Chen JY, Kuo CM. 2007. Three different hepcidins from tilapia, *Oreochromis mossambicus*: analysis of their expressions and biological functions. *Mol Immunol* 44: 1922-1934.
- Hudson N, Franklin CE. 2002a. Effect of aestivation on muscle characteristics and locomotor performance in the green-striped burrowing frog *Cyclorana alboguttata*. *J Comp Physiol B*. 172: 177-182.
- Hudson NJ, Franklin CE. 2002b. Maintaining muscle mass during extended disuse: aestivating frogs as a model species. *J Exp Biol*. 205: 2297-2303.
- Inoki K, Li Y, Zhu T, Wu J, Guan KL. 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol*. 4: 648-657.
- Ishizuya-Oka A, Hasebe T, Shi YB. 2010. Apoptosis in amphibian organs during metamorphosis. *15*: 350-64.
- Jackman RW, Kandarian SC. 2004. The molecular basis of skeletal muscle atrophy. *Am J Physiol Cell Physiol*. 287 :C834-C843.
- James RS. 2010. Effects of aestivation on skeletal muscle performance. *Prog Mol Subcell Biol*. 49: 171-81.
- Janknecht R, Wells NJ, Hunter T. 1998. TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. *Genes Dev*. 12: 2114-2119.
- Janssens PA, Cohen PP. 1968. Biosynthesis of urea in the estivating African lungfish and in *Xenopus laevis* under conditions of water-shortage. *Comp Biochem Physiol*. 24: 887-898.
- Joanisse DR, Storey KB. 1996. Oxidative damage and antioxidants in *Rana sylvatica*, the freeze-tolerant wood frog. *Am J Physiol*. 271: R545-553.
- Jokumsen A, Weber RE. 1980. Haemoglobin-oxygen binding properties in the blood of *Xenopus laevis*, with special reference to the influences of estivation and of temperature and salinity acclimation. *J Exp Biol*. 86: 19-37.
- Jones JM, Gamperl AK, Farrell AP, Toews DP. 1997. Direct measurement of flow from the posterior lymph hearts of hydrated and dehydrated toads (*Bufo marinus*). *J Exp Biol*. 200: 1695-1702.

- Jozkowicz A, Plytycz B. 1998. Temperature but not season affects the transplantation immunity of anuran amphibians. *J Exp Zool.* 281: 58-64.
- Kandarian SC, Jackman RW. 2006. Intracellular signaling during skeletal muscle atrophy. *Muscle Nerve.* 33: 155-165.
- Kielgast J, Rödder D, Veith M, Lötters S. 2010. Widespread occurrence of the amphibian chytrid fungus in Kenya. *Anim Conserv.* 13: 36-43.
- Kim YO, Hong S, Nam BH, Lee JH, Kim KK, Lee SJ. 2005. Molecular cloning and expression analysis of two hepcidin genes from olive flounder *Paralichthys olivaceus*. *Biosci Biotechnol Biochem.* 69: 1411-1414.
- Kloos DU, Choi C, Wingender E. 2002. The TGF-beta – Smad network: introducing bioinformatic tools. *Trends Genet.* 18: 96-103
- Kollias HD, McDermott JC. 2008. Transforming growth factor-beta and myostatin signaling in skeletal muscle. *J Appl Physiol.* 104: 579-587.
- Kong S, Blennerhassett LR, McCauley RD, Hall JC. 1998. Ischaemia-reperfusion injury to the intestine. *Aust Nz J Surg.* 68: 554-561.
- Kowalik TF. 2002. Smad about E2F. TGFbeta repression of c-Myc via a Smad3/E2F/p107 complex. *Mol Cell.* 10: 7-8.
- Lakhal S, Talbot NP, Crosby A, Stoepker C, Townsend AR, Robbins PA, Pugh CW, Ratcliffe PJ, Mole DR. 2009. Regulation of growth differentiation factor 15 expression by intracellular iron. *Blood.* 113: 1555-1563.
- Lalani R, Bhasin S, Byhower F, Tarnuzzer R, Grant M, Shen R, Asa S, Ezzat S, Gonzalez-Cadavid NF. 2000. Myostatin and insulin-like growth factor-I and -II expression in the muscle of rats exposed to the microgravity environment of the NeuroLab space shuttle flight. *J Endocrinol.* 167: 417-428.
- Layne JR. 1995. Crystallization temperatures of frogs and their individual organs. *J Herpetol.* 29: 296-298.
- Layne JR, Lee RE. 1987. Freeze tolerance and the dynamics of ice formation in wood frogs (*Rana sylvatica*) from southern Ohio. *Can J Zool.* 65: 2062-2065.
- Layne JR, Lee RE, Heil TL. 1989. Freezing induced changes in the heart rate of wood frogs (*Rana sylvatica*). *Am J Physiol.* 257: R1046–R1049.
- Layne JR, Lee RE, Huang JL. 1990. Inoculation triggers freezing at high subzero temperatures in a freeze-tolerant frog (*Rana sylvatica*) and insect (*Eurosta solidaginis*). *Can J Zool.* 68: 506–510.
- Le NT, Richardson DR. 2002. The role of iron in cell cycle progression and the proliferation of neoplastic cells. *Biochim Biophys Acta.* 1603: 31-46.

Lee MR, Lee RE Jr, Strong-Gunderson JM, Minges SR. 1995. Isolation of ice nucleating active bacteria from the freeze-tolerant frog *Rana sylvatica*. *Cryobiology*. 32: 358-365.

Lee SJ, McPherron AC. 2001. Regulation of myostatin activity and muscle growth. *Proc Natl Acad Sci U*. 98: 9306-9311.

Light L. 1991. Habitat selection of *Rana pipiens* and *Rana sylvatica* during exposure to warm and cold temperatures. *Am Midl Nat*. 125: 259-268.

Lips KR, Middleton EM, Orchard SA, Rand AS. 2001. Amphibian declines and environmental change: use of remote-sensing data to identify environmental correlates. *Conserv Biol*. 15: 903-913.

Liu F, Hata A, Baker JC, Doody J, Cárcamo J, Harland RM, Massagué J. 1996. A human Mad protein acting as a BMP-regulated transcriptional activator. *Nature*. 381: 620-623.

Mahida YR, Rose F, Chan WC. 1997. Antimicrobial peptides in the gastrointestinal tract. *Gut*. 40: 161-163.

Malik AI. 2009. Cellular adaptation to dehydration stress: Molecular adaptations for dehydration tolerance in the African clawed frog, *Xenopus laevis*. Ph.D. thesis, Department of Biology, Carleton University.

Malik AI, Storey KB. 2009. Activation of extracellular signal-regulated kinases during dehydration in the African clawed frog, *Xenopus laevis*. *J Exp Biol*. 212: 2595-2603.

Malik AI, Storey KB. 2011. Transcriptional regulation of antioxidant enzymes by FoxO1 under dehydration stress. *Gene*. [Epub ahead of print]

Malvin GM, Hood L, Sanchez M. 1992. Regulation of blood flow through ventral pelvic skin by environmental water and NaCl in the toad *Bufo woodhousei*. *Physiol Zool*. 65: 540-553.

Malyszko J. 2009. Hemojuvelin: the hepcidin story continues. *Kidney Blood Press Res*. 32: 71-76.

Mangoni ML, Miele R, Renda TG, Barra D, Simmaco M. 2001. The synthesis of antimicrobial peptides in the skin of *Rana esculenta* is stimulated by microorganisms. *FASEB J*. 15: 1431-1432.

Maniero GD, Carey C. 1997. Changes in selected aspects of immune function in the leopard frog, *Rana pipiens*, associated with exposure to cold. *J Comp Physiol B*. 167: 256-263.

Mantle BL, Hudson NJ, Harper GS, Cramp RL, Franklin CE. 2009. Skeletal muscle atrophy occurs slowly and selectively during prolonged aestivation in *Cyclorana alboguttata* (Gunther 1867). *J Exp Biol*. 212: 3664-3672.

- Marxsen J, Zoppini A, Wilczek S. 2010. Microbial communities in streambed sediments recovering from desiccation. *FEMS Microbiol Ecol.* 71: 374-86.
- Massagué J, Wotton D. 2000. Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J.* 19: 1745-1754.
- Matutte B, Storey KB, Knoop FC, Conlon JM. 2000. Induction of synthesis of an antimicrobial peptide in the skin of the freeze-tolerant frog, *Rana sylvatica*, in response to environmental stimuli. *FEBS Letters.* 483: 135-138.
- Mbachu EM, Klein LV, Rubin BB, Lindsay TF. 2004. A monoclonal antibody against cytokine-induced neutrophil chemoattractant attenuates injury in the small intestine in a model of ruptured abdominal aortic aneurysm. *J Vasc Surg.* 39: 1104-1111.
- McBean RL, Goldenstein L. 1970a. Renal function during osmotic stress in the aquatic toad *Xenopus laevis*. *Am J Physiol.* 219: 1115-1123.
- McBean RL, Goldenstein L. 1970b. Accelerated synthesis of urea in *Xenopus laevis* during osmotic stress. *Am J Physiol.* 219: 1124-1130.
- McDade TW, Reyes-García V, Tanner S, Huanca T, Leonard WR. 2008. Maintenance versus growth: investigating the costs of immune activation among children in lowland Bolivia. *Am J Phys Anthropol.* 136: 478-484.
- McFarlane C, Plummer E, Thomas M, Hennebry A, Ashby M, Ling N, Smith H, Sharma M, Kambadur R. 2006. Myostatin induces cachexia by activating the ubiquitin proteolytic system through an NF-kappaB-independent, FoxO1-dependent mechanism. *J Cell Physiol.* 209: 501-514.
- McGaw IJ. 2005. Does feeding limits cardiovascular modulation in the Dungeness crab, *Cancer magister* during hypoxia? *J Exp Biol.* 208 :83-91.
- McMullen DC, Hallenbeck JM. 2010. Regulation of Akt during torpor in the hibernating ground squirrel, *Ictidomys tridecemlineatus*. *J Comp Physiol B.* 180: 927-934.
- McNally JD, Sturgeon CM, Storey KB. 2003. Freeze-induced expression of a novel gene, *fr47*, in the liver of the freeze-tolerant wood frog, *Rana sylvatica*. *Biochim Biophys Acta.* 27: 183-191.
- McNally JD, Wu SB, Sturgeon CM, Storey KB. 2002. Identification and characterization of a novel freezing inducible gene, *li16*, in the wood frog *Rana sylvatica*. *FASEB J.* 16: 902-904.
- McNamara JM, Buchanan KL. 2005. Stress, resource allocation and mortality. *Behav Ecol.* 16: 1008-1017.
- McPherron AC, Lee SJ. 1997. Double muscling in cattle due to mutations in the myostatin gene. *Proc Natl Acad Sci U S A.* 94: 12457-12461.

- Mehal WZ, Azzaroli F, Crispe IN. 2001. Immunology of the healthy liver: old questions and new insights. *Gastroenterology*. 120: 250-260.
- Mîinea CP, Sano H, Kane S, Sano E, Fukuda M, Peränen J, Lane WS, Lienhard GE. 2005. AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. *Biochem J*. 391: 87-93.
- Miodoński AJ, Bigaj J, Mika J, Plytycz B. 1996. Season-specific thymic architecture in the frog, *Rana temporaria*: SEM studies. *Dev Comp Immunol*. 20: 129-137.
- Miyazono K. 2000. TGF- β signalling by Smad proteins. *Cytokine Growth F R*. 11: 15-32.
- Moore KS, Bevins CL, Brasseur MM, Tomassini N, Turner K, Eck H, Zasloff M. 1991. Antimicrobial peptides in the stomach of *Xenopus laevis*. *J Biol Chem*. 266: 19851-19857.
- Moret Y. 2003. Explaining variable costs of the immune response: selection for specific versus non-specific immunity and facultative life history change. *Oikos*. 102: 213-216.
- Mosher DS, Quignon P, Bustamante CD, Sutter NB, Mellersh CS, Parker HG, Ostrander EA. 2007. A mutation in the myostatin gene increases muscle mass and enhances racing performance in heterozygote dogs. *PLoS Genet*. 3: e79.
- Muir TJ, Costanzo JP, Lee RE Jr. 2007. Osmotic and metabolic responses to dehydration and urea-loading in a dormant, terrestrially hibernating frog. *J Comp Physiol B*. 177: 917-926.
- Nagarajan RP, Zhang J, Li W, Chen Y. 1999. Regulation of Smad7 promoter by direct association with Smad3 and Smad4. *J Biol Chem*. 274: 33412-33418.
- Nakao A, Afrakhte M, Moren A, Nakayama T, Christian J, Heuchel R, Itoh S, Kawabata M, Heldin NE, Heldin CH, Ten Dijke P. 1997. Identification of Smad7, a TGF β -inducible antagonist of TGF- β signalling. *Nature*. 389: 631-635.
- Nakayama T, Berg LK, Christian JL. 2001. Dissection of inhibitory Smad proteins: both N- and C-terminal domains are necessary for full activities of *Xenopus* Smad6 and Smad7. *Mech Dev*. 100: 251-262.
- Nakazawa H, Yukuhiro F, Furukawa S, Sagisaka A, Tanaka H, Ishibashi J, Yamakawa M. 2003. Spontaneous synthesis of an antibacterial peptide linked to ecdysis in Lepidopteran insects. *J Insect Biotechnol Sericol*. 72: 133-137.
- Nemeth E, Preza GC, Jung CL, Kaplan J, Waring AJ, Ganz T. 2005. The N-terminus of hepcidin is essential for its interaction with ferroportin: structure-function study. *Blood*. 107: 328-333.

- Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, Ganz T. 2004. IL-6 mediates hypoferrremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest.* 113: 1271-1276.
- Neviere RR, Li FY, Singh T, Myers ML, Sibbald W. 2000. Endotoxin induces a dose-dependent myocardial cross-tolerance to ischemia-reperfusion injury. *Crit Care Med.* 28: 1439-44.
- Nicolás FJ, De Bosscher K, Schmierer B, Hill CS. 2004. Analysis of Smad nucleocytoplasmic shuttling in living cells. *J Cell Sci.* 117: 4113-4125.
- Nieder Korn JY, Stern ME, Pflugfelder SC, De Paiva CS, Corrales RM, Gao J, Siemasko K. 2006. Desiccating stress induces T cell-mediated Sjögren's syndrome-like lacrimal keratoconjunctivitis. *J Immunol.* 176: 3950-3957.
- Ohmura H, Wakahara M. 1998. Transformation of skin from larval to adult types in normally metamorphosing and metamorphosis-arrested salamander, *Hynobius retardatus*. *Differentiation.* 63: 238-246.
- Ohnuma A, Conlon JM, Iwamuro S. 2010. Differential expression of genes encoding preprobrevinin-2, preproalustrin-2, and preproanatrutin-2 in developing larvae and adult tissues of the mountain brown frog *Rana ornativentris*. *Comp Biochem Physiol C Toxicol Pharmacol.* 151: 122-130.
- Ohnuma A, Conlon JM, Kawasaki H, Iwamuro S. 2006. Developmental and triiodothyronine-induced expression of genes encoding preprotemporins in the skin of Tago's brown frog *Ranatagoi*. *Gen Comp Endocrinol.* 146: 242-250.
- O'Neill ED, Wilding JP, Kahn CR, Van Remmen H, McArdle A, Jackson MJ, Close GL. 2010. Absence of insulin signalling in skeletal muscle is associated with reduced muscle mass and function: evidence for decreased protein synthesis and not increased degradation. *Age (Dordr).* 32: 209-222.
- Padhi A, Verghese B. 2007. Evidence for positive Darwinian selection on the hepcidin gene of Perciform and Pleuronectiform fishes. *Mol Divers.* 11: 119-130.
- Park CH, Valore EV, Waring AJ, Ganz T. 2001. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem.* 276: 7806-7810.
- Pasquair LD, Haimovich J. 1976. The antibody response during amphibian ontogeny. *Immunogenetics.* 3: 381-391.
- Pinder AW, Burggren WW. 1986. Ventilation and partitioning of oxygen uptake in the frog *Rana pipiens*: effects of hypoxia and activity. *J Exp Biol.* 126: 453-468.
- Pinder AW, Storey KB, Ultsch GR. 1992. Estivation and hibernation. In *Environmental Physiology of the Amphibians* (ed. M. E. Feder and W. W. Burggren), pp. 250-274. Chicago: University of Chicago Press.

- Pounds JA, Fogden PL, Campbell JH. 1999. Biological response to climate change on a tropical mountain. *Nature*. 398: 611-615.
- Powers SK, Kavazis AN, McClung JM. 2007. Oxidative stress and disuse muscle atrophy. *J Appl Physiol*. 102: 2389-2397.
- Quinn ZA, Yang CC, Wrana JL, McDermott JC. 2001. Smad proteins function as co-modulators for MEF2 transcriptional regulatory proteins. *Nucleic Acids Res*. 29: 732-742.
- Raffel TR, Rohr JR, Kiesecker JM, Hudson PJ. 2006. Negative effects of changing temperature on amphibian immunity under field conditions. *Funct Ecol*. 20: 819-828.
- Reardon K A, Davis J, Kapsa RM, Choong P, Byrne E. 2001. Myostatin, insulin-like growth factor-1, and leukemia inhibitory factor mRNAs are upregulated in chronic human disuse muscle atrophy. *Muscle Nerve*. 24: 893-899.
- Redan JA, Rush BF, Lysz TW, Smith S, Machiedo GW. 1990. Organ distribution of gut-derived bacteria caused by bowel manipulation or ischemia. *Am J Surg*. 159: 85-99.
- Regard E, Taurog A, Nakashima T. 1978. Plasma thyroxine and triiodothyronine levels in spontaneously metamorphosing *Rana catesbeiana* tadpoles and in adult anuran amphibians. *Endocrinology*. 102: 674-684.
- Reguly T, Wrana JL. 2003. In or out? The dynamics of Smad nucleocytoplasmic shuttling. *Trends Cell Biol*. 13: 216-220.
- Reilly DS, Tomassini N, Bevins CL, Zasloff M. 1994a. A Paneth cell analogue in *Xenopus* small intestine expresses antimicrobial peptide genes: conservation of an intestinal host-defense system. *J Histochem Cytochem*. 42: 697-704.
- Reilly DS, Tomassini N, Zasloff M. 1994b. Expression of magainin antimicrobial peptide genes in the developing granular glands of *Xenopus* skin and induction by thyroid hormone. *Dev Biol*. 162: 123-133.
- Reisz-Porszasz S, Bhasin S, Artaza JN, Shen R, Sinha-Hikim I, Hogue A, Fielder T J, Gonzalez-Cadavid N F. 2003. Lower skeletal muscle mass in male transgenic mice with muscle-specific overexpression of myostatin. *Am J Physiol*. 285: E876-E888.
- Richards-Zawacki CL. 2010. Thermoregulatory behaviour affects prevalence of chytrid fungal infection in a wild population of Panamanian golden frogs. *Proc Biol Sci*. 277: 519-28.
- Rinaldi AC. 2002. Antimicrobial peptides from amphibian skin: an expanding scenario. *Curr Opin Chem Biol*. 6: 799-804.
- Robert J, Ohta Y. 2009. Comparative and developmental study of the immune system in *Xenopus*. *Dev Dyn*. 238: 1249-1270.

Rodrigues PN, Vázquez-Dorado S, Neves JV, Wilson JM. 2006. Dual function of fish hepcidin: response to experimental iron overload and bacterial infection in sea bass (*Dicentrarchus labrax*). *Dev Comp Immunol*. 30: 1156-1167.

Rohr JR, Raffel TR. 2010. Linking global climate and temperature variability to widespread amphibian declines putatively caused by disease. *Proc Natl Acad Sci USA*. 107: 8269–8274.

Rollins-Smith LA. 1998. Metamorphosis and the amphibian immune system. *Immunol Rev*. 166: 221-230.

Rollins-Smith LA, Barker KS, Davis AT. 1997. Involvement of glucocorticoids in the reorganization of the amphibian immune system at metamorphosis. *Dev Immunol*. 5: 145-52.

Rollins-Smith LA, Conlon JM. 2005. Antimicrobial peptide defenses against chytridiomycosis, an emerging infectious disease of amphibian populations. *Dev Comp Immunol*. 29: 589-598.

Rollins-Smith LA, Reinert LK, O'Leary CJ, Houston LE, Woodhams DC. 2005. Antimicrobial peptide defenses in amphibian skin. *Integr Comp Biol*. 45: 137-142.

Romspert AP. 1975. Osmoregulation of the African clawed frog, *Xenopus laevis*, in hypersaline media. *Comp Biochem Physiol A*. 54: 207-210

Ross S, Hill CS. 2008. How the Smads regulate transcription. *Int J Biochem Cell Biol*. 40: 383-408.

Rotstein OD. 2000. Pathogenesis of multiple organ dysfunction syndrome: gut origin, protection, and decontamination. *Surg Infect (Larchmt)*. 1: 217-225.

Ruben LN, Edwards BF, Rising J. 1977. Temperature and variation of the function of complement and antibody of Amphibia. *Experientia*. 33: 1522-1524.

Rubinsky B, Wong STS, Hong JS, Gilbert J, Roos M, Storey KB. 1994. ¹H magnetic resonance imaging of freezing and thawing in freeze-tolerant frogs. *Am J Physiol*. 266: R1771–R1777.

Sakamori R, Takehara T, Tatsumi T, Shigekawa M, Hikita H, Hiramatsu N, Kanto T, Hayashi N. 2010. STAT3 signaling within hepatocytes is required for anemia of inflammation in vivo. *J Gastroenterol*. 45: 244-248.

Sartori R, Milan G, Patron M, Mammucari C, Blaauw B, Abraham R, Sandri M. 2009. Smad2 and 3 transcription factors control muscle mass in adulthood. *Am J Physiol Cell Physiol*. 296: C1248-257.

Schadich E, Cole AL, Squire M, Mason D. 2010. Skin peptides of different life stages of Ewing's tree frog. *Exp Zool A Ecol Genet Physiol*. 313: 532-537.

- Schimel J, Balser TC, Wallenstein M. 2007. Microbial stress-response physiology and its implications for ecosystem function. *Ecology*. 88: 1386-1994.
- Schmid WD. 1982. Survival of frogs in low temperature. *Science*. 215: 697-698.
- Schreiber AM, Brown DD. 2003. Tadpole skin dies autonomously in response to thyroid hormone at metamorphosis. *Proc Natl Acad Sci U S A*. 18: 1769-1774.
- Sears R, Nuckolls F, Haura E, Taya Y, Tamai K, Nevins JR. 2000. Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. *Genes Dev*. 14: 2501-2514.
- Sekelsky JJ, Newfeld SJ, Raftery LA, Chartoff EH, Gelbart WM. 1995. Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in *Drosophila melanogaster*. *Genetics*. 139: 1347-1358.
- Serrano AL, Muñoz-Cánoves P. 2010. Regulation and dysregulation of fibrosis in skeletal muscle. *Exp Cell Res*. 316: 3050-3058.
- Seymour RS. 1973. Energy metabolism of dormant spadefoot toads (*Scaphiopus*). *Copeia*. 1973: 435-445.
- Shao C, Liu M, Wu X, Ding F. 2007. Time-dependent expression of myostatin RNA transcript and protein in gastrocnemius muscle of mice after sciatic nerve resection. *Microsurgery*. 27: 487-4793.
- Shavlakadze T, Grounds M. 2006. Of bears, frogs, meat, mice and men: complexity of factors affecting skeletal muscle mass and fat. *Bioessays*. 28: 994-1009.
- Sheik CS, Beasley WH, Elshahed MS, Zhou X, Luo Y, Krumholz LR. 2011. Effect of warming and drought on grassland microbial communities. *ISME J*. [Epub ahead of print]
- Shi J, Camus AC. 2006. Hepcidins in amphibians and fishes: Antimicrobial peptides or iron-regulatory hormones? *Dev Comp Immunol*. 30: 746-755.
- Shoemaker VH. 1992. 'Exchange of water, ions, and respiratory gases in terrestrial amphibians', in Feder, M.E. and Burggren, W.W. (eds) *Environmental Physiology of the Amphibians*, Chicago: University of Chicago Press, pp. 125–159.
- Shoemaker VH, Nagy KA. 1977. Osmoregulation in amphibians and reptiles. *Annu Rev Physiol*. 39: 449-471.
- Simmaco M, Mangoni ML, Boman A, Barra D, Boman HG. 1998. Experimental infections of *Rana esculenta* with *Aeromonas hydrophila*: a molecular mechanism for the control of the normal flora. *Scand J Immunol*. 48: 357-363.
- Simons RK, Maier RV, Lennard ES. 1987. Neutrophil function in a rat model of endotoxin-induced lung injury. *Arch Surg*. 122: 197-203.

Smith VDE, Jackson CM. 1931. The changes during dessication and rehydration in the body and organs of the leopard frog (*Rana pipiens*). Biol Bull 60: 80-93.

Sodhi NS, Bickford D, Diesmos A, Lee TM, Koh LP, Brook BW, Sekercioglu CH, Bradshaw JA. 2008. Measuring the meltdown: drivers of global amphibian extinction and decline. PLoS ONE. 3: e1636.

Souchelnytskyi S, Tamaki K, Engström U, Wernstedt C, ten Dijke P, Heldin CH. 1997. Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-beta signaling. J Biol Chem. 272: 28107-28115.

Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyeva Y, Kline WO, Gonzalez M, Yancopoulos GD, Glass DJ. 2004. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol Cell. 14: 395-403.

Storey KB. 2002. Life in the slow lane: molecular mechanisms of estivation. Comp Biochem Physiol A. 133: 733-754.

Storey KB, Baust JG, Wolanczyk JP. 1992. Biochemical modification of plasma ice nucleating activity in a freeze-tolerant frog. Cryobiology. 29: 374-384.

Storey KB, Storey JM. 1986. Freeze tolerant frogs: Cryoprotectants and tissue metabolism during freeze/thaw cycles. Can J Zool. 64: 49-56.

Storey KB, Storey JM. 1992. Natural freeze tolerance in ectothermic vertebrate. Ann Rev Physiol. 54: 619-637.

Storey KB, Storey JM. 2004a. Physiology, biochemistry, and molecular biology of vertebrate freeze tolerance: the wood frog. In: Life in the Frozen State (Benson, E., Fuller, B., and Lane, N., eds.) CRC Press, Boca Raton, pp. 243-274.

Storey KB, Storey JM. 2004b. Metabolic rate depression in animals: transcriptional and translational controls. Biol Rev Camb Philos Soc. 79: 207-233.

Storey KB, Storey JM. 2007. Putting life on 'pause'--molecular regulation of hypometabolism. J Exp Biol. 210: 1700-1714.

Storey KB, Storey JM. 2010. Metabolic regulation and gene expression during estivation. In: Estivation: Molecular and Physiological Aspects (Navas, C.A. and Carvalho, J.E., eds), Progress in Molecular and Subcellular Biology, Springer, Heidelberg, Vol. 49, pp. 25-45.

Storey JM, Storey KB. 1985. Triggering of cryoprotectant synthesis by the initiation of ice nucleation in the freeze tolerant frog, *Rana sylvatica*. J Comp Physiol B. 156: 191-195.

- Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues AS, Fischman DL, Waller RW. 2004. Status and trends of amphibian declines and extinctions worldwide. *Science*. 306: 1783-1786.
- Suzuki K, Machiyama F, Nishino S, Watanabe Y, Kashiwagi K, Kashiwagi A, Yoshizato K. 2009. Molecular features of thyroid hormone-regulated skin remodeling in *Xenopus laevis* during metamorphosis. *Dev Growth Differ*. 51: 411-427.
- Takase M, Imamura T, Sampath TK, Takeda K, Ichijo H, Miyazono K, Kawabata M. 1998. Induction of Smad6 mRNA by bone morphogenetic proteins. *Biochem Biophys Res Commun*. 244: 26-29.
- Tanno T, Bhanu NV, Oneal PA, Goh SH, Staker P, Lee YT, Moroney JW, Reed CH, Luban NL, Wang RH, Eling TE, Childs R, Ganz T, Leitman SF, Fucharoen S, Miller JL. 2007. High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. *Nat Med*. 13: 1096-1101.
- Tattersall GJ, Boutilier RG. 1997. Balancing hypoxia and hypothermia in cold-submerged frogs. *J Exp Biol*. 200: 1031-1038.
- Tattersall GJ, Boutilier RG. 1999. Behavioural oxy-regulation by cold-submerged frogs in heterogeneous oxygen environments. *Can J Zoolog*. 77: 843-850.
- Tattersall GJ, Ultsch GR. 2008. Physiological ecology of aquatic overwintering in ranid frogs. *Biol Rev Camb Philos Soc*. 83: 119-140.
- Tazuke Y, Drongowski RA, Teitelbaum DH, Coran AG. 2003. The effect of hypoxia on permeability and bacterial translocation in Caco-2 adult and I-407 fetal enterocyte cell culture models. *Pediatr Surg Int*. 19: 316-20.
- Tessier SN, Storey KB. 2010. Expression of myocyte enhancer factor-2 and downstream genes in ground squirrel skeletal muscle during hibernation. *Mol Cell Biochem*. 344: 151-162.
- Tinsley RC, Kobel HR. 1996. The biology of *Xenopus*. Zoological Society of London, Clarendon Press, Oxford.
- Tobin JF, Celeste AJ. 2005. Myostatin, a negative regulator of muscle mass: implications for muscle degenerative diseases. *Curr Opin Pharmacol*. 5: 328-332.
- Topper JN, DiChiara MR, Brown JD, Williams AJ, Falb D, Collins T, Gimbrone MA Jr. 1998. CREB binding protein is a required coactivator for Smad-dependent, transforming growth factor beta transcriptional responses in endothelial cells. *Proc Natl Acad Sci USA*. 95: 9506-9511.
- Trendelenburg AU, Meyer A, Rohner D, Boyle J, Hatakeyama S, Glass DJ. 2009. Myostatin reduces Akt/TORC1/p70S6K signaling, inhibiting myoBLAST differentiation and myotube size. *Am J Physiol Cell Physiol*. 296: C1258-1270.

- Turnage RH, Guice KS, Oldham KT. 1994. Endotoxemia and Remote Organ Injury Following Intestinal Reperfusion *Journal of Surgical Research*. 56: 571-578.
- Tselepis C, Ford SJ, McKie AT, Vogel W, Zoller H, Simpson R, Diaz Castro J, Iqbal TH, Ward D. 2010. Characterization of the transition-metal-binding properties of hepcidin. *Biochem J*. 427: 289-296.
- van Dam EM, Govers R, James DE. 2005. Akt activation is required at a late stage of insulin-induced GLUT4 translocation to the plasma membrane. *Mol Endocrinol*. 19: 1067-1077.
- van der Hoven B, Nabuurs M, van Leengoed LA, Groeneveld AB, Thijs LG. 2001. Gut luminal endotoxin reduces ischemia-reperfusion injury of the small gut in germ-free pigs. *Shock*. 16: 28-32.
- Vanable JW, Jr. 1964. Granular gland development during *Xenopus laevis* metamorphosis. *Dev Biol*. 10: 331-357.
- Verga Falzacappa, Casanovas G, Hentze MW, Muckenthaler MU. 2008. A bone morphogenetic protein (BMP)-responsive element in the hepcidin promoter controls HFE2-mediated hepatic hepcidin expression and its response to IL-6 in cultured cells. *J Mol Med*. 86: 531-540.
- Wabnitz PA, Walters H, Tyler MJ, Wallace JC, Bowie JH. 1998. First record of host defence peptides in tadpoles. The magnificent tree frog *Litoria splendida*. *J Pept Res*. 52: 477-481.
- Wackerhage H, Ratkevicius A. 2008. Signal transduction pathways that regulate muscle growth. *Essays Biochem*. 44: 99-108.
- Walia B, Wang L, Merlin D, Sitaraman SV. 2003. TGF-beta down-regulates IL-6 signaling in intestinal epithelial cells: critical role of SMAD-2. *FASEB J*. 17: 2130-2132.
- Wang H, Lafdil F, Kong X, Gao B. 2011a. Signal transducer and activator of transcription 3 in liver diseases: a novel therapeutic target. *Int J Biol Sci*. 7: 536-550.
- Wang H, Lafdil F, Wang L, Park O, Yin S, Niu J, Miller AM, Sun Z, Gao B. 2011b. Hepatoprotective versus Oncogenic Functions of STAT3 in Liver Tumorigenesis. *Am J Pathol*. [Epub ahead of print].
- Wang KJ, Cai JJ, Cai L, Qu HD, Yang M, Zhang M. 2009. Cloning and expression of a hepcidin gene from a marine fish (*Pseudosciaena crocea*) and the antimicrobial activity of its synthetic peptide. *Peptides*. 30: 638-646.
- Wang RH, Li C, Xu X, Zheng Y, Xiao C, Zerfas P, Cooperman S, Eckhaus M, Rouault T, Mishra L, Deng CX. 2005. A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. *Cell Metab*. 6: 399-409.

Watt KI, Jaspers RT, Atherton P, Smith K, Rennie MJ, Ratkevicius A, Wackerhage H. 2010. SB431542 treatment promotes the hypertrophy of skeletal muscle fibers but decreases specific force. *Muscle Nerve*. 41: 624-629.

Wehling M, Cai B, Tidball JG. 2000. Modulation of myostatin expression during modified muscle use. *FASEB J*. 14: 103-110.

West NH, Burggren WW. 1984. Control of pulmonary and cutaneous blood flow in the toad, *Bufo marinus*. *Am J Physiol*. 247: R884-R894.

West TG, Boutilier RG. 1998. Metabolic suppression in anoxic frog muscle. *J Comp Physiol B*. 168: 273-280.

Whitelaw PF, Hesketh JE. 1992. Expression of c-myc and c-fos in rat skeletal muscle. Evidence for increased levels of c-myc mRNA during hypertrophy. *Biochem J*. 281: 143-147.

Wierenga AT, Schuringa JJ, Eggen BJ, Kruijer W, Vellenga E. 2002. Down regulation of IL-6-induced STAT3 tyrosine phosphorylation by TGF-beta1 is mediated by caspase-dependent and -independent processes. *Leukemia*. 16: 675-682.

Wilz M, Haldmaier G. 2000. Comparison of hibernation, estivation and daily torpor in the edible dormouse, *Glis glis*. *J Comp Physiol B*. 170: 511-521.

Winmill RE, Chen AK, Hedrick MS. 2005. Development of the respiratory response to hypoxia in the isolated brainstem of the bullfrog *Rana catesbeiana*. *J Exp Biol*. 208: 213-222.

Wójcik S, Nogalska A, Engel WK, Askanas V. 2008. Myostatin and its precursor protein are increased in the skeletal muscle of patients with Type-II muscle fibre atrophy. *Folia Morphol (Warsz)*. 67: 6-12.

Wolanczyk JP, Baust JG, Storey KB. 1990a. Seasonal ice nucleating activity in the freeze-tolerant frog, *Rana sylvatica*. *Cryo Lett*. 11: 143-150.

Wolanczyk JP, Storey KB, Baust JG. 1990b. Nucleating activity in the blood of the freeze-tolerant frog, *Rana sylvatica*. *Cryobiology*. 27: 328-335.

Wolowczuk I, Verwaerde C, Viltart O, Delanoye A, Delacre M, Pot B, Grangette C. 2008. Feeding our immune system: impact on metabolism. *Clin Dev Immunol*. 2008: 639803.

Woodhams DC, Rollins-Smith LA, Alford RA, Simon MA, Harris RN. 2007. Innate immune defenses of amphibian skin: antimicrobial peptides and more. *Anim Conserv*. 10: 425-428.

Wrighting DM, Andrews NC. 2006. Interleukin-6 induces hepcidin expression through STAT3. *Blood*. 108: 3204-3209.

- Wu KJ, Polack A, Dalla-Favera R. 1999. Coordinated regulation of iron-controlling genes, H-ferritin and IRP2, by c-MYC. *Science*. 283: 676-679.
- Wu S, De Croos JN, Storey KB. 2008. Cold acclimation-induced up-regulation of the ribosomal protein L7 gene in the freeze tolerant wood frog, *Rana sylvatica*. *Gene*. 424: 48-55.
- Wu S, Storey JM, Storey KB. 2009. Phosphoglycerate kinase 1 expression responds to freezing, anoxia, and dehydration stresses in the freeze tolerant wood frog, *Rana sylvatica*. *J Exp Zool A Ecol Genet Physiol*. 311: 57-67.
- Xu DZ, Lu Q, Kubicka R, Deitch EA. 1999. The effect of hypoxia/reoxygenation on the cellular function of intestinal epithelial cells. *J Trauma*. 46:280-285.
- Xu Q, Cheng CH, Hu P, Ye H, Chen Z, Cao L, Chen L, Shen Y, Chen L. 2008. Adaptive evolution of hepcidin genes in antarctic notothenioid fishes. *Mol Biol Evol*. 25: 1099-1112.
- Yagi K, Furuhashi M, Aoki H, Goto D, Kuwano H, Sugamura K, Miyazono K, Kato M. 2002. c-myc is a downstream target of the Smad pathway. *J Biol Chem*. 277: 854-861.
- Yang M, Wang KJ, Chen JH, Qu HD, Li SJ. 2007. Genomic organization and tissue-specific expression analysis of hepcidin genes from black porgy (*Acanthopagrus schlegelii* B). *Fish Shellfish Immunol*. 23: 1060-1071.
- Yaoita Y, Brown DD. 1990. A correlation of thyroid hormone receptor gene expression with amphibian metamorphosis. *Genes Dev*. 4: 1917-1924.
- Yeh E, Cunningham M, Arnold H, Chasse D, Monteith T, Ivaldi G, Hahn WC, Stukenberg PT, Shenolikar S, Uchida T, Counter CM, Nevins JR, Means AR, Sears R. 2004. A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. *Nat Cell Biol*. 6: 308-318.
- Yoshimura A, Muto G. 2011. TGF- β function in immune suppression. *Curr Top Microbiol Immunol*. 350: 127-47.
- Yoshizato K. 2007. Molecular mechanism and evolutionary significance of epithelial-mesenchymal interactions in the body- and tail-dependent metamorphic transformation of anuran larval skin. *Rev Cytol*. 260: 213-60.
- Zasloff M. 1992. Antibiotic peptides as mediators of innate immunity. *Curr Opin Immunol*. 4: 3-7.
- Zawel L, Dai JL, Buckhaults P, Zhou S, Kinzler KW, Vogelstein B, Kern SE. 1998. Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol Cell*. 4: 611-617.
- Zheng X, de Paiva CS, Li DQ, Farley WJ, Pflugfelder SC. 2010. Desiccating stress promotion of Th17 differentiation by ocular surface tissues through a dendritic cell-mediated pathway. *Invest Ophthalmol Vis Sci*. 6: 3083-3091.

Zhi-Yong S, Dong YL, Wang XH. 1992. Bacterial translocation and multiple system organ failure in bowel ischemia and reperfusion. *J Trauma*. 32: 148-53.

Zhu X, Hadhazy M, Wehling M, Tidball JG, McNally EM. 2000. Dominant negative myostatin produces hypertrophy without hyperplasia in muscle. *FEBS Lett*. 474: 71-75.

Zhu X, Topouzis S, Liang LF, Stotish RL. 2004. Myostatin signaling through Smad2, Smad3 and Smad4 is regulated by the inhibitory Smad7 by a negative feedback mechanism. *Cytokine*. 26: 262-272.

Zierath JR, Tsao TS, Stenbit AE, Ryder JW, Galuska D, Charron MJ. 1998. Restoration of hypoxia-stimulated glucose uptake in GLUT4-deficient muscles by muscle-specific GLUT4 transgenic complementation. *J Biol Chem*. 273: 20910-20915.