Western Graduate&PostdoctoralStudies

Western University
Scholarship@Western

Electronic Thesis and Dissertation Repository

9-12-2018 3:00 PM

Mechanisms Underlying Freeze Tolerance in the Spring Field Cricket, *Gryllus veletis*

Jantina Toxopeus, *The University of Western Ontario*

Supervisor: Sinclair, Brent J., *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biology

© Jantina Toxopeus 2018

Follow this and additional works at: https://ir.lib.uwo.ca/etd

Part of the Biology Commons, Cellular and Molecular Physiology Commons, Comparative and Evolutionary Physiology Commons, and the Entomology Commons

Recommended Citation

Toxopeus, Jantina, "Mechanisms Underlying Freeze Tolerance in the Spring Field Cricket, *Gryllus veletis*" (2018). *Electronic Thesis and Dissertation Repository*. 5708.
https://ir.lib.uwo.ca/etd/5708

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlswadmin@uwo.ca.

Abstract

Freeze tolerance has evolved repeatedly across insects, facilitating survival in low temperature environments. Internal ice formation poses several challenges, but the mechanisms that mitigate these challenges in freeze-tolerant insects are not well understood. To better understand how insects survive freezing, I describe a novel laboratory model, the spring field cricket *Gryllus veletis* (Orthoptera: Gryllidae). Following acclimation to six weeks of decreasing temperature and photoperiod (mimicking autumn), *G. veletis* juveniles becomes moderately freeze-tolerant, surviving freezing at -8 °C for up to one week, and surviving temperatures as low as -12 °C. Acclimation is associated with increased control of the temperature and location of ice formation, accumulation of cryoprotectant molecules (*myo*-inositol, proline, and trehalose) in hemolymph and fat body tissue, metabolic rate suppression, and differential expression of more than 3,000 genes in fat body tissue. To test cryoprotectant function, I increase their concentration in *G. veletis* hemolymph (*via* injection) and freeze isolated fat body tissue with exogenous cryoprotectants. I show that cryoprotectants improve survival of freeze-tolerant *G. veletis* (proline), their fat body cells (*myo*-inositol), or both (trehalose) under otherwise lethal conditions, suggesting limited functional overlap of these cryoprotectants. However, no cryoprotectant (alone or in combination) can confer freeze tolerance on freeze-intolerant *G. veletis* or their cells. During acclimation, *G. veletis* upregulates genes encoding cryoprotectant transmembrane transporters, antioxidants, and molecular chaperones, which may protect cells during freezing and thawing. In addition, acclimated *G. veletis* upregulates genes encoding lipid metabolism enzymes, and cytoskeletal proteins and their regulators, which I hypothesize promote membrane and cytoskeletal remodelling. To investigate the function of these genes in freeze tolerance, I develop a method to knock down gene expression in *G. veletis* using RNA interference. I knock down expression of three genes (encoding a cryoprotectant transporter, an antioxidant, and a cytoskeletal regulator), laying the ground work for others to test whether and how these genes contribute to mechanisms underlying freeze tolerance. By using a combination of descriptive and manipulative experiments in an

appropriate laboratory model, I improve our understanding of the factors that contribute to insect freeze tolerance.

Keywords

acclimation, cold tolerance, cryoprotectants, insects, mechanisms, metabolome, overwintering, RNAi, thermal biology, transcriptome

Co-Authorship Statement

Chapter 1 was published as a review article in *Biological Reviews* (see Appendix D for reprint permission). I am the first author, and co-author Brent J. Sinclair (BJS) contributed to conceiving and writing this review article.

Chapter 2 is in preparation for publication. I am the first author, and Alexander H McKinnon (AHM), Tomáš Štětina (TS), Kurtis F. Turnbull (KFT), Vladimír Koštál (VK) and BJS are co-authors. AHM developed the acclimation protocol that induces freeze tolerance in *Gryllus veletis*, and completed much of the initial characterization of this freeze tolerance as part of his MSc thesis (lethal limits, osmolality, ion concentrations). TS and KFT helped design and collect data for the respirometry experiments. VK contributed to design and interpretation of the metabolomics dataset. BJS contributed to experimental conception, design and interpretation. I conceived of and conducted the cell survival assays, metabolomics, and cryoprotectant manipulation experiments, and analyzed and visualized data from all experiments in this chapter. I drafted the manuscript together with BJS and contributions from all authors.

Chapter 3 is in preparation for publication. I will be the first author, with Lauren E. Des Marteaux (LED) and BJS as co-authors. LED helped with differential gene expression analysis, interpretation, and figure preparation. I prepared samples for RNA-Seq, and assembled and annotated the transcriptome. BJS and I contributed to experimental conception, design and interpretation. I drafted the manuscript, with contributions from BJS and LED.

Chapter 4 is in preparation for publication. I will be the first author, with BJS as co-author. BJS and I contributed to experimental conception, design and interpretation. I conducted and analyzed all experiments in this chapter. I drafted the manuscript, with contributions from BJS.

Acknowledgments

I would like to thank my academic mentors, past and present. I came into this Ph.D. with a solid scientific foundation, thanks to David Garbary and Tom MacRae, my Honours and Master's advisors (respectively). Brent Sinclair's mentorship during my Ph.D. has pushed me to develop further as a researcher, for which I am grateful. He got me hooked on freeze tolerance, and I have really enjoyed exploring this important topic during my Ph.D. I would like to thank Vladimír Koštál for hosting me in his lab at the Czech Academy of Sciences for part of my Ph.D. research, and all the guidance he provided. I would also like to thank my Ph.D. advisory committee members, Jim Staples and Louise Milligan, whose advice has been insightful and kind.

Much of this thesis was also made possible by the help and support of faculty and staff at Western and other institutions. At Western, thank you to: Hillary Bain, Arzie Chant, Carol Curtis, Sherri Fenton, and Diane Gauley for their efficient administration of the Biology department and graduate program; Chris Guglielmo and Jim Staples for the continued use of their laboratory equipment; and Steve Bartlett, Carrie Hamilton, and Karen Nygard, who keep the Biotron facilities running smoothly. At the Czech Academy of Sciences, thank you to: Irena Vacková and Jarča Korbelová for their technical support, and Martin Moos and the Laboratory of Analytical Biochemistry for assistance processing my metabolomics samples.

I have many academic peers to thank, as well as my supportive friends and family outside of academia. I am eternally grateful to former Sinclair lab members, Ruth Jakobs and Zander McKinnon, who showed me the ropes when I was a very new thermal biologist. I'd especially like to thank Laura Ferguson, whose initial observations on overwintering *Gryllus veletis* sparked the use of this cricket as a freeze tolerance model. Further, I enjoyed collaborating with and learning from my other lab mates: Susan Anthony, John Ciancio, Lauren Des Marteaux, Jackie Lebenzon, Yanira Jiménez Padilla, and Kurtis Turnbull. I would also like to thank Honza Rozsypal, Tomáš Štětina, and rest of the Koštál lab crew for making me feel welcome in their lab. I appreciated the social and

intellectual support from my peers in the Biology graduate program, particularly those in BGS 2035, the Physiology and Biochemistry stream, and members of the Mustang Bioinformatics Club (with special shout outs to Tian Wu and Maryam Jangjoo). This Ph.D. would have been a lot more challenging without you!

One of the highlights of my Ph.D. has been the undergraduate students who have helped with my research – I enjoyed watching you grow as scientists, and am thankful for all the hours you put into research projects with me. I'd especially like to thank Ose Akioyamen, Aisa Kuper-Psenicnik, Claire Newman, Sarah Vo and Meng Zhang, for their hard work on their (and my) experiments. I am grateful to many lab assistants, for their help with cricket maintenance, including Irene Alfred, Haonan (Johnny) Jiang, Michelle Lim, Nicole Martin-Kenny, Aldina Sertovic, and Adam Smith.

I am grateful for financial support from a Canada Graduate Scholarship (Natural Sciences and Engineering Research Council of Canada; NSERC), an Ontario Graduate Scholarship, and the University of Western Ontario Department of Biology. I would also like to thank the Company of Biologists and NSERC for travel grants to support my work in other labs. This support provided important financial security, facilitating my professional development.

Table of Contents

Abstract i

Co-Authorship Statement iii

Acknowledgments iv

Table of Contents vi

List of Tables xi

List of Figures xiv

List of Appendices xvii

List of Abbreviations xviii

Chapter 1 1

1 Mechanisms underlying insect freeze tolerance 1

1.1 Introduction 1

1.2 Freezing is a dynamic process 8

1.2.1 Cooling 8

1.2.2 Ice nucleation and propagation 9

1.2.3 Changes while in the frozen state 12

1.2.4 Thawing and recovery 13

1.3 Challenges associated with cooling, freezing and thawing 14

1.3.1 Low temperatures impair cellular function 14

1.3.2 Mechanical damage during freezing and thawing 17

1.3.3 Damage caused by freeze-induced cellular dehydration 18

1.3.4 Damage due to metabolic limitation 19

1.3.5 Limits of freeze tolerance: life and death when frozen 20

1.4 Mechanisms conferring freeze tolerance 22

1.4.1 Controlling ice formation and propagation reduces mechanical damage . 23

1.4.2 Controlling ice content reduces damage due to dehydration 25

1.4.3 Stabilizing cells and macromolecules reduces cell damage and death 28

1.4.4 Managing biochemical processes reduces damage from harmful metabolites 29

1.4.5 Repair and recovery of physiological function post-thaw 30

1.5 Ecology and evolution of freeze tolerance 32

1.5.1 Pressures that select for freeze tolerance: three non-exclusive hypotheses 33

1.5.2 Routes to evolve freeze tolerance 35

1.5.3 Insect freeze tolerance in a changing climate 38

1.6 New hypotheses and relevant tools 38

1.6.1 Understanding the processes and challenges of freezing 39

1.6.2 Models and experimental manipulations for understanding freeze tolerance 42

1.7 Conclusion 44

1.8 Thesis overview 45

1.9 References 47

Chapter 2 62

2 Cryoprotectant molecules differentially enhance freeze tolerance of the spring field cricket *Gryllus veletis* 62

2.1 Introduction 62

2.2 Materials and methods 65

2.2.1 Rearing and acclimation conditions 65

2.2.2 Measuring metabolic rate and water loss rate 67

2.2.3 Determining freeze tolerance 68

2.2.4 *In vivo* cellular freeze tolerance 69

2.2.5 *Ex vivo* cellular freeze tolerance 70

2.2.6 Biochemical composition of hemolymph and tissues 70

2.2.7 Ice nucleation manipulations 73

2.2.8 *In vivo* manipulations of putative cryoprotectants 73

2.2.9 *Ex vivo* manipulations of putative cryoprotectants 74

2.3 Results and discussion 74

2.3.1 Acclimation induces freeze tolerance in *G. veletis* nymphs 74

2.3.2 Freeze-tolerant *G. veletis* control ice nucleation 78

2.3.3 Acclimated *G. veletis* elevate hemolymph osmolality and alter metabolism to accumulate potential cryoprotectants 81

2.3.4 Exogenous cryoprotectants do not confer freeze tolerance 88

2.3.5 Cryoprotectant-specific enhancement of freeze tolerance in acclimated *G. veletis* 90

2.3.6 Mechanisms underlying mortality at the lethal limits 94

2.4 Conclusions 95

2.5 References 96

Chapter 3 102

3 How crickets become freeze tolerant: the transcriptomic underpinnings of acclimation in *Gryllus veletis* 102

3.1 Introduction 102

3.2 Materials and methods 104

3.2.1 Study animals 104

3.2.2 RNA extraction, cDNA library preparation, and sequencing 105

3.2.3 *De novo* transcriptome assembly and annotation 106

3.2.4 Differential gene expression analysis 106

3.3 Results and discussion 107

3.3.1 Transcriptome summary 107

3.3.2 Differential gene expression early and late in acclimation may contribute to freeze tolerance 108

3.3.3 Transcriptional regulation of metabolism and cell cycle activity 117

3.3.4 Low molecular weight cryoprotectants and their transporters 118

3.3.5 Upregulation of cytoprotective genes during acclimation 120

3.3.6 Potential cellular remodelling during acclimation 122

3.3.7 Regulation of acclimation 123

3.3.8 Acclimation processes lacking transcriptional support 124

3.3.9 How does differential gene expression during acclimation confer freeze tolerance? 126

3.4 Conclusions 129

3.5 References 130

Chapter 4 138

4 Development of a method to knock down gene expression in *Gryllus veletis* using RNA interference 138

4.1 Introduction 138

4.1.1 RNA interference 138

4.1.2 Model system and genes of interest 140

4.1.3 Objectives 143

4.2 Materials and methods 143

4.2.1 Animals and rearing 143

4.2.2 dsRNA synthesis and injection 143

4.2.3 Validation of dsRNA penetration into fat body tissue 147

4.2.4 Validation of knockdown 149

4.3 Results 151

4.3.1 dsRNA persists in fat body tissue for up to three days 151

4.3.2 RNAi reduces transcript abundance of *Ferritin*, *Supervillin*, and *Tret-1* 151

4.4 Discussion 156

4.4.1 RNAi efficacy in *G. veletis* is construct-specific 156

4.4.2 Leveraging RNAi to understand freeze tolerance 158

4.5 Conclusions 160

4.6 References 161

Chapter 5 165

5 General Discussion 165

5.1 The value of descriptive and functional approaches in understanding freeze tolerance 166

5.1.1 Descriptive studies with '-omics' technologies 166

5.1.2 Functional studies to test the mechanisms underlying freeze tolerance . 168

5.2 Revisiting hypotheses about the mechanisms underlying insect freeze tolerance 169

5.2.1 Controlling ice, stabilizing molecules, and regulating metabolism 169

5.2.2 How do the timing and interactions of mechanisms influence freeze tolerance? 174

5.3 Approaches to characterize the processes and challenges of freeze tolerance ... 175

5.3.1 Characterizing processes associated with freeze tolerance 176

5.3.2 Characterizing challenges associated with freeze tolerance 178

5.4 Concluding remarks 181

5.5 References 182

Appendices 186

Curriculum Vitae 247

List of Tables

Table 1.1. Examples of freeze-tolerant (FT) insects described herein, grouped by Order (bold) and Family. 3

Table 1.2. Putative cryoprotectants associated with insect freeze tolerance. 6

Table 1.3. Cytoprotective proteins predicted to facilitate insect freeze tolerance. 7

Table 1.4. Hypotheses (H) concerning the potential causes of lethal limits in freeze-tolerant insects. 21

Table 1.5. Techniques and tools for measuring parameters that will inform our understanding of processes, challenges, and mechanisms of insect freeze tolerance. 41

Table 2.1. Metabolic pathway analysis indicating which pathways appear to have altered activity in freeze-tolerant *G. veletis* fat body, based on metabolite concentrations measured at zero and six weeks of acclimation. 87

Table 3.1. Summary of the *Gryllus veletis* transcriptome *de novo* assembly. 110

Table 3.2. Selected genes upregulated in *Gryllus veletis* during acclimation whose putative function in freeze tolerance is discussed in text. 115

Table 3.3. Selected genes downregulated in *Gryllus veletis* during acclimation whose putative function in freeze tolerance is discussed in text. 116

Table 4.1. Target genes for RNA interference (RNAi) in this study. 142

Table 4.2. Primers used to amplify templates for double stranded RNA (dsRNA) construct synthesis, including the annealing temperature (T_A) and number of cycles (Cyc) in the PCR reaction, and the predicted length (in base pairs; bp) of the PCR product... 145

Table 4.3. Primers used for quantitative PCR (qPCR) and gene-specific primed (GSP) cDNA synthesis, including the annealing temperature (T_A,) used in qPCR cycle or cDNA synthesis, and the efficiencies of qPCR primers. 150

Table 5.1. Predictions for experiments that test the hypothesized mechanisms underlying freeze tolerance in *Gryllus veletis*. 171

Table 5.2. Predicted responses of freeze-intolerant (FI) and freeze-tolerant (FT) *Gryllus veletis* during the processes of cooling, freezing, thawing, and recovery, which are hypothesized to be passive or active.. 177

Table A.1. Freeze tolerance of fifth-instar *Gryllus veletis* following acclimation, acclimatization, or laboratory manipulations. 189

Table A.2. Gas exchange and water loss parameters in freeze-tolerant (FI) and freeze-intolerant (FT) fifth instar juvenile *Gryllus veletis*. 190

Table A.3. Melting point (T_m), thermal hysteresis (TH) and ice crystal morphology of hemolymph samples from freeze-tolerant and freeze-intolerant fifth-instar juvenile *Gryllus veletis*, determined by nanolitre osmometry. 191

Table A.4. Hemolymph concentrations (mM) of metabolites, as determined by GC/LC-MS and GC-FID. 192

Table A.5. Fat body concentrations (nmol/mg fresh mass) of metabolites, as determined by GC/LC-MS and GC-FID. 194

Table A.6. Generalized linear models testing the effect of elevated cryoprotectant concentrations on survival of freeze-tolerant (FT) and freeze-intolerant (FI) crickets and their fat body cells. 196

Table B.1. Summary of 16 RNA-Seq libraries, before and after trimming (removing sequences with fewer than 15 base pairs; bp). 203

Table B.2. Selected genes of interest that were not differentially expressed in *Gryllus veletis* fat body during acclimation. 204

Table B.3. Selected genes upregulated in *Gryllus veletis* fat body during acclimation, in addition to those discussed in the text. 207

Table B.4. Selected genes of interest that were abundant, but not differentially expressed, in *Gryllus veletis* fat body during acclimation. 211

List of Figures

Figure 1.1. Insect body temperature, and physical processes in an insect as the environment cools and rewarms. 9

Figure 1.2. Documented sites of ice nucleation in freeze-tolerant insects, with examples. 10

Figure 1.3. Model of extracellular freezing in freeze-tolerant insects. 12

Figure 1.4. (above) Summary of the challenges associated with cooling, freezing and thawing, and mechanisms for addressing those challenges. 17

Figure 1.5. Relationship between hemolymph osmolality and ice content. 27

Figure 2.1. Acclimation and acclimatization regimes that induce freeze tolerance in *Gryllus veletis.* 66

Figure 2.2. (A) CO_2 emission ($\dot{V}CO_2$) and (B) water loss rate (WLR) of freeze-intolerant (FI) and freeze-tolerant (FT) *Gryllus veletis* at two temperatures. 76

Figure 2.3. Lethal limits of freeze-tolerant (FT) *Gryllus veletis*..... 77

Figure 2.4. Fat body cell (FBC) survival following freezing (A) *in vivo* and (B) *ex vivo*.79

Figure 2.5. Supercooling points (SCPs) of *Gryllus veletis* and its tissues. 80

Figure 2.6. Hemolymph and fat body metabolite composition during acclimation. 83

Figure 2.7. Putative cryoprotectant accumulation in hemolymph and fat body during acclimation. 84

Figure 2.8. Hemolymph composition of freeze-tolerant (FT) and freeze-intolerant (FI) *Gryllus veletis*. 85

Figure 2.9. Effect of cryoprotectant injection on post-thaw survival of (A) freeze-intolerant (FI) *Gryllus veletis* and (B) their fat body cells (FBCs) frozen *ex vivo*..... 89

Figure 2.10. Effect of cryoprotectant injection on post-thaw survival of (A) freeze-tolerant (FT) *Gryllus veletis* and (B) their fat body cells (FBCs) frozen *ex vivo*..... 92

Figure 3.1. Acclimation alters patterns of gene expression in *Gryllus veletis* fat body. 111

Figure 3.2. (above) Relative enrichment of GO terms differentially expressed in *Gryllus veletis* fat body during control and acclimation conditions. 113

Figure 3.3. Summary of KEGG pathways differentially regulated in *Gryllus veletis* fat body during control or acclimation conditions. 114

Figure 3.4. (above) Candidate mechanisms of freeze tolerance acclimation in *Gryllus veletis* fat body tissue. 128

Figure 4.1. Methods work flow for RNA interference (RNAi) in *Gryllus veletis*..... 146

Figure 4.2. Agarose gel of dsRNA templates and dsRNA constructs. 152

Figure 4.3. Agarose gel of *GFP* and *HSP70* dsRNA in *Gryllus veletis* fat body post-injection, amplified by GSP RT-PCR. 153

Figure 4.4. Transcript abundance of RNAi targets (A) *Ferritin*, (B) *HSP70*, (C) *P5C reductase*, (D) *Supervillin*, and (E) *Trehalose transporter Tret-1* three days after injection with dsRNA. 154

Figure 4.5. Relative abundance of *P5C reductase* transcripts in *Gryllus veletis* fat body, following injection with 0 μg, 0.5 μg, 1.5 μg, or 3 μg *P5CR* dsRNA. 155

Figure 5.1. Predictions of how challenges change with (A, B) temperature and (C, D) time when *Gryllus veletis* is frozen, and the hypothesized relationship between challenges and mortality at the lethal limits. 180

Figure A.1. Example respirometry traces from *Gryllus veletis*. 197

Figure A.2. Representative micrographs used to determine *Gryllus veletis* fat body cell (FBC) survival. 198

Figure A.3. Lethal limits of freeze-tolerant fat body cells (FBCs) frozen *ex vivo*. 199

Figure A.4. Ice crystal morphology of *Gryllus veletis* hemolymph is unaffected by acclimation. 200

Figure A.5. Injection elevates hemolymph cryoprotectant concentrations. 201

Figure A.6. Survival of fat body cells (FBCs) frozen *ex vivo* in physiologically-relevant concentrations of exogenous cryoprotectants. 202

Figure B.1. Differential gene expression of the 'alanine, aspartate and glutamate metabolism' KEGG pathway components in *Gryllus veletis* acclimated for three weeks relative to zero week controls. 212

Figure B.2. Differential gene expression of the 'PPAR signaling' KEGG pathway components in *Gryllus veletis* acclimated for six weeks relative to zero week controls. 213

Figure B.3. Differential gene expression of the 'MAPK signaling' KEGG pathway components in *Gryllus veletis* (A) acclimated or (B) maintained under control conditions for six weeks relative to zero week controls. 214

List of Appendices

Appendix A: Chapter 2 supplementary material 186

Appendix B: Chapter 3 supplementary material..... 203

Appendix C: Chapter 4 supplementary material..... 228

Appendix D: Reprint permissions..... 239

List of Abbreviations

20HE – 20- hydroxyecdysone

acTAG – acetylated triacylglycerol

AFP – antifreeze protein

ANOVA – analysis of variance

ANCOVA – analysis of covariance

ADP – adenosine diphosphate

AMP – adenosine monophosphate

AQP – aquaporin

ATP – adenosine triphosphate

BLAST – basic local alignment search tool

bp – base pair

BUSCO – benchmark universal single copy orthologs

cAMP – cyclic adenosine monophosphate

cDNA – complementary deoxyribonucleic acid

CRISPR – clustered regularly interspaced short palindromic repeats

Ct – cycle threshold

CWL – cuticular water loss

CYP – cytochrome P450

DAPI – 4',6-diamidino-2-phenylindole

ddH_2O – distilled, deionized water

DNA – deoxyribonucleic acid

EGLP - entomoglyceroporin

dsRNA – double-stranded RNA

FBC(s) – fat body cell(s)

FI – freeze-intolerant

FID – flame ionization detection

FT – freeze-tolerant

GAGE – generally applicable gene-set enrichment

GC – gas chromatography

GFP – green fluorescent protein

GLP – aquaglyceroporin

GO – gene ontology

GFP – gene-specific primer

HSP – heat shock protein

INA – ice-nucleating agent

IIF – intracellular ice formation

JH – juvenile hormone

KEGG – Kyoto encyclopedia of genes and genomes

L:D – hours of light:dark

LC – liquid chromatography

Lt – lethal time

LLT – lower lethal temperature

MAPK – mitogen-activated protein kinase

MCV – minimum cell volume

miRNA – micro ribonucleic acid

mRNA – messenger ribonucleic acid

MS – mass spectrometry

P5CR – Pyrroline-5-carboxylate reductase

PC – principal component

PCA – principal components analysis

PCR – polymerase chain reaction

PEPCK – phosphoenolpyruvate carboxykinase

PEG – polyethylene glycol

PFAM – protein family

PPAR – peroxisome proliferator-activated receptor

Q_{10} – rate of change as a function of temperature

RdRp – RNA-dependent RNA polymerase

RH – relative humidity

RI – recrystallization inhibitor

RISC – RNA-induced silencing complex

RNA – ribonucleic acid

RNAi – RNA interference

ROS – reactive oxygen species

RT-PCR – reverse transcriptase PCR

RT-qPCR – reverse transcriptase quantitative PCR

RWL – respiratory water loss

SCD – stearoyl CoA dehydrogenase

SCP – supercooling point

siRNA – short interfering RNA

TCA – tricarboxylic acid

TH – thermal hysteresis

T_A – annealing temperature

T_m – melting point

Tret-1 – trehalose transporter 1

$\dot{V}CO_2$ – rate of CO_2 production

WLR – water loss rate

Chapter 1

1 Mechanisms underlying insect freeze tolerance

A version of this chapter is published as a review article in *Biological Reviews* (Toxopeus and Sinclair, 2018, doi: 10.1111/brv.12425). This synthetic review is a substantial component of my thesis work. I have modified some of the phrasing to conform to thesis formatting regulations, and to link this chapter to subsequent chapters in the thesis. In Sections 1.1 to 1.6, I review the background information relevant to the experimental chapters (as is typical in a thesis general introduction). In addition, I synthesize this information to generate a theoretical framework for studying freeze tolerance, highlighting many hypotheses that I hope will guide the field for years to come. In Sections 1.7 and 1.8, I describe the focus of my experimental chapters, in which I test a subset of these hypotheses.

1.1 Introduction

Many terrestrial insects encounter temperatures low and sustained enough to freeze their body fluids. Insects can employ a range of strategies to mitigate this risk (Lee, 2010; Sømme, 1999), including behavioural avoidance (*via* migration or burrowing), reducing the likelihood of freezing by promoting supercooling (freeze avoidance), removing freezable water (cryoprotective dehydration; e.g. Elnitsky et al., 2008), and modifying the body fluid composition to prevent ice crystallization (vitrification; e.g. Sformo et al., 2010). Perhaps the most striking insect cold-tolerance strategy, however, is freeze tolerance, whereby insects tolerate the conversion of as much as 82 % of their body water into internal ice (Lee, 2010; Ramløv and Westh, 1993). This strategy was first described by Réaumur (1736) nearly 300 years ago, yet the mechanisms underlying insect freeze tolerance are not fully understood. In this chapter, I review our knowledge of freeze tolerance from molecular underpinnings to evolutionary processes, and develop a framework to guide future investigations.

Internal ice formation can cause cellular dehydration and mechanical damage, and restricts the opportunity to maintain homeostasis or respond to environmental challenges

(Lee, 2010). Nevertheless, freeze tolerance has clearly evolved multiple times (Dennis et al., 2015; Sinclair and Chown, 2010; Walters et al., 2009b) in a diversity of insects (see Table 1.1 for the freeze-tolerant insect examples discussed herein). However, due to the taxonomically and geographically diverse nature of freeze tolerance, it is unclear whether mechanisms underlying freeze tolerance are consistent across species.

Indeed, ice formation and the conditions under which insects tolerate that ice vary among freeze-tolerant species. The temperature at which ice formation begins (the supercooling point, SCP) can range from -1 °C (e.g. *Chymomyza costata* larvae in contact with ice; Koštál et al., 2011) to -54 °C (e.g. *Pytho deplanatus*, an alpine beetle; Ring, 1982). Some insects survive intracellular ice formation (IIF; e.g. larvae of the wasp *Cephus cinctus*; Salt, 1961), while others restrict ice formation to extracellular spaces (e.g. New Zealand alpine weta *Hemideina maori*; Sinclair and Wharton, 1997). Once frozen, freeze-tolerant insects die if cooled to their lower lethal temperature (LLT) or held in the frozen state for their lethal time (Lt). The LLT ranges from moderate (e.g. -11.5 °C, *Pringleophaga marioni*; Klok and Chown, 1997) to extreme (e.g. < -196 °C, *C. costata*; Koštál et al., 2011) low temperatures, and the Lt from several days (e.g. 7 days at -8 °C, *Gryllus veletis*; McKinnon, 2015) to many months (e.g. >205 days at -10 °C, *Cryptocercus punctulatus*; Hamilton et al., 1985).

The presence and extent of freeze tolerance varies by life stage and season. For example, only the overwintering prepupae of the goldenrod gall fly (*Eurosta solidaginis*) are freeze tolerant (Storey and Storey, 2013). These seasonal changes may be centrally regulated by the neuroendocrine system (Xu et al., 1990), and often coincide with programmed diapause and seasonal changes in diet. By contrast, other insects, such as the New Zealand alpine cockroach *Celatoblatta quinquemaculata*, maintain year-round freeze tolerance, but nevertheless have a lower LLT during winter (Sinclair, 1997). Some of these changes can be very rapid; for example, the LLT of *Belgica antarctica* decreases after a brief cold exposure (1 h at -5 °C; Teets et al., 2008).

Table 1.1. Examples of freeze-tolerant (FT) insects described herein, grouped by Order (bold) and Family. Asterisks denote model species or candidate model species for studying the mechanisms and/or evolution of freeze tolerance.

Insect	FT stage	Example of:	References
Blattaria			
Blattidae			
Celatoblatta quinquemaculata	Nymph	Year-round freeze tolerance, survivable intracellular ice formation (IIF)	Sinclair (1997); Worland et al. (2004)
Cryptocercidae			
Cryptocercus punctulatus	Adult	Long lethal time (Lt), 205 days	Hamilton et al. (1985)
Coleoptera			
Carabidae			
Pterostichus spp.	Adult	Genus with FT and freeze-avoidant members	Miller (1969); Rossolimo (1997)
Cucujidae			
Cucujus clavipes	Larva	Multiple cold-tolerance strategies	Duman (1984); Sformo et al. (2010)
Promecheilidae			
Hydromedion sparsutum	Larva	Elevated metabolic rate post-thaw	Block et al. (1998b)
Perimylops antarcticus	Larva	Elevated metabolic rate post-thaw	Block et al. (1998b)
Pyrochroidae			
Dendroides canadensis	Larva	Antifreeze protein (AFP) accumulation	Duman (1980)
Pythidae			
Pytho deplanatus	Larva	Very low supercooling point (SCP)	Ring (1982)
Scarabaeidae			
Osmoderma eremicola	Larva	Ice content	Storey et al. (1993)
Tenebrionidae			
Eleodes blanchardi	Adult	Relating SCP and ice content to mortality	Zachariassen et al. (1979b)
Upis ceramboides	Adult	Extreme low-temperature survival	Miller (1978)
Diptera			
Chironomidae			
Belgica antarctica	Larva	Freeze tolerance, cryoprotective dehydration	Elnitsky et al. (2008)
Drosophilidae			
Chymomyza amoena	Diapausing larva	Ice formation	Sinclair et al. (2009)
*Chymomyza costata**	Diapausing larva	Inoculative freezing, very low lower lethal temperature (LLT), -196 °C	Koštál et al. (2011)
Drosophila melanogaster	Quiescent larva	Proline confers freeze tolerance	Koštál et al. (2012)
Heleomyzidae			
Heleomyza borealis	Dormant larva	Survive high ice contents (>80 %)	Worland et al. (2000)

Table 1.1 continued

Insect	FT stage	Example of:	References
Diptera			
Mycetophilidae			
Exechia nugatoria	Adult	Compartmentalized freezing	Sformo et al. (2009)
Tephritidae			
*Eurosta solidaginis**	Diapausing prepupa	Ice-formation dynamics, microRNA (miRNA) synthesis when frozen	Courteau et al. (2012); Lee and Lewis (1985)
Tipulidae			
Tipula trivittata	Larva	Cryoprotectant accumulation	Duman et al. (1985); Knight and Duman (1986)
Hymenoptera			
Cephidae			
Cephus cinctus	Larva	Survivable IIF	Salt (1961)
Vespidae			
Vespula maculata	Adult (queen)	Hemolymph ice-nucleating agents (INAs)	Duman et al. (1984)
Orthoptera			
Anostostomatidae			
*Hemideina maori**	Adult	High ice content (>80 %), osmotic cellular dehydration	Ramløv and Westh (1993); Sinclair and Wharton (1997)
Gryllidae			
*Gryllus veletis**	Late-instar nymph	Short Lt, 7 days	McKinnon (2015)
Prophalangopsidae			
Cyphoderris monstrosa	Late-instar nymph	Avoid chill coma during freezing	Toxopeus et al. (2016)
Phasmatodea			
Diapheromeridae			
Niveaphasma annulata	Adult	Variance in freeze tolerance among populations	Dennis et al. (2015)
Lepidoptera			
Cossidae			
Cossus cossus	Larva	Buffered overwintering microhabitat	Li (2016)
Crambidae			
Chilo suppressalis	Diapausing larva	Aquaporin (AQP) function	Izumi et al. (2007)
Erebidae			
Pyrrharctia isabella	Diapausing larva	Low metabolic rate, immune activity post-thaw	Marshall and Sinclair (2011)
Tineidae			
Pringleophaga marioni	Larva	Respiration while frozen	Sinclair et al. (2004)
Plecoptera			
Nemouridae			
Nemoura arctica	Nymph	Aquatic insect; makes glycerol while frozen	Walters et al. (2009b)

To withstand the challenges associated with freezing, many freeze-tolerant insects accumulate cryo- and cyto-protectants. Cryoprotectants are hypothesized to protect against the direct effects of low temperatures and ice (Table 1.2) and cytoprotectants generally to preserve cell structure and integrity [Table 1.3; see Storey and Storey (2013); Tattersall et al. (2012); Zachariassen (1985); Zachariassen and Kristiansen (2000) for reviews]. For example, overwintering *Chilo suppressalis* accumulate glycerol and ice-nucleating agents (INAs; Izumi et al., 2006; Tsumuki and Konno, 1991), both of which are cryoprotectants thought to facilitate freeze tolerance (Table 1.2). Conversely, *H. maori* does not accumulate glycerol (Ramløv et al., 1992), and not all populations of *H. maori* produce hemolymph INAs (Sinclair et al., 1999). Similarly *Dendroides canadensis* accumulate substantial quantities of hemolymph antifreeze proteins (AFPs; Duman, 1980), whereas *C. quinquemaculata* exhibits no evidence of hemolymph AFPs (Wharton et al., 2009). This suggests that no one cryoprotectant or class of cryoprotectants is either necessary or sufficient to allow survival of internal ice formation, and casts doubt on the prospect of a unified set of mechanisms underlying freeze tolerance.

In this chapter I seek to reframe our understanding of the mechanisms underlying insect freeze tolerance away from identifying specific or general classes of ‘freeze tolerance molecules’ to focus instead on the inherently dynamic processes of freezing and thawing. Freeze-tolerant insects must withstand the conversion of their body water into ice, maintain cellular integrity while frozen, and re-establish homeostasis and organismal integrity upon thawing. I then place cryo- and cytoprotective molecules within this framework, considering their role in protecting against or mitigating the challenges associated with cooling, freezing and thawing. I discuss the ecological and evolutionary context of insect freeze tolerance. Finally, I introduce the experimental chapters of this thesis, in which I investigate the mechanisms that may contribute to cryo- and cytoprotection of freeze-tolerant *G. veletis*

Table 1.2. Putative cryoprotectants associated with insect freeze tolerance.

Cryoprotectant	Hypothesized function	Example in freeze-tolerant insect(s)
Low molecular weight metabolites		
Polyols (e.g. glycerol, sorbitol)	Increase 'bound' water, colligatively reduce ice content (Lee, 2010), and reduce probability of intracellular ice formation (IIF)	*Pyrrharctia isabella* accumulate over 800 mM hemolymph glycerol (Marshall and Sinclair, 2011)
Sugars (e.g. trehalose)	Stabilize macromolecules (e.g. proteins) *via* direct interaction with them/their hydration shell (Crowe et al., 1984)	*Hemideina maori* accumulate up to 300 mM hemolymph trehalose in the winter (Neufeld and Leader, 1998)
Amino acids (e.g. proline, arginine)	Stabilize macromolecules *via* direct interaction with them/their hydration shell (Arakawa and Timasheff, 1983) Prevent protein aggregation by physical buffering (Rudolph and Crowe, 1986)	High *in vivo* concentrations of proline and arginine increase freeze tolerance of *Chymomyza costata* (Koštál et al., 2011) and confer freeze tolerance on *Drosophila melanogaster* (Koštál et al., 2016; Koštál et al., 2012)
Lipids		
Antifreeze glycolipids	Prevent recrystallization of ice (Duman, 2015)	*Upis ceramboides* accumulate glycolipid with antifreeze properties (Walters et al., 2009a)
Acetylated triacylglycerols (acTAGs)	Improve survival of IIF (Marshall et al., 2014)	*Eurosta solidaginis* accumulate acTAGs prior to winter (Marshall et al., 2014)
Ice-binding proteins		
Ice-nucleating agents (INAs)	Control initiation of ice formation (Zachariassen, 1985)	*H. maori* (Wilson and Ramløv, 1995) have hemolymph INAs
Recrystalliz-ation inhibitors (RIs)	Control ice crystal size and shape (Duman and Horwath, 1983; Zachariassen and Kristiansen, 2000)	Antifreeze proteins (AFPs) from *Dendroides canadensis* inhibit ice recrystallization *in vitro* (Knight and Duman, 1986)
Transport proteins		
Aquaporins (AQPs)	Facilitate water movement out of cells during freezing, reducing IIF (Storey and Storey, 2013); facilitate water movement into cells during thawing	AQP inhibition increases freeze injury of *E. solidaginis* (Philip et al., 2008) and *Chilo suppressalis* (Izumi et al., 2007) tissues
Cryoprotectant transporters	Facilitate cryoprotectant redistribution during freezing, improving cellular survival (Storey and Storey, 2013)	Glycerol movement into *C. suppressalis* fat body cells during freezing minimizes freeze injury (Izumi et al., 2006)

Table 1.3. Cytoprotective proteins predicted to facilitate insect freeze tolerance. n/a indicates molecules for which I am unaware of any studies in freeze-tolerant insects.

Proteins	Hypothesized function	Example in freeze-tolerant insect(s)
Antioxidants	Reduce oxidative damage (Storey and Storey, 2010)	Antioxidant enzymes in *Belgica antarctica* (Lopez-Martinez et al., 2008), *Eurosta solidaginis* (Joanisse and Storey, 1994)
Cell adhesion proteins	Maintain tissue integrity (Des Marteaux et al., 2017); reduce ice formation between cells	n/a
Chaperones (e.g. heat shock proteins, HSPs)	Protect macromolecules; prevent protein aggregation (Storey and Storey, 2013)	HSPs in *Chilo suppressalis* (Lu et al., 2014), *E. solidaginis* (Zhang et al., 2011), *B. antarctica* (Rinehart et al., 2006)
Chelators	Reduce damage due to high ion concentrations (ion binding; Storey and Storey, 2010)	Ferritin (iron chelator) in *E. solidaginis* (Storey and Storey, 2010)
Cytochrome P450s	Reduce oxidative damage (Poupardin et al., 2010); general detoxification/ repair (Scott and Wen, 2001)	Several cytochrome P450s in *B. antarctica* (Lopez-Martinez et al., 2009)
Cytoskeletal protein isoforms, and cytoskeletal regulators	Maintain cell structure/integrity at low temperatures (resist depolymerization; Storey and Storey, 2013)	T-complex protein 1 (cytoskeletal chaperone) in *E. solidaginis* (Zhang et al., 2011), altered expression of *B. antarctica* cytoskeletal isoforms (Li et al., 2009)
Disordered proteins (e.g. dehydrins)	Reduce dehydration stress [e.g. late embryogenesis abundant (LEA) proteins (Toxopeus et al., 2014)]	Putative cryoprotective dehydrin in *E. solidaginis* (Pruitt et al., 2007)
Sirtuins (Sir2 proteins)	General stress resistance (Jung et al., 2016; Preyat and Leo, 2013), e.g. reduce oxidative stress	n/a

1.2 Freezing is a dynamic process

To be freeze tolerant, an insect must survive ice formation, maintain function (or capacity for recovery) while exposed to cold, and recover function after thawing. Freeze tolerance research has generally focused on the effects of ice itself (reviewed by Lee, 2010; Pegg, 2010; Ramløv, 2000; Storey and Storey, 2013; Zachariassen, 1985). However, low temperatures irrespective of ice formation (reviewed by Koštál, 2010; Lee, 2010; Overgaard and MacMillan, 2017; Ramløv, 2000), and thawing (reviewed in the mammalian cryopreservation context by Pegg, 2010) also pose serious challenges to cellular integrity. Here, I frame cooling, freezing, and thawing as processes, and subsequently identify the challenges and mechanisms associated with them.

1.2.1 Cooling

Physiological responses and biophysical changes begin accruing in insects during cooling, prior to ice formation. Most insects supercool: their body fluids remain liquid at temperatures below the melting point of those fluids (Fig. 1.1). Low temperatures impair most biological processes, with well-explored consequences for ion and water balance (Overgaard and MacMillan, 2017). Cold passively impacts the physico-chemical parameters of the internal *milieu*: pH increases by approximately 0.02 pH units per 1 °C decrease in temperature; O_2 solubility increases (but CO_2 solubility is relatively temperature insensitive); and the viscosity of cellular and extracellular fluids increases with cooling (Somero et al., 2017). Cold can activate cellular signalling pathways (Teets et al., 2008; Teets et al., 2013), and stimulate altered transcription and translation (Štětina et al., 2018; Storey and Storey, 2013), including differential regulation of microRNAs (miRNAs; Courteau et al., 2012). Thus, the cooling process can both perturb homeostasis and be a signal for physiological changes to protect against ice formation.

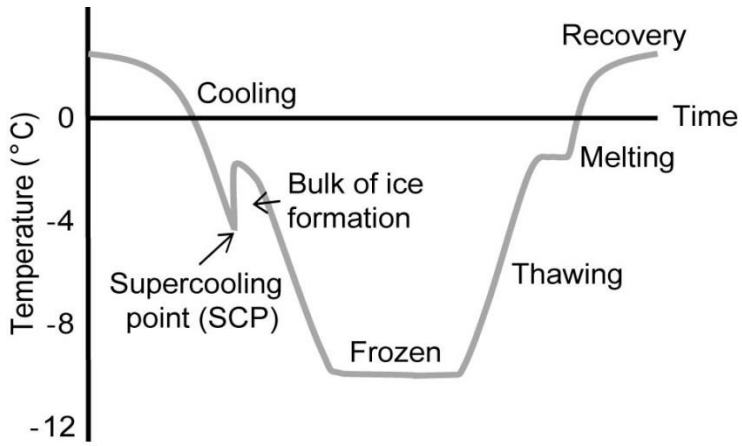

Figure 1.1. Insect body temperature and physical processes in an insect as the environment cools and rewarms.

1.2.2 Ice nucleation and propagation

Ice formation is likely a 'passive' process, i.e. driven by simple physical and chemical principles. However, some freeze-tolerant animals have an 'active' response to freezing. For example, the wood frog *Lithobates sylvaticus* (formerly *Rana sylvatica*) increases metabolic rate when ice formation begins (Sinclair et al., 2013b). In addition, both the wood frog (Storey and Storey, 1984) and enchytraeid worms (Pedersen and Holmstrup, 2003) mobilize glucose stores upon freezing. Conversely, the freeze-tolerant insect *P. marioni* does not appear to increase metabolic rate during freezing (Sinclair et al., 2004), suggesting that the process of freezing in insects may be passive. I discuss these passive processes here.

Ice formation is nucleated at the SCP when sufficient water molecules are arranged into an ice-like structure to form an ice crystal (Fig. 1.1; Lee, 2010). The probability of homogeneous (spontaneous) nucleation in insects is low at temperatures above about -20 °C (Zachariassen et al., 2004b). Ice crystals are excellent nucleators and are often responsible for nucleation, especially from the environment (Fig. 1.2). For example, contact with external ice increases the SCP of *C. costata* from -20 °C to -1 °C (Shimada and Riihimaa, 1988) and is necessary for freeze tolerance in this species. Many biological molecules are INAs, some of which can be extremely efficient (Fig. 1.2). Endogenous

INAs are produced by the insect, and may include proteins (Duman and Horwath, 1983; Wilson and Ramløv, 1995), other organic macromolecules such as lipoproteins (Duman et al., 1985), or inorganic crystals (e.g. $CaPO_4$ in *E. solidaginis* Malpighian tubules; Mugnano et al., 1996). Exogenous nucleators – apart from ice – can include bacteria (Worland and Block, 1999) and fungi (Tsumuki et al., 1992), and plant material, such as algae (Worland and Lukešová, 2000), which may be either external or in the gut.

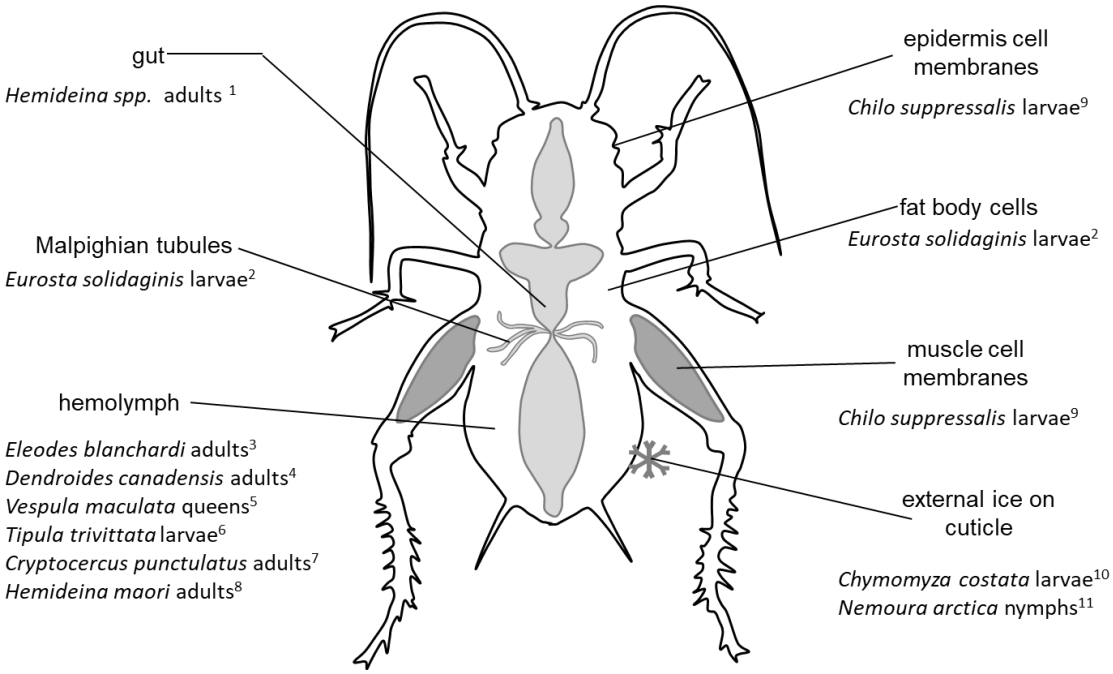

Figure 1.2. Documented sites of ice nucleation in freeze-tolerant insects, with examples (denoted by superscripts) from: 1, Sinclair et al. (1999); 2, Mugnano et al. (1996); 3, Zachariassen and Hammel (1976); 4, Duman (1980); 5, Duman et al. (1984); 6, Duman et al. (1985); 7, Hamilton et al. (1985); 8, Wilson and Ramløv (1995); 9, Tsumuki and Konno (1991); 10, Shimada and Riihimaa (1988); 11, Walters et al. (2011).

Following ice nucleation, ice propagates throughout the insect (Sinclair et al., 2009). The bulk of ice formation occurs at the SCP, generating an exotherm due to heat released by ice formation (Fig. 1.1; Sinclair et al., 2015). The exotherm duration may range from a few seconds in the approximately 1 mg larvae of the drosophilid *Chymomyza amoena* (Sinclair et al., 2009) to an hour or more in a 7 g *H. maori* weta (Ramløv et al., 1992). Ice formation (and heat release) continues beyond the exotherm, until the insect reaches equilibrium ice content. For example, *E. solidaginis* takes approximately 48 h to reach equilibrium ice content at -23 °C, despite a relatively brief (< 5 min) observable exotherm (Lee and Lewis, 1985). Equilibrium ice content increases with decreasing temperature: even after completion of freezing at one temperature, fluctuations in temperature will lead to changes in ice content (Lundheim, 2002). Equilibrium ice content also depends on the availability of 'freezable water' – less ice will form as the osmolality of a solution increases (Storey and Storey, 1988; Tattersall et al., 2012; Zachariassen et al., 1979a).

The extracellular freezing model (Fig. 1.3) is the predominant model of ice formation in freeze-tolerant insects (Scholander et al., 1953; Zachariassen, 1985). In this model, ice forms extracellularly. Solutes are excluded from the growing ice, and the osmotic pressure of the unfrozen fraction of the hemolymph consequently increases, dehydrating cells *via* osmosis (Asahina et al., 1954; Izumi et al., 2006). This osmotic dehydration of cells continues until the cytoplasm is at equilibrium with the unfrozen fraction of the hemolymph (Sinclair and Wharton, 1997). Once in equilibrium, the cytoplasm is theoretically unfreezable by virtue of its osmotic pressure, and IIF is avoided. However, the final distribution of ice varies considerably among freeze-tolerant insects: survivable IIF has been documented in cells from many freeze-tolerant insects (Sinclair and Renault, 2010), including fat body cells of *C. cinctus* (Salt, 1961) and *E. solidaginis* (Lee et al., 1993), and *C. quinquemaculata* midgut cells (Worland et al., 2004). The mechanisms allowing IIF survival are not understood, but in the Antarctic nematode *Panagrolaimus davidi*, ice is restricted to the cytoplasm and osmotically dehydrates organelles in a process analogous to the extracellular freezing model (Wharton et al., 2005a).

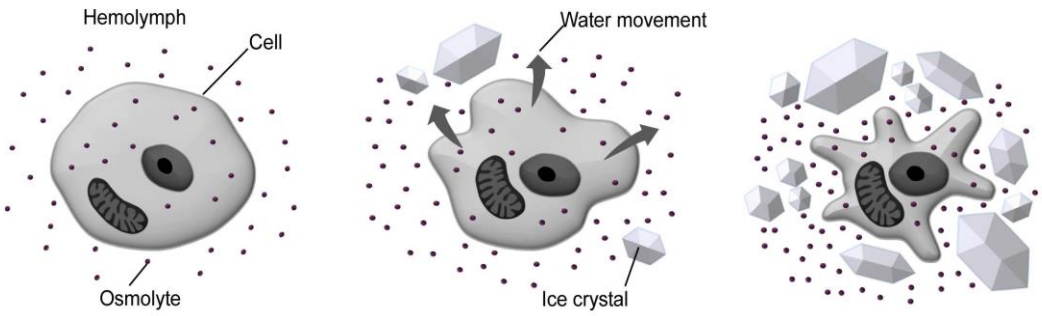

Figure 1.3. Model of extracellular freezing in freeze-tolerant insects. Ice is nucleated extracellularly, osmotically dehydrating cells and preventing internal ice formation.

Several factors may impact ice propagation. Whether ice is restricted to the hemolymph or propagates into cells will depend on the rate of ice formation: external ice nucleation at high subzero temperatures and slow cooling prevent IIF in *C. cinctus* and *C. quinquemaculata* cells, while nucleation at lower temperatures results in faster ice formation and propagation into cells (Salt, 1961; Worland et al., 2004). Dehydration may also limit ice propagation: at the SCP, ice propagates through the abdomen (71 % water content) of *Exechia nugatoria*, while the head and thorax (47 % water content) remain unfrozen (Sformo et al., 2009). If ice is nucleated externally (e.g. *via* contact with external ice, or ice nucleators in the gut), it must propagate across epithelia, i.e. the cuticle and epidermal epithelia or gut epithelia (Sinclair and Renault, 2010). One potential route for paracellular ice movement across gut epithelia is the rectal paracellular channels, which are wide enough to accommodate ice crystals (e.g. 10 nm in *Gryllus pennsylvanicus*; Des Marteaux et al., 2018). For a review of biophysical factors that affect ice growth and movement, see Mazur (2010). Ice can also propagate between cells (Berger and Uhrik, 1996), which may be facilitated by (but does not require) gap junctions (Acker et al., 2001).

1.2.3 Changes while in the frozen state

The conversion of the bulk of body water into ice precludes hemolymph circulation, and presumably therefore excretion and endocrine communication. However, even at

equilibrium ice content, the frozen state is not necessarily static: insects die when kept frozen at an otherwise-survivable temperature for a critical time (Lt), suggesting that changes occur after completion of ice formation. Ice structure changes over time: recrystallization, the accretion of water molecules onto large ice crystals from smaller crystals, occurs readily at temperatures above about -8 °C (Knight and Duman, 1986; Knight et al., 1988; Ramløv et al., 1996). Cellular activity may continue in frozen insects, as indicated by detectable CO_2 production (Irwin and Lee, 2002; Sinclair et al., 2004), ATP consumption (Storey and Storey, 1985), changes in miRNA expression (Courteau et al., 2012), and accumulation of metabolites such as glycerol (Michaud et al., 2008; Walters et al., 2009b), alanine (Michaud et al., 2008) and lactate (Storey and Storey, 1985). In addition, frozen insects continue to lose ion balance across cell membranes and epithelial layers, probably due to equilibration of ion gradients through leak channels, or slow leak of calcium ions from storage (Boardman et al., 2011; Kristiansen and Zachariassen, 2001; Štětina et al., 2018). Thus, frozen insects, at least at ecologically relevant temperatures, are not 'cryopreserved' in a static state.

1.2.4 Thawing and recovery

A frozen insect will begin to thaw when the environmental temperature increases above its melting point (Fig. 1.1). In some cases, the insect is active immediately post-thaw (Sinclair et al., 2004), implying that freeze-tolerant insects restore biological processes during thawing. However, many frozen insects (e.g. *C. costata*; Koštál et al., 2011) remain inactive post-thaw for minutes to days, during which the insect is presumably recovering physiological function, and/or repairing freeze injury. Some insects that are active post-thaw (e.g. *C. cinctus* frozen to -15 °C) may still die within the next few days (Salt, 1961), suggesting that recovery of movement (the typical method for determining 'survival') does not imply complete recovery from freezing. In addition, insects may appear to recover from freezing, but experience sub-lethal effects that prevent development to the next life-history stage (Štětina et al., 2018).

Despite their probable importance, our understanding of thawing and recovery are limited. Thawing appears to be passive: there is no apparent change in adenylate charge (ATP availability) during thawing in *E. solidaginis* (Storey and Storey, 1985), nor is

metabolic rate elevated during thawing in *P. marioni* (Sinclair et al., 2004). Thawing may thus be a simple reversal of the processes associated with cooling and freezing: ice content decreases and cells rehydrate (Zachariassen, 1985). However, because recrystallization is energetically favoured with increasing temperature, ice crystals can grow during thawing, even though overall ice content is decreasing (Mazur, 2010). In addition, thawing is unlikely to be spatially or temporally uniform. I expect that peripheral body regions will warm more quickly and should thaw earlier and at lower ambient temperatures than the abdomen and thorax. Thus, any challenges associated with thawing (e.g. osmotic stress) may occur at different times and intensities across the insect. During recovery, freeze-tolerant insects presumably expend energy to restore homeostasis (e.g. ion gradients), and recognize and repair any damage that occurred during freezing and thawing (see Section 1.4.5).

1.3 Challenges associated with cooling, freezing and thawing

Freeze-tolerant insects must tolerate the physiological and biophysical impacts of low temperatures, freezing, and thawing. Here I draw from theory on insect cold tolerance, mammalian cryopreservation, and the physical chemistry of ice to speculate on the nature of these challenges. I summarize the effects of low temperatures and ice, and how they challenge survival in Fig. 1.4.

1.3.1 Low temperatures impair cellular function

During cooling and freezing, low temperatures likely inhibit macromolecular function, impairing membrane- and protein-dependent cellular processes, and causing damage (Fig. 1.4). Enzymes are less flexible in the cold, which decreases binding affinity (or prevents enzyme–substrate binding altogether) thus impairing function (Somero et al., 2017). This reduced enzyme function will decrease metabolic capacity, reducing ATP availability, potentially increasing anaerobic metabolism, and facilitating the accumulation of harmful metabolic intermediates and by-products that damage macromolecules (Storey and Storey, 1988; Watson and Morris, 1987). Reduced antioxidant enzyme function, as well as inhibited function of the mitochondrial electron

transport system, can lead to reactive oxygen species (ROS) accumulation and oxidative damage to macromolecules (Gulevsky et al., 2006; Lalouette et al., 2011; Rojas and Leopold, 1996; Somero et al., 2017). Reduced protein function will truncate cellular responses to stressors (Ramløv, 2000), for example by slowing transcriptional and translational machinery (Farewell and Neidhardt, 1998; Miguel et al., 2013), or dissociating multi-subunit proteins (e.g. cytoskeletal polymers) necessary for structural integrity or intracellular transport (Des Marteaux et al., 2018). The structural stabilization of proteins by the hydrophobic effect declines at low temperatures, potentially causing irreversible denaturation (Dias et al., 2010; Marqués, 2006), leading to both functional failure and cytotoxic aggregates of denatured proteins (Korsloot et al., 2004).

Low temperatures reduce membrane fluidity (Somero et al., 2017), likely impairing the function of membrane-bound proteins, and processes such as endo- and exocytosis that depend on membrane fluidity. Membrane-associated enzymes, such as ATP-dependent ion pumps, have reduced activity in the cold – which is associated with loss of ion homeostasis (Koštál et al., 2007) and accumulation of chilling injury in chill-susceptible insects (MacMillan et al., 2015). Very low temperatures cause membrane phase transitions from a fluid to a crystalline (gel) state, potentially disrupting cells and causing death. This loss of fluidity occurs at the gel-transition temperature, which is determined by membrane composition itself (Hazel, 1995). Thus, low temperatures alone are challenging, accounting for the majority of insects which are killed by cold-induced injuries unrelated to ice formation (Sinclair et al., 2015).

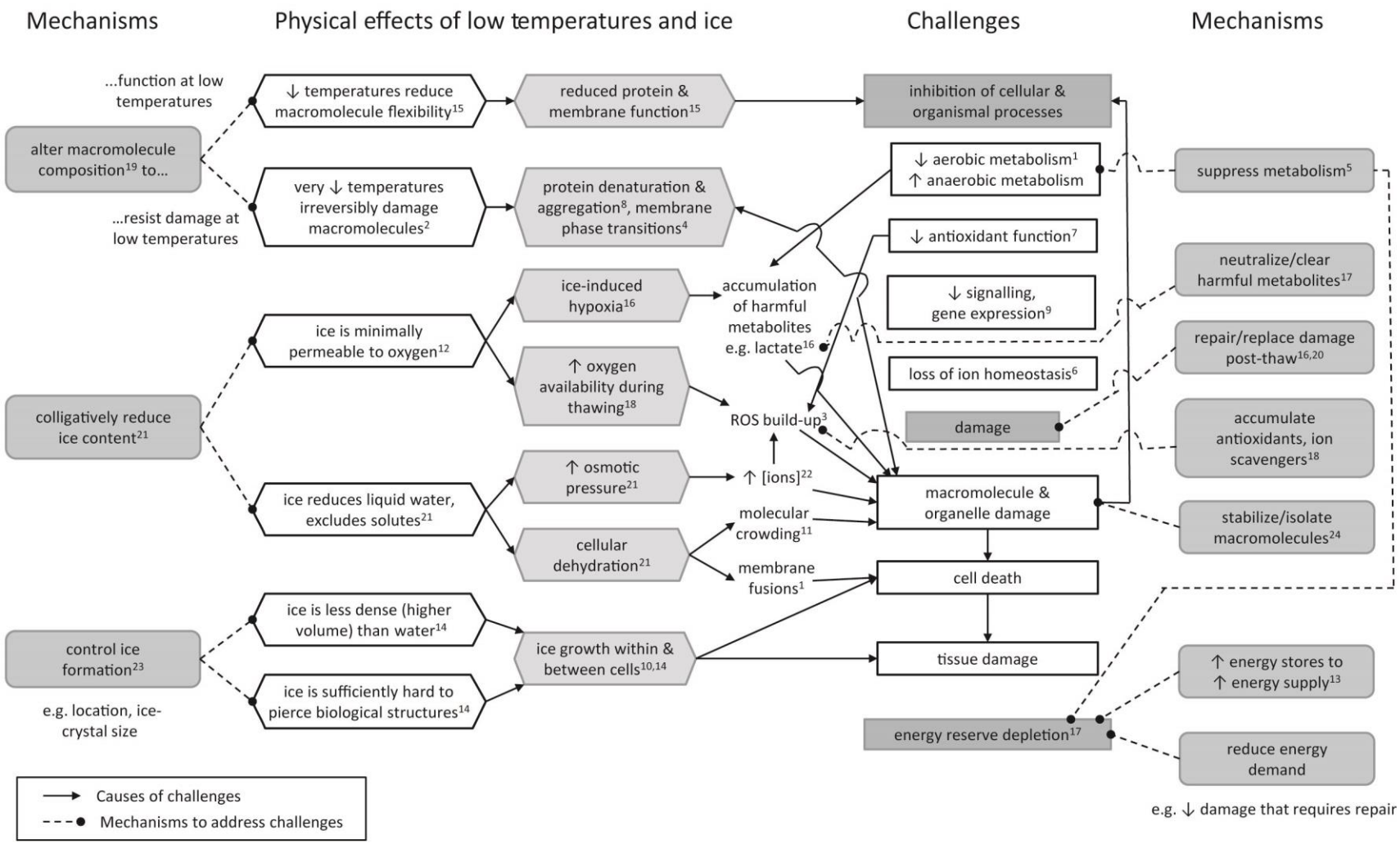
Mechanisms
Physical effects of low temperatures and ice
Challenges
Mechanisms
...function at low temperatures
alter macromolecule composition[19] to...
...resist damage at low temperatures
↓ temperatures reduce macromolecule flexibility[15]
very ↓ temperatures irreversibly damage macromolecules[2]
reduced protein & membrane function[15]
protein denaturation & aggregation[8], membrane phase transitions[4]
inhibition of cellular & organismal processes
↓ aerobic metabolism[1]
↑ anaerobic metabolism
↓ antioxidant function[7]
↓ signalling, gene expression[9]
loss of ion homeostasis[6]
damage
suppress metabolism[5]
neutralize/clear harmful metabolites[17]
repair/replace damage post-thaw[16,20]
accumulate antioxidants, ion scavengers[18]
stabilize/isolate macromolecules[24]
colligatively reduce ice content[21]
ice is minimally permeable to oxygen[12]
ice reduces liquid water, excludes solutes[21]
ice-induced hypoxia[16]
↑ oxygen availability during thawing[18]
↑ osmotic pressure[21]
cellular dehydration[21]
accumulation of harmful metabolites e.g. lactate[16]
ROS build-up[3]
↑ [ions][22]
molecular crowding[11]
membrane fusions[1]
macromolecule & organelle damage
cell death
tissue damage
control ice formation[23]
e.g. location, ice-crystal size
ice is less dense (higher volume) than water[14]
ice is sufficiently hard to pierce biological structures[14]
ice growth within & between cells[10,14]
energy reserve depletion[17]
↑ energy stores to ↑ energy supply[13]
reduce energy demand
e.g. ↓ damage that requires repair
Causes of challenges
Mechanisms to address challenges

Figure 1.4. (above) Summary of the challenges associated with cooling, freezing and thawing, and mechanisms for addressing those challenges. ↑ indicates high (physical effects, challenges) or increase (mechanisms); ↓ indicates low (physical effects, challenges) or reduce (mechanisms); ROS, reactive oxygen species. These hypotheses are supported by the following references, indicated by superscript numbers on the figure: 1, Crowe et al. (1984); 2, Dias et al. (2010); 3, Doelling et al. (2014); 4, Hazel (1995); 5, Irwin and Lee (2002); 6, Koštál et al. (2007); 7, Lalouette et al. (2011); 8, Marqués (2006); 9, Miguel et al. (2013); 10, Pegg (2010); 11, Ramløv (2000); 12, Scholander et al. (1953); 13, Sinclair (2015); 14, Sinclair and Renault (2010); 15, Somero et al. (2017); 16, Štětina et al. (2018); 17, Storey and Storey (1985); 18, Storey and Storey (2010); 19, Tattersall et al. (2012); 20, Teets and Denlinger (2013); 21, Zachariassen (1985); 22, Zachariassen et al. (2004a); 23, Table 1.2 (and references therein) for examples of cryoprotectants that are hypothesized to contribute to controlling ice formation; 24, Tables 1.2 and 1.3 (and references therein) for examples of how low molecular weight metabolites (Table 1.2), and chaperones and disordered proteins (Table 1.3) are hypothesized to contribute to stabilizing macromolecules.

1.3.2 Mechanical damage during freezing and thawing

Internal ice formation kills most insects (Sinclair et al., 2015), and probably damages cells/tissues even in freeze-tolerant insects (Collins et al., 1997; Izumi et al., 2005; Marshall and Sinclair, 2011; Worland et al., 2004; Yi and Lee, 2003). Two properties of ice can cause mechanical stress (Fig. 1.4): ice is less dense than water (i.e. when water crystallizes, it expands), and ice is of sufficient hardness to pierce/tear biological tissue (i.e. ice crystal growth may cause shear stress). Internal ice formation can cause whole-body distension (e.g. *C. amoena* dorsal area increases by up to 5.5 % with freezing; Sinclair et al., 2009) and damage – the latter presumably dependent on ice location and quality (crystal size and shape). Ice formation may rupture cells, for example by expansion of intracellular ice, or compromise tissue integrity if ice forms between cells (Pegg, 2010; Sinclair and Renault, 2010; Storey and Storey, 1988). Extracellular ice may have minimal impact on organismal or cell viability in freeze-tolerant insects (Asahina et al., 1954); although if extracellular ice recrystallizes it may damage cells, as seen for thawing mammalian cells (Pegg, 2010). Freeze-tolerant insects therefore likely control both the location and quality (e.g. size or shape) of ice crystals.

1.3.3 Damage caused by freeze-induced cellular dehydration

Frozen insects lose very little water to the environment (Lundheim and Zachariassen, 1993; Sinclair et al., 2013a). However, internal ice formation reduces available liquid water inside the animal (i.e. decreases water activity; Bradley, 2009), resulting in increased hemolymph and cytoplasmic solute concentrations (increased osmotic stress) and low cellular water content (dehydration stress) (Fig. 1.4; Lee, 2010; Zachariassen, 1985). When ice melts, the decreased osmotic pressure could also cause damage if cell rehydration is too rapid ('osmotic shock,' as reviewed by Elliott et al., 2017). Thus, both dehydration and/or osmotic stress could damage cells in frozen and thawed insects (Pegg, 2010; Worland et al., 2004; Yi and Lee, 2003).

Increased osmotic pressure associated with freezing (i.e. freeze concentration) might destabilize proteins and damage cell membranes, causing cell death (Lee, 2010). The reduced water availability will increase the concentrations of individual solutes (Lee, 2010), including cations (Zachariassen et al., 2004a), which can have specific consequences. Increased [H^+] will decrease pH, which can alter protein structure and stability (Harrison, 2001). Cations such as Ca^{2+} alter signalling (Teets et al., 2013), often in a concentration-dependent manner, and can activate processes such as apoptosis (Orrenius et al., 2003). Others, such as Fe^{2+} will exacerbate ROS formation (e.g. *via* the Fenton reaction; Storey and Storey, 2013), increasing oxidative damage. High concentrations of some trace metal ions (Cu^{2+}, Mg^{2+}) may have toxic effects (Zachariassen et al., 2004a). Hyperkalemia (high extracellular [K^+]) disrupts muscle function and causes injury in chilled locusts (MacMillan et al., 2014), but freeze-tolerant *C. costata* appear to restore ion balance rapidly after freezing (Štětina et al., 2018), and the consequences of high cation concentrations in frozen insects warrant further exploration. Concentration of other solutes may also cause damage: trehalose can crystallize (and potentially cause mechanical damage) under freezing conditions (Wen et al., 2016), although this has not yet been demonstrated *in vivo*.

Freezing may damage cells by dehydrating them below a critical 'minimum cell volume' (MCV) threshold that precludes cellular recovery (Lee, 2010). Cellular dehydration will cause molecular crowding (Ramløv, 2000), increasing the probability of unfavourable

intracellular interactions that can damage macromolecules (e.g. between proteins, and between proteins and ions or ROS). Dehydration can also reduce structural stability of macromolecules (e.g. by removing the hydration shell), as demonstrated *in vitro* for globular proteins (Morisaku et al., 2014; Prestrelski et al., 1993), and membranes (Crowe et al., 1984). Extreme cytosolic volume loss can lead to potential shear stress on the cytoskeleton and cell–cell adhesions, or cause cell membrane fusions that result in cell death (Li et al., 2009). The effect of freezing on macromolecule stability and its consequences (e.g. protein aggregation) have not been examined in freeze-tolerant insects.

1.3.4 Damage due to metabolic limitation

Gases do not diffuse well through ice, and freezing may therefore impose hypoxia or anoxia (Fig. 1.4; Scholander et al., 1953). Frozen insects accumulate anaerobic end products such as lactate, succinate, and alanine (Michaud et al., 2008; Storey and Storey, 1985; Storey et al., 1981), suggesting a shift to anaerobic metabolism. However, the larger tracheae of frozen *C. amoena* do not collapse (Sinclair et al., 2009), and frozen *E. solidaginis* (Irwin and Lee, 2002) and *P. marioni* (Sinclair et al., 2004) appear to exchange CO_2 with their environment. Thus, it is unclear whether frozen insects are only partially hypoxic (I hypothesize that this could vary among tissues), or if being frozen is accompanied by a facultative shift to anaerobiosis. Lack of aerobic metabolism is associated with adenylate charge reduction in frozen *E. solidaginis* (Storey and Storey, 1985), which may impede any energy-requiring processes during recovery.

If oxygen supply is restricted in frozen insects, then thawing – especially given the efficient tracheal system – likely rapidly increases oxygen availability to tissues. This influx of oxygen has been considered analogous to ischemia-reperfusion injury (Storey and Storey, 2013), and could therefore be accompanied by a large increase in the formation of potentially damaging ROS. Repeatedly frozen *E. solidaginis* accumulate more oxidative damage than *E. solidaginis* frozen and thawed once, potentially reflecting reperfusion injury from multiple thaw events (Doelling et al., 2014).

1.3.5 Limits of freeze tolerance: life and death when frozen

Most freeze-tolerant insects cannot survive being frozen indefinitely: they die after a critical period of time (Lt) or below a threshold temperature (their LLT). This implies that the challenges of surviving freezing can be exacerbated while the insect is frozen. I suggest parameters that may determine these lethal limits in Table 1.4.

One explanation for these lethal thresholds is that some molecules, organelles, or tissues may be more susceptible to the challenges of the frozen state than others (e.g. differential damage of tissues observed by Marshall and Sinclair, 2011; Yi and Lee, 2003). By this logic, damage to these 'weak links' accrues in a time- or temperature-dependent manner, resulting in whole-animal mortality. Lethal freezing in *E. solidaginis* is associated with damage to nuclei (in the brain), mitochondria (in Malpighian tubules), and myofilaments (in the muscle; Collins et al., 1997) – suggesting these cellular components (and tissues) are most sensitive to freezing stress, but the mechanisms of this damage are unclear. Alternately, the LLT could be simply associated with direct effects of temperature on macromolecules (i.e. unrelated to ice; Table 1.4, Hypothesis 1). For example, cell death at the LLT in *H. maori* (Sinclair and Wharton, 1997) suggests that membranes are damaged, which could be caused by cold-induced membrane phase transitions, or dissociation/denaturation of the cytoskeleton (e.g. Des Marteaux et al., 2018).

The quantity, quality, and distribution of ice change with both time and temperature (Lee, 2010; Ramløv, 2000). In particular, the increase in ice content with decreasing temperature will exacerbate osmotic and dehydration stress, and could contribute to mechanical damage/distortion (Table 1.4, Hypothesis 2). This hypothesis of a critical ice content at the LLT is supported in *E. solidaginis* (c. 66 %; Lee and Lewis, 1985), but ice content does not differ between non-lethal and lethal freezing temperatures in *H. maori* (c. 82 %; Ramløv and Westh, 1993) or *Heleomyza borealis* (c. 80 %; Worland et al., 2000). While recrystallization over time (especially at high subzero temperatures; Mazur, 2010) could mechanically damage cells or tissues, there is no evidence for or against a role for recrystallization in the Lt of freeze-tolerant insects (Table 1.4, Hypothesis 3). Finally, the location of ice may change with temperature or time, and ice propagation into particularly weak cells or tissues could define the lethal limits (Table 1.4, Hypothesis 4).

Table 1.4. Hypotheses (H) concerning the potential causes of lethal limits in freeze-tolerant insects. Arrows, ↑ and ↓, indicate increase(s) and decrease(s), respectively. Both ↓ lower lethal temperature (LLT) and ↑ lethal time (Lt) imply increased freeze tolerance.

Challenge	Hypothesis	Prediction
H1. Low temperature		
Low temperatures destabilize proteins and membranes	Death at the LLT occurs at a critical level of macromolecule/cell damage	Mechanisms that stabilize macromolecules (e.g. accumulating proline, trehalose, chaperones) ↓ LLT
H2. Ice content		
High ice content increases osmotic/ dehydration stress	Death at the LLT occurs when cells dehydrate below a minimum critical volume (MCV)	Mechanisms that reduce ice content (e.g. accumulating colligative cryoprotectants) reduce cell dehydration; ↓ LLT
High ice content increases mechanical damage	Death at the LLT occurs when ice content above a critical threshold causes mechanical damage	Mechanisms that reduce ice content (e.g. accumulating colligative cryoprotectants) reduce mechanical damage from ice; ↓ LLT
H3. Ice quality		
Recrystallization mechanically damages cells	Death at the Lt occurs at a critical level of mechanical damage due to ice crystal growth while frozen	Mechanisms that control ice size [e.g. recrystallization inhibitors (RIs)] reduce mechanical damage from ice crystals; ↑ Lt (e.g. at moderate subzero temperatures)
H4. Ice location		
Intracellular ice formation (IIF) and extracellular ice formation within tissues cause mechanical damage	Death at the LLT occurs when ice propagates into (weak link) cells or tissues, which may occur at high ice content	Mechanisms that reduce ice content (e.g. accumulating colligative cryoprotectants) and control ice location [e.g. ice-nucleating agents (INAs), tight intercellular junctions] improve cell and insect survival; ↓ LLT
H5. Accumulation of harmful metabolites		
Accumulation of harmful metabolic wastes while frozen damages cells/tissues	Death at the Lt occurs at a critical waste [e.g. reactive oxygen species (ROS), lactate] accumulation threshold, defining the Lt	Metabolic suppression reduces ROS and other waste production; ↑ Lt. Mechanisms that remove/neutralize these products (e.g. antioxidants, buffers) improve cell survival; ↑ Lt
H6. Metabolic activity		
Depletion of energy reserves in the frozen state precludes recovery/repair	Death at the Lt occurs at a critical energy reserve (e.g. adenylate charge) depletion	Energy reserve accumulation, metabolic suppression and/or overwintering at low temperatures (lower metabolic rate) slows energy reserve depletion; ↑ Lt

Alternately, the Lt could be associated with a threshold accumulation of toxic products [e.g. ROS (Joanisse and Storey, 1996) and lactate (Storey and Storey, 1985)] or unrepaired macromolecular damage (Table 1.4, Hypothesis 5). Depletion of adenylate charge (energy reserves) while frozen (Storey and Storey, 1985) could also limit recovery (Table 1.4, Hypothesis 6). For example, prolonged freezing may result in loss of ion homeostasis (Boardman et al., 2011; Kristiansen and Zachariassen, 2001), and considerable energy stores may be required to restore neuromuscular function post-thaw. If these energy stores are compromised prior to recovery, then the lethal limits may be a consequence of disrupted thawing/recovery processes. Thus, metabolic processes could drive the Lt either by producing too many by-products, or through depletion of energy resources required for recovery; either way I predict that the Lt should be positively correlated with the strength of metabolic suppression.

1.4 Mechanisms conferring freeze tolerance

Freeze-tolerant insects prevent or repair damage caused by low temperatures and ice, and preserve (or recover post-thaw) the cellular and organismal processes important for survival. Most of the putative cryo- and cytoprotectants that may contribute to these strategies (Tables 1.2 and 1.3) have been identified by correlating an accumulation of those molecules with the acquisition of freeze tolerance, usually across seasons (e.g. Baust and Lee, 1981; Marshall et al., 2014; Philip and Lee, 2010). Glycerol and other polyols are the best-known low molecular weight cryoprotectants associated with freeze tolerance (Miller and Smith, 1975; Salt, 1957; Walters et al., 2009b). However, the free amino acid proline (Koštál et al., 2011; Leader and Bedford, 1978; Ramløv, 1999) and lipid-related cryoprotectants (Marshall et al., 2014; Sinclair and Marshall, 2018; Walters et al., 2009a) were identified more recently, and because they were not included in earlier screens of potential cryoprotectants, their prevalence among freeze-tolerant species is unclear. Furthermore, our understanding of the proteins involved in freeze tolerance is incomplete. Ice-binding proteins [INAs (Knight and Duman, 1986); recrystallization inhibitors (RIs; Wilson and Ramløv, 1995)], heat shock proteins (HSPs; Lee et al., 1995; Lu et al., 2014; Rinehart et al., 2006; Zhang et al., 2011), and aquaporins (AQPs; Goto et al., 2011; Izumi et al., 2006; Philip et al., 2008; Yi et al., 2011) are all associated with

freeze tolerance, but most were identified *via* targeted exploration rather than an untargeted ('-omics'-style) approach that might identify unexpected molecules. Thus, the identification of cryoprotectants in freeze-tolerant species (Tables 1.2 and 1.3) has been biased towards *a priori* expectations, limiting the extent to which we can identify novel molecules associated with freeze tolerance. In Chapters 2 and 3, I use metabolomics and transcriptomics (respectively) to identify a broad range of molecules that are associated with freeze tolerance in *G. veletis.*

It is challenging to ascribe functional roles to these molecular correlates of freeze tolerance based on descriptive studies alone. The most informative cryoprotectant studies manipulate the function of these molecules. For example, I infer that AQPs facilitate freeze tolerance because their inhibition by mercuric chloride (loss-of-function) reduces survival of frozen cells from *B. antarctica* (Yi et al., 2011), *C. suppressalis* (Izumi et al., 2007) and *E. solidaginis* (Philip et al., 2008). Proline is clearly cryoprotective, given that elevated concentrations (gain-of-function) confer freeze tolerance on *Drosophila melanogaster* (Koštál et al., 2016). In Chapters 2 and 4, I design experiments to test the function of putative cryoprotectants and genes associated with *G. veletis* freeze tolerance.

In this section I eschew a focus on (groups of) cryo- and cytoprotectants, and instead discuss their function within a theoretical framework of hypothesized strategies and mechanisms underlying insect freeze tolerance – these mechanisms are summarized in Fig. 1.4. I hope that this strategy/mechanisms paradigm will facilitate a hypothesis-driven approach to unravelling the role of cryoprotectants and freeze tolerance in general.

1.4.1 Controlling ice formation and propagation reduces mechanical damage

I hypothesize that freeze-tolerant insects control ice crystal location and size to minimize mechanical damage (Fig. 1.4). Ice location could be controlled by modifying the site of ice nucleation (e.g. *via* INAs), by physical barriers such as cell–cell tight junctions, and by the redistribution of water during freezing (e.g. *via* AQPs), while ice-binding proteins (e.g. AFPs or RIs) could alter ice quality. In Chapter 2, I investigate whether freeze-tolerant *G. veletis* accumulates INAs or other ice-binding proteins, and if these function

in facilitating survival of internal ice formation. In Chapter 3, I determine whether freeze-tolerant *G. veletis* transcriptionally-upregulate any of these factors.

Ice-nucleating agents initiate ice formation, and differential distribution of INAs will thus localize ice formation, perhaps to compartments that are robust to ice-induced mechanical damage. A core hypothesis is that INAs confine ice to extracellular spaces (Fig. 1.3), preventing IIF (and the associated mechanical damage) by facilitating osmotic dehydration of cells (Lee, 2010; Zachariassen, 1985). In addition to controlling the location of ice, INAs elevate the temperature at which ice formation begins (Sømme and Zachariassen, 1981; Zachariassen and Kristiansen, 2000). This promotes slow formation of large ice crystals (Salt, 1961), which can promote osmotic dehydration by extending the time available for water and cryoprotectants to redistribute during freezing (Storey and Storey, 1988). High SCPs can substantially improve survival of internal ice, for example *C. costata* is freeze tolerant if ice formation is nucleated above -2 °C, but not at -20 °C (Shimada and Riihimaa, 1988). However, a high SCP is not sufficient for freeze tolerance: for example, *Eleodes blanchardi* beetles held at +20 °C retain their high SCP (-6.5 °C), but lose their freeze tolerance (Zachariassen and Hammel, 1976).

This model of osmotic cellular dehydration to avoid IIF depends on transmembrane water movement during ice formation. The bulk of water movement across cell membranes is through AQPs, which may also transport glycerol (aquaglyceroporins, GLPs; or entomoglyceroporins, EGLPs) and other small polar molecules (Finn et al., 2015; Hub and De Groot, 2008), and likely facilitate intracellular accumulation of low molecular weight cryoprotectants (Izumi et al., 2007). If AQPs are inhibited (e.g. by mercuric chloride), cells from *C. suppressalis* and *E. solidaginis* do not survive freezing (Izumi et al., 2007; Philip et al., 2008). Water moves very quickly through AQPs (Izumi et al., 2006), which will be particularly important for osmotic dehydration if ice formation is rapid (e.g. if nucleated at low temperatures; Duman and Horwath, 1983). While AQPs may be important for preventing IIF, these transporters may facilitate other dynamic processes associated with freeze tolerance (e.g. glycerol distribution, recovery during thawing), and are necessary for general cellular homeostasis. I also note that avoiding IIF

is not necessary for freeze tolerance, but the prevalence of, and mechanisms underlying, survival of IIF are unknown (Sinclair and Renault, 2010).

Ice-binding proteins can regulate ice crystal shape and size (Fig. 1.4). The insect AFPs examined to date inhibit recrystallization, at least *in vitro* (Table 1.2; Horwath et al., 1996; Knight and Duman, 1986; Walters et al., 2009a; Wharton et al., 2009). This recrystallization inhibition may reduce damage due to ice crystal growth at high subzero temperatures (Mazur, 2010). Thus, while AFPs prevent growth of nascent ice crystals in many freeze-avoidant species *via* thermal hysteresis (TH) activity (i.e. non-colligative depression of freezing point), AFPs in freeze-tolerant insects are hypothesized to modify the behaviour of existing ice crystals (Duman, 2001; Walters et al., 2011). I note that RIs do not always have TH activity (e.g. in the nematode *P. davidi*; Wharton et al., 2005a), and that some freeze-tolerant insects do not exhibit RI activity [e.g. *Tipula trivittata* (Knight and Duman, 1986) and *H. maori* (Ramløv et al., 1996)]. Thus, it is not clear if recrystallization is unimportant for some freeze-tolerant species, or if we cannot comprehensively identify the mechanisms that regulate it, and I suggest characterizing RI activity in more freeze-tolerant species (e.g. *via* the high-throughput gold nanoparticle aggregation assay; Mitchell et al., 2015). I am not aware of *in vivo* observations of the quality of ice or the recrystallization process in freeze-tolerant insects.

1.4.2 Controlling ice content reduces damage due to dehydration

The proportion of water that is converted into ice depends on solute concentration, temperature, and time. Because insects cannot control temperature or time spent frozen, we assume that frozen insects are at their equilibrium ice content in nature. Freeze-tolerant insects may reduce equilibrium ice content by accumulating low molecular weight cryoprotectants (Fig. 1.4, Table 1.2; Rozsypal et al., 2018; Storey and Storey, 1988; Tattersall et al., 2012; Zachariassen et al., 1979a), which act as compatible osmolytes (Somero et al., 2017). The concentration of these low molecular weight cryoprotectants is assumed to equilibrate between intracellular and extracellular spaces, and therefore high hemolymph osmolality will be associated with low ice content (Fig. 1.5). Minimizing ice content should (by reducing cellular volume loss through

dehydration) reduce shrinkage and molecular crowding in the (unfrozen) cells of the frozen insect, improving survival.

In practice, the link between ice content and survival is not entirely clear. For example, freeze-tolerant *E. blanchardi* appear uninjured when up to 65 % of body water freezes, while freeze-sensitive morphs are injured at an ice content of only 55 % (Zachariassen et al., 1979b). Furthermore, frozen *H. maori* (Ramløv, 1999; Ramløv and Westh, 1993) and *H. borealis* (Worland et al., 2000) have ice contents above 80 % (Fig. 1.5), which should lead to substantial dehydration stress. In addition, while proline accumulation correlates with lower ice content in *C. costata*, vitrification (rather than ice content) appears to be the strongest predictor of freeze tolerance at low temperatures (Rozsypal et al., 2018). This suggests that ice content (and dehydration of cells below a critical MCV) is not the sole determinant of frozen insect survival. There are surprisingly few ice content studies, and no cell volume measurements, in freeze-tolerant insects. I suggest that additional data are required to critically evaluate the relationship between ice content, cell volume, and mortality of frozen insects.

An alternative to avoiding dehydration stress may be to mitigate it, for example by accumulating anhydroprotectant molecules (e.g. trehalose) that stabilize macromolecules and cells under low water activity (Crowe et al., 1987). These anhydroprotectants could enhance freeze-tolerant insect survival at concentrations too low to alter ice content substantially. For example, the approximately 40–85 mM proline accumulated by overwintering *H. maori* (Neufeld and Leader, 1998; Ramløv, 1999) likely has only a minimal impact on ice content, but could facilitate survival by directly protecting macromolecules (see Section 1.3.3) under the (presumably intense) desiccating conditions associated with >80 % ice content. In Chapter 2, I measure hemolymph osmolality of freeze-tolerant and freeze-intolerant *G. veletis*, identify the source of elevated osmolality, and discuss the potential impact on ice content and dehydration tolerance.

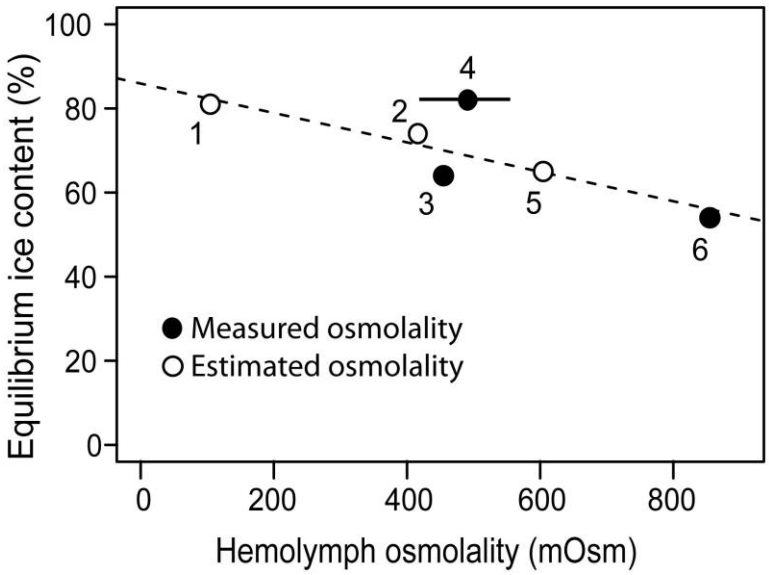

Figure 1.5. Relationship between hemolymph osmolality and ice content. Values of equilibrium ice content and hemolymph osmolality are from: 1, *Heleomyza borealis* (Worland et al., 2000); 2, *Celatoblatta quinquemaculata* (Block et al., 1998a); 3, *Osmoderma eremicola* (Storey et al., 1993); 4, *Hemideina maori* (Ramløv, 1999; Ramløv and Westh, 1993); 5, *Eurosta solidaginis* (Lee and Lewis, 1985; Morrissey and Baust, 1976); 6, *Pyrrharctia isabella* (Layne and Blakeley, 2002). Most ice contents were measured at temperatures between -8 °C and -10 °C, with the exception of *E. solidaginis* (-25 °C). Filled circles, hemolymph osmolality measured by osmometry; open circles, hemolymph osmolality calculated from sum of hemolymph concentrations of sugars and polyols. When multiple osmolality measurements were available, the range is indicated by a solid line. Dashed line indicates regression line through the data (r^2 = 0.62, P = 0.06).

1.4.3 Stabilizing cells and macromolecules reduces cell damage and death

Like all ectotherms, insects can mitigate the cellular effects of low temperatures by altering their macromolecules (Fig. 1.4; Somero et al., 2017; Storey and Storey, 2013; Tattersall et al., 2012). These changes include alterations in membrane composition to maintain fluidity (and integrity) at low temperatures, e.g. higher concentrations of polyunsaturated phospholipids in *C. costata* (Koštál et al., 2003). Insects may also accumulate more flexible protein isoforms that retain function and are less likely to denature or aggregate at low temperatures. For example, *Cucujus clavipes* have winter-specific cytoskeletal protein isoforms (Carrasco et al., 2011). While the cellular stresses associated with cold are common to both freeze-tolerant and freeze-avoidant insects, here I focus on how freeze-tolerant insects protect against the specific cellular stresses associated with internal ice formation, including low water availability, and dehydration-induced molecular crowding and cell shrinkage (Fig. 1.4).

Because freezing reduces water availability, freeze-tolerant insects must counteract the destabilizing effect of dehydration on membranes (including organelle and vesicle membranes) and proteins. These macromolecules can be stabilized by direct or indirect (*via* the hydration shell) interaction with low molecular weight metabolites such as trehalose, proline, and other amino acids (Anchordoguy et al., 1988; Arakawa and Timasheff, 1982; Arakawa and Timasheff, 1983; Crowe et al., 1987; Rudolph and Crowe, 1985; Tsvetkova et al., 1991), several of which have been reported in freeze-tolerant insects (Table 1.2). In Chapter 2, I screen for potential low molecular weight cryoprotectants using metabolomics, and investigate cryoprotectant function in *G. veletis* freeze tolerance.

The molecular crowding associated with ice formation could promote unfavourable interactions among macromolecules, e.g. aggregation of denatured proteins. Freeze-tolerant insects may therefore accumulate molecules that refold, remove, or isolate denatured proteins. Proline (Rudolph and Crowe, 1986) and arginine (Arakawa and Tsumoto, 2003; Das et al., 2007) may reduce protein aggregation by forming chains/clusters to physically buffer proteins from each other (Koštál et al., 2016). I

speculate that freeze-tolerant insects may also accumulate intrinsically disordered proteins (Table 1.3), which prevent protein aggregation under cold and dehydrating conditions (Newman et al., 2017; Toxopeus et al., 2014). Several freeze-tolerant insects accumulate HSPs (Lu et al., 2014; Rinehart et al., 2006; Zhang et al., 2011). These molecular chaperones (Table 1.3) can prevent denaturation and/or aggregation under osmotic stress and low temperatures, and may refold denatured proteins (King and MacRae, 2015), which will both maintain cellular function and reduce macromolecular damage. In Chapter 3, I screen the *G. veletis* transcriptome for cytoprotective proteins, and discuss the extent to which their upregulation is associated with freeze tolerance.

Freezing-induced cell shrinkage could place the cell membrane, cytoskeleton, and cell–cell junctions under shear or strain stress. Well-documented changes in membrane composition [e.g. increased proportion of phosphatidylethanolamine (Izumi et al., 2009) or increased membrane sterol fraction (Koštál et al., 2013)] likely increase membrane flexibility and prevent rupture during shrinkage. Proteomic studies of *B. antarctica* (Li et al., 2009) and transcriptomic studies of freeze-intolerant insects (Clark et al., 2009; Des Marteaux et al., 2017; MacMillan et al., 2016) suggest that changes in cellular/tissue modelling are imporant for stress tolerance. I speculate that freeze-tolerant insects accumulate alternative isoforms of cytoskeletal and cell adhesion proteins that withstand the tensions associated with cell dehydration, or accumulate regulators of these proteins. Conversely, freeze-tolerant insects may not prevent cytoskeleton depolymerization at low temperatures, but rather accumulate chaperones that promote cytoskeleton reassembly post-freeze, as suggested by upregulation of chaperone T-complex protein 1 (TCP-1) in *E. solidaginis* (Storey and Storey, 2013). In Chapter 3, I discuss whether differential gene expression in freeze-tolerant *G. veletis* may regulate membrane and cytoskeleton remodelling.

1.4.4 Managing biochemical processes reduces damage from harmful metabolites

Because the frozen state is not static, freeze-tolerant insects may need to neutralize or prevent the production of harmful/toxic metabolites such as ROS and metabolic end products (e.g. lactate and uric acid; Fig. 1.4). The metabolic suppression accompanying

diapause in many overwintering insects (Hahn and Denlinger, 2011), and specifically associated with freezing (Irwin and Lee, 2002; Marshall and Sinclair, 2012), likely reduces production of these metabolites. To remove harmful metabolites, *B. antarctica* upregulates detoxifying cytochrome P450 enzymes when recovering from dehydration (Lopez-Martinez et al., 2009), and I expect to see similar responses during thawing of freeze-tolerant insects (Table 1.3). Overwintering *E. solidaginis* accumulate ion-scavenging proteins (e.g. ferritin) that likely reduce ROS formation, and also glutathione and antioxidant enzymes (e.g. superoxide dismutase) that neutralize ROS once produced (Table 1.3; Joanisse and Storey, 1998; Storey and Storey, 2010). Sirtuins are also important in ROS detoxification (Merksamer et al., 2013), but have not, to my knowledge, been explored in association with freeze tolerance. Similarly, strategies to mitigate the effects of accumulated anaerobic end products, such as lactate and alanine (e.g. lactate clearance in *E. solidaginis;* Storey and Storey, 1985) in frozen insects remain to be explored. In Chapter 2, I test whether freeze-tolerant *G. veletis* suppress their metabolic rate. I use both metabolomics (Chapter 2) and transcriptomics (Chapter 3) to investigate whether freeze-tolerant crickets alter metabolic pathway activity. I also screen the *G. veletis* transcriptome for genes whose upregulation could decrease the accumulation of harmful metabolites (Chapter 3).

I also anticipate disruption of non-metabolic processes with freezing, which freeze-tolerant insects may prevent, or recover post-thaw. For example, high Ca^{2+} concentrations due to cell dehydration will disrupt cell signalling (Zachariassen et al., 2004a), which freeze-tolerant insects may mitigate by accumulating ion chelators (Table 1.3). Other processes may be unavoidably disrupted, such as loss of membrane potentials (e.g. due to reduced activity of ion pumps; Overgaard and MacMillan, 2017) and reduced intracellular trafficking (e.g. due to high cytoplasmic viscosity in the frozen state; Lee, 2010; Zachariassen, 1985). These processes must instead be recovered during or post-thaw.

1.4.5 Repair and recovery of physiological function post-thaw

During or after thawing, insects must restore physiological function. If the freezing and thawing processes caused injuries, this damage must be repaired. Surprisingly, there is

almost no data on mechanisms of damage and repair in freeze-tolerant insects, so I am largely confined to speculation on how they recover at the whole-animal and cellular level, as well as how they recognize and repair damage (Fig. 1.4).

Many physiological processes depend on appropriate ion gradients across cell membranes and epithelia, including neuroendocrine function (neuron membrane potentials), locomotion (muscle membrane potentials), and digestion and excretion (ion gradients across gut and Malpighian tubule epithelia; Bradley, 2009). Thus, if ion and water balance were disrupted during freezing [as suggested by Boardman et al. (2011); Kristiansen and Zachariassen (2001); Štětina et al. (2018); but not Williams and Lee (2011)], recovery from freezing may parallel recovery from chill coma (MacMillan et al., 2012). That is, I predict that ATP-motivated ion transport is required to re-establish ion balance post-thaw, a process that is likely energetically demanding.

Because organismal integration is likely disrupted in frozen insects, I expect recovery processes to be regulated locally (e.g. by intracellular changes in [Ca^{2+}]; Teets et al., 2013) until integration of those systems (e.g. *via* neuroendocrine function) are restored. Although intracellular signalling is likely important for this regulation, freeze-tolerant insects may have to blunt/moderate signalling during thawing, when cell volume increases rapidly modify intracellular ion (and other signalling molecule) concentrations (Zachariassen et al., 2004a). Other processes that may be involved in restoring cellular homeostasis include refolding and/or reassembling denatured proteins (using chaperones such as HSPs; Štětina et al., 2018; Storey and Storey, 2013), and clearing metabolites accumulated during/prior to freezing, which may include harmful end products [e.g. in *C. costata* (Štětina et al., 2018) and *E. solidaginis* (Storey and Storey, 1985)], as discussed above, as well as cryoprotectants (e.g. glucose in the wood frog *L. sylvaticus*; Costanzo and Lee, 2013). I hypothesize that freeze-tolerant insects may remove these metabolites *via* catabolism, as well as whole-organism processes such as excretion and/or storage.

If damage occurred during freezing, recognition and repair of damage will be required (Fig. 1.4). The recovering insect may identify cellular damage *via* several markers, including accumulation of damaged proteins, disturbance of the cellular redox state, and

alterations in ion concentrations (e.g. Ca^{2+}) that alter signal transduction (Korsloot et al., 2004). Irreparably damaged cells may undergo apoptosis (programmed cell death) or necrosis (unregulated cell death) during recovery (Korsloot et al., 2004). Post-thaw, this cellular injury may activate immune responses (Sinclair et al., 2013a), and stimulate cell proliferation to repair tissue (Smith et al., 1990). Alternatively, insects may repair damaged cells, for example by removing damaged macromolecules to the proteasome, or organelles *via* autophagy (Štětina et al., 2018; Teets and Denlinger, 2013). These cellular components will need to be replaced, and I speculate that recovery from freezing could involve several waves of prioritized repair.

I expect both recovery and repair to be energetically costly. The beetles *Hydromedion sparsutum, Perimylops antarcticus* (Block et al., 1998b) and *E. blanchardi* (Zachariassen et al., 1979b) appear to have elevated metabolic rates post-thaw, whereas larvae of the lepidopteran *P. marioni* (Sinclair et al., 2004) and *Pyrrharctia isabella* (Marshall and Sinclair, 2011) do not. This discrepancy could arise from differences in methods (earlier studies used closed-system respirometry, whereas later studies used more sensitive open-flow systems), from a phylogenetic signal (beetles *versus* moths), or because repair and recovery is metabolically costly in some species but not in others. If repair and recovery are energetically costly, then I expect freeze-tolerant insects to manage those energy demands by ensuring an adequate energy supply (Sinclair, 2015; Sinclair and Marshall, 2018), and reducing overall energy demand during the freezing and thawing processes, by suppressing metabolism (e.g. in diapause; Irwin and Lee, 2002) and minimizing the need to replace/repair cells and macromolecules by sufficiently protecting them during freezing and thawing (Fig. 1.4).

1.5 Ecology and evolution of freeze tolerance

Sinclair and Chown (2010) identified freeze-tolerant Orthoptera, Blattaria, Coleoptera, Hymenoptera, Lepidoptera and Diptera, to which we can now add Phasmatodea (Dennis et al., 2015) and Plecoptera (Walters et al., 2009b), representing approximately one third of insect orders. Making this summary at the Order level is somewhat misleading, since there is ample evidence that freeze tolerance has evolved multiple times within most of these orders (Sinclair and Chown, 2010). Within the radiation of New Zealand stick

insects, for example, freeze tolerance has evolved at least twice (and freeze avoidance the same number of times), and within one species (*Niveaphasma annulata*) only five of the six populations studied were freeze tolerant (Dennis et al., 2015). Here, I discuss selective pressures and potential routes that allow insects to evolve freeze tolerance, and the implications for freeze tolerance under climate change.

1.5.1 Pressures that select for freeze tolerance: three non-exclusive hypotheses

1.5.1.1 Extreme low temperatures

The physical limit for maintaining aqueous solutions in a supercooled (i.e. liquid) state is around -58 °C (e.g. Miller, 1982). Some alpine habitats, and continental sub-Arctic and temperate habitats [notably the Yukon (Danks et al., 1997), Siberia (Li, 2016) and interior Alaska (Miller, 1982)] that have high insect abundance and diversity regularly experience air temperatures below this limit. While some insects likely survive by selecting buffered microhabitats [e.g. *Cossus cossus* caterpillars in soil, under snow cover (Li, 2016)], others are clearly exposed to ambient temperatures [e.g. *Upis ceramboides* overwinters under tree bark, above the snow line, exposed to -50 °C (Miller, 1978)]. This imposes strong selection pressure for either cryoprotective dehydration (e.g. *B. antarctica*; Elnitsky et al., 2008), vitrification (e.g. *C. clavipes*; Sformo et al., 2010) or freeze tolerance. Bale (1996) inferred that freeze tolerance was the most extreme form of cold tolerance (i.e. at one end of the continuum), and many insects from these extremely cold environments are indeed freeze tolerant (Li, 2016; Miller, 1982; Turnock and Fields, 2005). However Sinclair (1999) showed that the range of LLTs in freeze-tolerant insects was similar to that of freeze-avoidant insects, implying that freeze tolerance is not the only viable strategy for surviving extremely low temperatures.

1.5.1.2 High risk of freezing

I expect selection for freeze tolerance in species at high risk of inoculative freezing. Insects exposed to ice in their microhabitats (e.g. those encased in ice, or exposed to frozen soil) are susceptible to inoculative ice formation (Pedersen and Holmstrup, 2003; Ramløv, 1999; Zachariassen et al., 2004b). In these circumstances, freeze tolerance may

be advantageous compared to either cryoprotective dehydration (Elnitsky et al., 2008; Holmstrup, 2014) or resisting ice formation through an impermeable cuticle and AFPs (Crosthwaite et al., 2011). Aquatic insects might be particularly exposed to these conditions: at least one aquatic insect (*Nemoura arctica*) is freeze tolerant (Walters et al., 2009b), and I expect that Odonata (Sawchyn and Gillott, 1975) and Trichoptera (Olsson, 1981) that overwinter encased in ice are also freeze tolerant. Freeze tolerance may also be prevalent in wetland insects in the Arctic and sub-Arctic, where there is a high diversity of Diptera whose cold tolerance has been only sparsely investigated (Danks et al., 1994; Ring, 1982). Similarly, insects with gut floras that produce INAs (e.g. gut bacteria of *H. sparsutum*; Worland and Block, 1999), or that do not clear their gut of food prior to cold exposure (e.g. *Hemideina* spp.; Sinclair et al., 1999) may be prone to inoculation from the gut.

Sinclair et al. (2003) proposed that environments with unpredictable, year-round likelihood of freezing events select for insects to remain active, e.g. to take advantage of warm spells between freezes. In turn, this selects against significant preparation for winter, such as entering diapause and/or clearing the gut. If the gut remains full, then there is a strong likelihood of ice nucleation, leading to selection for freeze tolerance. Alpine and sub-Antarctic environments in the Southern Hemisphere meet the criteria for frequent, unpredictable freeze events, and have a correspondingly high proportion of freeze-tolerant species (Sinclair et al., 2003; Sinclair and Chown, 2005a). This situation also applies to the strong daily variations in temperature on tropical high mountains, where there is also an apparent preponderance of freeze-tolerant insects (Sømme et al., 1996; Sømme and Zachariassen, 1981).

1.5.1.3 Physiological advantages of being frozen

Many insects overwintering in temperate and polar environments do not feed (although see Sinclair and Chown, 2005a), so energy and water stores over winter may be non-renewable. A cold-tolerance strategy that reduces energy drain or water loss should therefore be advantageous. Frozen insects lose less water to the dry winter environment than unfrozen insects at the same temperature (Danks, 2000; Irwin and Lee, 2002; Ring and Danks, 1994). Similarly, there is some evidence that frozen insects have lower

metabolic rates than their unfrozen counterparts at the same temperature (Irwin and Lee, 2002; Sinclair et al., 2004), and that this metabolic suppression allows frozen insects to save energy over an entire winter (Marshall and Sinclair, 2012).

Overwintering insects can experience significant mortality from pathogens and parasites, or can bear the eggs or larvae of parasitoids. If freeze-tolerant insects can withstand being frozen better than their pathogens, parasites, or parasitoids, then freeze tolerance may be a strategy to reduce pathogen or parasite loads. There is ample evidence of immune activation or modification during winter (Ferguson and Sinclair, 2017), and at least one freeze-tolerant insect (*P. isabella*) has greater resistance to fungal pathogens after freezing exposure, implying an activation of the immune system (Marshall and Sinclair, 2011). A survey of cold-tolerance strategies of Arctic sawflies and their hymenopteran parasitoids concluded that both the hosts and parasitoids are freeze tolerant (Humble, 2006). In addition, Tyrrell et al. (1994) showed that nematode intestinal parasites can survive freezing of their *H. maori* host (and other nematodes are also significantly cold tolerant, see, e.g. Wharton, 1995). However, there is variation in the freeze tolerance of entomopathogenic nematodes that could make host freeze tolerance advantageous in some instances (Shapiro-Ilan et al., 2014). To my knowledge, the effect of freezing (rather than just cold) on fungal or bacterial pathogens has not been explored in freeze-tolerant insects. Thus, there is considerable scope for investigating the role of pathogens in the evolution of freeze tolerance.

1.5.2 Routes to evolve freeze tolerance

The evolutionary path to freeze avoidance is intuitive: insects that freeze die, those with subtle improvements in maintaining their body fluids in a liquid state will survive, and if that improvement is heritable, then it will be passed onto their offspring. To make the transition to withstanding internal ice formation is more difficult: many insects are killed by even a small amount of internal ice (Sinclair and Chown, 2010). Here I examine two (non-mutually exclusive) routes towards evolving freeze tolerance: pre-adaptation to related stresses and partial freeze tolerance.

1.5.2.1 Pre-adaptation to cold and/or desiccation

Desiccation tolerance, freeze avoidance and freeze tolerance share many protective molecules (Tables 1.2 and 1.3), both large (e.g. TH proteins, HSPs) and small (e.g. glycerol, proline). Similarly, freeze tolerance and desiccation tolerance share many characteristics, at both the organismal level (Ring and Danks, 1994), and at the cellular level, where the model of extracellular freezing implies intracellular desiccation (Fig. 1.3). Thus, insects already adapted to dry and/or cold environments could co-opt mechanisms of desiccation or cold tolerance to facilitate survival of internal ice.

Many terrestrial insects are physiologically adapted to dry environments (Sømme, 2012). At the extremes, there are insects which can withstand complete loss of body water (Sakurai et al., 2008), as well as those that exploit dehydration as a freeze-avoidance strategy [*B. antarctica* (Elnitsky et al., 2008); and *C. clavipes* (Sformo et al., 2010)]. Thus, it is plausible that the biochemical and cellular mechanisms for freeze tolerance evolved *via* cross tolerance for desiccation (Sinclair et al., 2013a). For example, *B. antarctica* can employ both freeze tolerance and cryoprotective dehydration (Elnitsky et al., 2008; Lee et al., 2006), which supports a link, at least in this case. However, although anhydrobionts tolerate extensive cellular dehydration (Sakurai et al., 2008), increased desiccation tolerance at the organismal level is not necessarily associated with increased desiccation tolerance at the cellular level. Among-species variation in desiccation tolerance in *Drosophila* is largely driven by changes in water loss rates (Rajpurohit et al., 2008) or initial water content (Gibbs and Matzkin, 2001). In addition, dehydrated insects preferentially lose hemolymph volume to preserve cellular volume (Barton-Browne, 1964). Clearly, more work comparing the capacity of insect cells to tolerate dehydration (I predict that freeze-tolerant species should have high capacity) is necessary to understand the relationship between cold and desiccation tolerance.

Alternately, freeze tolerance could arise by co-opting adaptations, such as polyol cryoprotectants and AFPs, present in already cold-hardy lineages. If the cryoprotectants in freeze-avoidant animals provide adequate protection from internal ice formation, then this transition appears relatively straightforward. This may explain the shift from freeze avoidance to freeze tolerance in species like *C. costata* that rely on inoculative freezing

(Shimada and Riihimaa, 1988), and the strategy shift in the other direction in *D. canadensis* (Horwath and Duman, 1984). Freeze-avoidant species with very high hemolymph cryoprotectant concentrations may survive IIF because their low content of freezable water should minimize ice content. This property could explain the existence of freeze-tolerant species with very low SCPs (Ring, 1982) and provide another route of transition to freeze tolerance (K.E. Zachariassen, personal communication). Under this hypothesis, I predict that some set of freeze-tolerant species have very cold-tolerant freeze-avoidant ancestors; unfortunately, there has been relatively little exploration of the evolution of cold tolerance in phylogenies with temperate or polar (rather than tropical or sub-tropical) ancestries, so I cannot yet evaluate the strength of this hypothesis.

1.5.2.2 Partial freeze tolerance

Many insects are partially freeze tolerant; that is, they will survive the initiation of ice formation (and consequently a small amount of ice in their body), but are then killed if that ice formation progresses beyond some threshold (Sinclair, 1999). Sinclair (1999) and Voituron et al. (2002) suggest that this partial freeze tolerance could be an evolutionary route to freeze tolerance: individuals that are exposed to brief periods in the cold (e.g. the variable habitats of the southern hemisphere or tropical high mountains; Sinclair et al., 2003) might have differential mortality, conferring a selective advantage on those that are better able to withstand ice formation, leading to the evolution of freeze tolerance. Partial freezing can only occur when environmental cold exposure is shorter than the duration of ice formation. I therefore hypothesize that this evolutionary pathway to freeze tolerance is more likely in (large) species where ice formation takes a long time [e.g. stick insects (Dennis et al., 2015); *Hemideina* spp. (Sinclair et al., 1999)], making partial freezing likely in nature.

This pattern of partial freeze tolerance begetting freeze tolerance is supported in New Zealand stick insects, in which partial freeze tolerance is a widespread – and possibly ancestral – trait (Dennis et al., 2015). However, there is relatively little evidence that partial freeze tolerance is heritable. Morey et al. (2013) attempted to select for partial freeze tolerance by removing *Epiphyas postvittana* (light brown apple moth) larvae at their SCP. Although the SCP distribution shifted slightly (suggesting that SCP is

heritable), there was no significant change in freeze tolerance. Damage caused by partial ice formation has not been well explored, but could include mechanical damage from ice, as well as damage caused by dehydration/osmotic stress (see Sections 1.3.2 and 1.3.3). I am unaware of any other selection experiments on freeze tolerance in insects, although this approach could yield valuable information about the evolution of physiological traits (Gibbs, 1999).

1.5.3 Insect freeze tolerance in a changing climate

Climate change will affect the frequency and intensity of extreme temperature events, as well as the duration of winter and the timing of these extreme cold events [see Williams et al. (2015) for a comprehensive review]. An increase in the frequency of extreme cold events (which may be a result of increased weather variability or of reduced snowpack leading to more exposure to freeze–thaw; Marshall and Sinclair, 2012; Williams et al., 2015) could have contrasting implications for freeze-tolerant insects. If repeated freezing comes with a substantial cost (Bale et al., 2001; Brown et al., 2004; Marshall and Sinclair, 2011; Sinclair and Chown, 2005b), then increased frequency of freezing events will be detrimental to freeze-tolerant insects compared to their freeze-avoidant counterparts. Conversely, if unpredictable freezing events favour freeze tolerance, then freeze tolerance may remain advantageous, as it appears to be for insects in the low alpine zone of New Zealand (Sinclair, 2001). The energy savings associated with freeze tolerance could hypothetically increase survival if increased precipitation leads to longer winters; however, microclimate temperatures beneath snow are too warm for insects to freeze in most habitats (Williams et al., 2015), so freeze-tolerant insects may not gain any advantage. Thus, the role of cold-tolerance strategy in predicting insect responses to changing climate will likely be both species- and habitat-specific.

1.6 New hypotheses and relevant tools

Molecules associated with freeze tolerance can be identified by comparing freeze-tolerant and freeze-intolerant individuals from different species (Joanisse and Storey, 1996), populations (Lee and Lewis, 1985), seasons (Baust and Lee, 1981) or laboratory treatments (Koštál et al., 2011). The advent of untargeted -omics approaches has (at least

in theory) facilitated the identification of a wider range of molecules (Courteau et al., 2012; Dennis et al., 2015; Poupardin et al., 2015). While continuing to identify and describe cryo- and cytoprotectants in freeze-tolerant insects is useful, lists of molecules or putative pathways do not lead automatically to an understanding of mechanisms. Indeed, it is unclear whether there is functional convergence of biochemically unrelated cryoprotectants (e.g. can proline and trehalose fill the same roles?) or whether unique cryoprotectants are required in different mechanisms. I argue that a thorough understanding of the mechanisms of freeze tolerance requires a better characterization of the processes and challenges associated with cooling, freezing, and thawing, and a critical assessment of how cryoprotectants modify those processes and mitigate the challenges.

1.6.1 Understanding the processes and challenges of freezing

Most of our empirical data about processes and challenges associated with cooling and rewarming are derived from studies on chill-susceptible insects (Overgaard and MacMillan, 2017), and mammalian cell cryopreservation (Pegg, 2010). However, cryopreservation conditions (vitrified cells in suspension) do not reflect those that insects experience in nature (e.g. cooling rates of < 1 °C min^{-1}; Sinclair, 1997; Sinclair, 2001). Similarly, freeze-tolerant insects appear to resist the challenges observed in chill-susceptible insects; for example, freeze-tolerant *Cyphoderris monstrosa* do not enter chill coma, but instead remain active at low temperatures until they freeze (Toxopeus et al., 2016). Thus, there is opportunity to focus efforts on how cooling, freezing and thawing alter biological processes and challenge survival in freeze-tolerant insects.

Internal ice formation is the most obviously unique feature of freeze tolerance, yet there have been only a few direct studies of the dynamic process of ice formation in real time. The existing studies on ice propagation have not identified the initial and final location of ice, or the size or quality of ice crystals, whether studied *in vivo* (e.g. synchrotron X-ray visualization; Sinclair et al., 2009) or *ex vivo* (e.g. live cell imaging; Sinclair and Wharton, 1997). *In silico* models of ice formation (e.g. Haji-Akbari and Debenedetti, 2017) are not yet scalable to whole animals (Li and Liu, 2010), but could be used to explore cellular dehydration and IIF (see Botkin et al., 2011; Fadda et al., 2011), and ice propagation (e.g. Lee et al., 1993; Worland et al., 2004) in real time (Table 1.5).

Given the difficulty in studying ice formation in real time, an alternative approach is to take ‘snapshots’ throughout the freezing process, and recreate the dynamic nature and responses. For example, freeze-substitution (e.g. Wharton et al., 2005b) could be used to pinpoint the location of ice at different time points in the freezing process (Table 1.5). In addition, snapshots of ice content, determined by calorimetry, can be used to quantify the amount of ice at different temperatures and times in the freezing process (Table 1.5; Koštál et al., 2012; Lee and Lewis, 1985; Ramløv and Westh, 1993). This ‘snapshot’ approach could be combined with -omics approaches to characterize dynamic responses by the insect to cooling, freezing, thawing, and recovery.

As an adjunct to understanding the ice formation process and cellular responses to it, we need to determine the cause of injury in insects that do not survive ice formation. To better understand the challenges associated with freezing, I encourage moving beyond binary measurements of freeze injury (e.g. cell death or survival), to document damage during the freeze–thaw process at the ultrastructural and macromolecular level (e.g. using techniques listed in Table 1.5). It should also be possible to detect responses to damage, including apoptosis and autophagy (Table 1.5). We should then determine the causes of this damage. For example, it would be valuable to test whether high ice contents (Fig. 1.5) are associated with cell/organismal death in freeze-tolerant insects (e.g. Lee and Lewis, 1985). Similarly, it should be possible to distinguish freeze-induced cellular damage caused by osmotic stress (e.g. high ion concentrations – as measured by fluorescent dyes, similar to Teets et al., 2013) or dehydration stress (e.g. membrane fusions determined by electron microscopy; Collins et al., 1997), rather than mechanical damage. By describing processes and challenges better, especially those associated with ice, I expect that we will be in a much better position to test how cryo- and cytoprotectants contribute to freeze tolerance by altering processes or mitigating challenges

Table 1.5. Techniques and tools for measuring parameters that will inform our understanding of processes, challenges, and mechanisms of insect freeze tolerance.

Technique/tool	Measurement	Reference
Processes: characterizing the freezing process		
Synchrotron X-ray imaging	Whole-body ice formation (real time)	Sinclair et al. (2009)
Cold stage microscopy	Intracellular ice formation (real time)	Sinclair & Wharton (1997)
Freeze substitution	Ice location	Wharton et al. (2005)
Calorimetry (e.g. differential scanning calorimetry)	Ice content	Rozsypal et al. (2018)
Transcriptomics and (phospho-)proteomics	Gene expression (and regulation thereof)	Courteau et al. (2012) and Chapter 3
Metabolomics	Cryoprotectant composition/location	Koštál et al. (2011)
Challenges: measuring damage/responses to damage		
Live–dead staining	Cell death or survival	Yi & Lee (2003) and Chapter 2
Electron microscopy	Ultrastructural changes/damage	Collins et al. (1997)
Protein misfolding assays	Protein aggregation	Gregoire et al. (2012)
Comet assay	DNA damage	Olive & Banáth (2006)
Membrane lipid peroxidation assay	Oxidative damage	Lopez-Martinez et al. (2008)
Protein carbonylation assay	Oxidative damage	Lopez-Martinez et al. (2008)
TUNEL assay	Apoptosis	Yi et al. (2007)
Monodansylcadaverine staining	Autophagy	Wu et al. (2011)
Fluorescent ion chelators	Intracellular ion concentrations (osmotic pressure)	Teets et al. (2013)
Mechanisms: cryoprotectant manipulations		
Injection/feeding	Increase cryoprotectant concentrations *in vivo*	Koštál et al. (2016) and Chapter 2
RNA interference	Knockdown protein synthesis	Scott et al. (2013) and Chapter 4
CRISPR-Cas9	Generate knockout mutants	Gratz et al. (2013)

1.6.2 Models and experimental manipulations for understanding freeze tolerance

Although the repeated evolution and complexity of freeze tolerance makes it difficult to draw generalizations about mechanisms and processes, within- and among-species comparisons are a potential tool to test mechanisms underlying freeze tolerance. However, I identify several caveats: 1) within-species comparisons of freeze-tolerant and freeze-intolerant morphs can be confounded by life history. For example, freeze-tolerant stages of *E. solidaginis* and *C. costata* are in diapause (Table 1.1) while freeze-intolerant stages are not. Thus, care must be taken to disentangle mechanisms associated with diapause from those associated with surviving internal ice formation. For example, upregulation of storage proteins in a freeze-tolerant insect is more likely to be an important component of diapause (Hahn and Denlinger, 2011), but unlikely to protect against low temperatures or ice. In species that are freeze tolerant only when in deep diapause, it may not be possible to disentangle diapause and freeze tolerance. For example, metabolic rate suppression may be important for developmental arrest (diapause) as well as for preventing metabolic dysregulation when frozen. 2) Among-species comparisons may be confounded by phylogeny. For example, the differences in post-thaw metabolic rate between *H. sparsutum*, *P. antarcticus* (Block et al., 1998b) and *E. blanchardi* (Zachariassen et al., 1979b) relative to *P. marioni* (Sinclair et al., 2004) and *P. isabella* larvae (Marshall and Sinclair, 2011) may reflect a phylogenetic signal (Coleoptera *versus* Lepidoptera; Table 1.1), rather than different post-thaw recovery processes. Correcting for phylogeny is theoretically straightforward (Garland et al., 2005), but practically difficult, since it requires freeze-tolerant species to be placed within a resolved phylogeny of many species for which cold tolerance has been explored (e.g. Dennis et al., 2015; Sinclair et al., 1999). With these caveats in mind, we can use within- and among-species comparisons to continue to identify putative cryo- and cytoprotectants, and generate hypotheses concerning the mechanisms by which they contribute to freeze tolerance.

To test hypotheses about the mechanisms underlying freeze tolerance, I suggest identifying Krogh models that allow laboratory manipulations, and the disentangling of

confounding factors such as life stage and diapause. Many putative cryo- and cytoprotectants are associated with freeze tolerance (Tables 1.2 and 1.3), yet their function remains in the realm of hypothesis. Experimental manipulation of these cryoprotectants is the most powerful approach to understanding their function, such as reducing cryoprotectant synthesis [e.g. *via* CRISPR/Cas9 technology (Gratz et al., 2013) or RNA interference (RNAi; Scott et al., 2013)] or increasing cryoprotectant abundance [e.g. by feeding (Koštál et al., 2016) or injection (Benoit et al., 2009; Rosendale et al., 2016)]. If cryoprotectant manipulations reduce or enhance freeze tolerance, we can conclude that they contribute to the mechanisms underlying freeze tolerance. We can also use these experiments to test whether different cryoprotectants contribute to similar mechanisms underlying freeze tolerance. As a caveat, these manipulative experiments cannot be applied to understand all mechanisms; for example, it would be challenging to modify the abundance of acetylated triacylglycerols in *E. solidaginis* (Marshall et al., 2014) because their synthesis is unknown, and manipulating the composition of (intracellularly) stored triacylglycerols is difficult, if not impossible. Because there is no 'magic bullet' molecule conferring freeze tolerance these manipulations will also need to unravel synergisms among cryo- and cytoprotectants, in the context of both cellular-level processes and whole-organism survival.

Of the many freeze-tolerant insects I have highlighted in this review, I identify several that are particularly promising as emerging model systems (denoted by asterisks in Table 1.1). A good model for within-species comparisons that avoid confounding effects of life history is *G. veletis*, which has both freeze-tolerant and freeze-intolerant morphs at the same life stage (late instar nymph; McKinnon, 2015), without any apparent diapause. I explore this model further in the remainder of my thesis. Both New Zealand stick insects (Dennis et al., 2015) and weta (Sinclair et al., 1999) are good systems for examining freeze tolerance among species: they have well-resolved phylogenies, and include freeze-tolerant, partially freeze-tolerant, and freeze-intolerant lineages. There is also extensive diversity in cold tolerance of Diptera and Coleoptera, which could be amenable to among-species comparisons. For example, there are both freeze-tolerant and freeze-avoidant species in the Holarctic carabid genus *Pterostichus* (Miller, 1969; Rossolimo, 1997). To manipulate cryo- and cytoprotectants experimentally, we must work with

species that can be reared in laboratories, such as *C. costata* and *G. veletis*. *Chymomyza costata* larvae are amenable to cryoprotectant manipulation *via* feeding (Koštál et al., 2011), and, once the in-progress genome sequencing is completed (V. Koštál, personal communication), CRISPR-Cas9 (e.g. Newman et al., 2017). *Gryllus veletis* are likely responsive to RNAi (as are other *Gryllus* spp.; Meyering-Vos et al., 2006), and are large enough (> 100 mg) to allow cryoprotectant concentrations to be manipulated easily *via* injection, and to perform tissue/cell-specific assays. I use these methods in Chapters 2 (cryoprotectant injection, cell survival assays) and 4 (RNAi). In addition, we can advance our understanding of freeze tolerance by identifying mechanisms by which freeze-intolerant model organisms can be converted to freeze-tolerant insects. This has been done with some success by feeding proline to *D. melanogaster* (Koštál et al., 2012), and could represent a gold standard for confirming the sufficiency of putative cryoprotectants or processes in freeze tolerance. Continued characterization of cold-tolerance strategies will likely reveal additional potential models, which will be critical in unravelling the complexity and diversity of mechanisms underlying freeze tolerance.

1.7 Conclusion

Freeze tolerance facilitates survival of low temperatures and unpredictable climates, and has evolved repeatedly across insects. However, we have limited understanding of the mechanisms that underlie survival of internal ice. I suggest that freeze tolerance is a complex cold tolerance strategy that requires surviving a process (cooling, freezing, thawing), and mitigating the associated challenges. I hypothesize that freeze-tolerant insects may use a variety of mechanisms to control ice, prevent or repair damage to cells and macromolecules, manage biochemical processes while frozen/thawing, and restore physiological processes post-thaw. Freeze tolerance likely evolved to facilitate survival in environments with extreme low temperatures and/or high risk of freezing, and in cases where freezing offers a physiological advantage (e.g. energy reserve management). This cold-tolerance strategy may have evolved in insects that were partially freeze-tolerant, or those already well adapted to stresses associated with low temperatures and dehydration. Refocusing freeze-tolerant research on hypothesis-driven studies in appropriate

laboratory models will help resolve this almost 300 year-old question of how insects survive internal ice.

1.8 Thesis overview

To better understand the mechanisms underlying freeze tolerance, in this thesis I further develop *G. veletis* as a laboratory model, generating and testing hypotheses about the mechanisms underlying freeze tolerance. I use both descriptive and functional approaches to determine:

1) physiological changes correlated with freeze tolerance; and
2) whether and how those changes function in freeze tolerance.

To do so, I have structured my thesis into four objectives, addressed over three chapters. Chapters 2-4 in this thesis were prepared as manuscripts for publication.

My first objective was to characterize how freeze tolerance alters the metabolome and other physiological parameters (metabolic rate, ice nucleation, hemolymph osmolality) of *G. veletis*, and to identify potential cryoprotective molecules. My second objective was to determine whether these potential cryoprotectants function in *G. veletis* freeze tolerance, facilitating survival of internal ice formation. In Chapter 2 ("Cryoprotectant molecules differentially enhance freeze tolerance of the spring field cricket *Gryllus veletis*"), I find that freeze-tolerant *G. veletis* protect their cells from freeze injury, accumulate potential ice-nucleating agents, and suppress their metabolic rate relative to freeze-intolerant crickets. Using metabolomics, I determine that freeze-tolerant crickets accumulate three putative low molecular weight cryoprotectants. By manipulating metabolite concentrations *in vivo* and *ex vivo*, I demonstrate that each cryoprotectant improves freeze tolerance, but appears to do so *via* different mechanisms, based on how these molecules impact survival of freeze-tolerant *G. veletis* at their lethal limits. I conclude these cryoprotectants function in freeze tolerance, but that they (alone or in combination) are not sufficient for freeze tolerance.

My third objective was to identify additional processes that may contribute to or regulate freeze tolerance by characterizing the transcriptome of freeze-tolerant *G. veletis*. In Chapter 3 ("How crickets become freeze tolerant: the transcriptomic underpinnings of

acclimation in *Gryllus veletis*"), I assemble a *de novo* transcriptome of *G. veletis*, and compare gene expression between freeze-intolerant crickets (maintained under rearing conditions) and freeze-tolerant crickets (acclimated for six weeks). I find that freeze-tolerant *G. veletis* differentially regulate many genes that may protect cells at low temperatures and in the presence of ice, including those that support the physiological changes described in Chapter 2, and those that may support processes not previously identified in this model. I conclude with hypotheses concerning how these genes contribute to the mechanisms underlying freeze tolerance, by protecting macromolecules and preserving cell integrity.

My final objective was to develop a method to knock down gene expression in *G. veletis*, providing a tool to test which genes and processes are necessary for surviving internal ice formation. In Chapter 4 ("Development of a method to knock down gene expression in *Gryllus veletis* using RNA interference"), I develop a RNA interference (RNAi) protocol for *G. veletis*. I reduce expression of three target genes that are upregulated in freeze-tolerant crickets (Chapter 3), and identify further protocol considerations to knock down other targets in *G. veletis.* I suggest several follow-up experiments using RNAi to test hypotheses about the roles of antioxidant defence, cytoskeletal stability, and cryoprotectant transport in freeze tolerance.

1.9 References

Acker, J. P., Elliott, J. A. and McGann, L. E. (2001). Intercellular ice propagation: experimental evidence for ice growth through membrane pores. *Biophysical Journal* **81**, 1389-1397.

Anchordoguy, T., Carpenter, J. F., Loomis, S. H. and Crowe, J. H. (1988). Mechanisms of interaction of amino acids with phospholipid bilayers during freezing. *Biochimica et Biophysica Acta* **946**, 299-306.

Arakawa, T. and Timasheff, S. N. (1982). Stabilization of protein structure by sugars. *Biochemistry* **21**, 6536-6544.

Arakawa, T. and Timasheff, S. N. (1983). Preferential interactions of proteins with solvent components in aqueous amino acid solutions. *Archives of Biochemistry and Biophysics* **224**, 169-177.

Arakawa, T. and Tsumoto, K. (2003). The effects of arginine on refolding of aggregated proteins: not facilitate refolding, but suppress aggregation. *Biochemical and Biophysical Research Communications* **304**, 148-152.

Asahina, E., Aoki, K. and Shinozaki, J. (1954). The freezing process of frost-hardy caterpillars. *Bulletin of Entomological Research* **45**, 329-339.

Bale, J. S. (1996). Insect cold hardiness: a matter of life and death. *European Journal of Entomology* **93**, 369-382.

Bale, J. S., Worland, M. R. and Block, W. (2001). Effects of summer frost exposures on the cold tolerance strategy of a sub-Antarctic beetle. *Journal of Insect Physiology* **47**, 1161-1167.

Barton-Browne, L. B. (1964). Water regulation in insects. *Annual Review of Entomology* **9**, 63-82.

Baust, J. G. and Lee, R. E. (1981). Divergent mechanisms of frost-hardiness in two populations of the gall fly, *Eurosta solidaginsis*. *Journal of Insect Physiology* **27**, 485-490.

Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. A., Lee, R. E. and Denlinger, D. L. (2009). Dehydration-induced cross tolerance of *Belgica antarctica* larvae to cold and heat is facilitated by trehalose accumulation. *Comparative Biochemistry and Physiology A* **152**, 518-523.

Berger, W. and Uhrik, B. (1996). Freeze-induced shrinkage of individual cells and cell-to-cell propagation of intracellular ice in cell chains from salivary glands. *Cellular and Molecular Life Sciences* **52**, 843-850.

Block, W., Wharton, D. A. and Sinclair, B. J. (1998a). Cold tolerance of a New Zealand alpine cockroach, *Celatoblatta quinquemaculata* (Dictyoptera, Blattidae). *Physiological Entomology* **23**, 1-6.

Block, W., Worland, M. R. and Bale, J. (1998b). Respiratory responses to chilling and freezing in two sub-antarctic insects. *Cryobiology* **37**, 163-6.

Boardman, L., Terblanche, J. S. and Sinclair, B. J. (2011). Transmembrane ion distribution during recovery from freezing in the woolly bear caterpillar *Pyrrharctia isabella* (Lepidoptera: Arctiidae). *Journal of Insect Physiology* **57**, 1154-1162.

Botkin, N. D., Hoffmann, K.-H. and Turova, V. L. (2011). Optimal control of ice formation in living cells during freezing. *Applied Mathematical Modelling* **35**, 4044-4057.

Bradley, T. J. (2009). Animal osmoregulation. New York: Oxford University Press, Inc.

Brown, C. L., Bale, J. S. and Walters, K. F. A. (2004). Freezing induces a loss of freeze tolerance in an overwintering insect. *Proceedings of the Royal Society B* **271**, 1507-1511.

Carrasco, M. A., Buechler, S. A., Arnold, R. J., Sformo, T., Barnes, B. M. and Duman, J. G. (2011). Elucidating the biochemical overwintering adaptations of larval *Cucujus clavipes puniceus*, a nonmodel organism, via high throughput proteomics. *Journal of Proteome research* **10**, 4634-4646.

Clark, M. S., Thorne, M. A. S., Purać, J., Burns, G., Hillyard, G., Popović, Ž. D., Grubor-Lajšić, G. and Worland, M. R. (2009). Surviving the cold: molecular analyses of insect cryoprotective dehydration in the Arctic springtail *Megaphorura arctica* (Tullberg). *BMC Genomics* **10**, 328.

Collins, S. D., Allenspach, A. L. and Lee, R. E. (1997). Ultrastructural effects of lethal freezing on brain, muscle and Malpighian tubules from freeze-tolerant larvae of the gall fly, *Eurosta solidaginis*. *Journal of Insect Physiology* **43**, 39-45.

Costanzo, J. P. and Lee, R. E. (2013). Avoidance and tolerance of freezing in ectothermic vertebrates. *Journal of Experimental Biology* **216**, 1961-1967.

Courteau, L. A., Storey, K. B. and Morin, P. (2012). Differential expression of microRNA species in a freeze tolerant insect, *Eurosta solidaginis*. *Cryobiology* **65**, 210-214.

Crosthwaite, J. C., Sobek, S., Lyons, D. B., Bernards, M. A. and Sinclair, B. J. (2011). The overwintering physiology of the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). *Journal of Insect Physiology* **57**, 166-173.

Crowe, J. H., Crowe, L. M., Carpenter, J. F. and Wistrom, C. A. (1987). Stabilization of dry phospholipid bilayers and proteins by sugars. *Biochemical Journal* **242**, 1-10.

Crowe, J. H., Crowe, L. M. and Chapman, D. (1984). Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science* **223**, 701-703.

Danks, H. V. (2000). Dehydration in dormant insects. *Journal of Insect Physiology* **46**, 837-852.

Danks, H. V., Downes, J. A., Larson, D. J. and Scudder, G. G. E. (1997). Insects of the Yukon: characteristics and history. In *Insects of the Yukon* (eds. H. V. Hanks and J. S. Downes), pp. 963-1013. Ottawa: Biological Survey of Canada.

Danks, H. V., Kukal, O. and Ring, R. A. (1994). Insect cold-hardiness: insights from the Arctic. *Arctic* **74**, 391-404.

Das, U., Hariprasad, G., Ethayathulla, A. S., Manral, P., Das, T. K., Pasha, S., Mann, A., Ganguli, M., Verma, A. K. and Bhat, R. (2007). Inhibition of protein aggregation: supramolecular assemblies of arginine hold the key. *PLoS One* **2**, e1176.

Dennis, A. B., Dunning, L. T., Sinclair, B. J. and Buckley, T. R. (2015). Parallel molecular routes to cold adaptation in eight genera of New Zealand stick insects. *Scientific Reports* **5**, 13965.

Des Marteaux, L. E., McKinnon, A. H, Udaka, H., Toxopeus, J. and Sinclair, B. J. (2017). Effects of cold-acclimation on gene expression in Fall field cricket (*Gryllus pennsylvanicus*) ionoregulatory tissues. *BMC Genomics* **18**, 357.

Des Marteaux, L. E., Stinziano, J. R. and Sinclair, B. J. (2018). Effects of cold acclimation on rectal macromorphology, ultrastructure, and cytoskeletal stability in *Gryllus pennsylvanicus* crickets. *Journal of Insect Physiology* **104**, 15-24.

Dias, C. L., Ala-Nissila, T., Wong-ekkabut, J., Vattulainen, I., Grant, M. and Karttunen, M. (2010). The hydrophobic effect and its role in cold denaturation. *Cryobiology* **60**, 91-99.

Doelling, A. R., Griffis, N. and Williams, J. B. (2014). Repeated freezing induces oxidative stress and reduces survival in the freeze-tolerant goldenrod gall fly, *Eurosta solidaginis*. *Journal of Insect Physiology* **67**, 20-27.

Duman, J. G. (1980). Factors involved in overwintering survival of the freeze tolerant beetle, *Dendroides canadensis*. *Journal of Comparative Physiology B* **136**, 52-59.

Duman, J. G. (1984). Change in overwintering mechanism of the Cucujid beetle, *Cucujus clavipes*. *Journal of Insect Physiology* **30**, 235-239.

Duman, J. G. (2001). Antifreeze and ice nucleator proteins in terrestrial arthropods. *Annual Review of Physiology* **63**, 327-357.

Duman, J. G. (2015). Animal ice-binding (antifreeze) proteins and glycolipids: an overview with emphasis on physiological function. *Journal of Experimental Biology* **218**, 1846-1855.

Duman, J. G. and Horwath, K. L. (1983). The role of hemolymph proteins in the cold tolerance of insects. *Annual Review of Physiology* **45**, 261-270.

Duman, J. G., Morris, J. P. and Castellino, F. J. (1984). Purification and composition of an ice nucleating protein from queens of the hornet, *Vespula maculata*. *Journal of Comparative Physiology B* **154**, 79-83.

Duman, J. G., Neven, L. G., Beals, J. M., Olson, K. R. and Castellino, F. J. (1985). Freeze-tolerance adaptations, including haemolymph protein and lipoprotein nucleators, in the larvae of the cranefly *Tipula trivittata*. *Journal of Insect Physiology* **31**, 1-8.

Elliott, G. D., Wang, S. and Fuller, B. J. (2017). Cryoprotectants: a review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. *Cryobiology* **76**, 74-91.

Elnitsky, M. A., Hayward, S. A. L., Rinehart, J. P., Denlinger, D. L. and Lee, R. E. (2008). Cryoprotective dehydration and the resistance to inoculative freezing in the Antarctic midge, *Belgica antarctica*. *Journal of Experimental Biology* **211**, 524-530.

Fadda, S., Cincotti, A. and Cao, G. (2011). Rationalizing the equilibration and cooling stages of cryopreservation: the effect of cell size distribution. *AIChE Journal* **57**, 1075-1095.

Farewell, A. and Neidhardt, F. C. (1998). Effect of temperature on in vivo protein synthetic capacity in *Escherichia coli*. *Journal of Bacteriology* **180**, 4704-4710.

Ferguson, L. V. and Sinclair, B. J. (2017). Insect immunity varies idiosyncratically during overwintering. *Journal of Experimental Zoology A* **327**, 222-234.

Finn, R. N., Chauvigné, F., Stavang, J. A., Belles, X. and Cerdà, J. (2015). Insect glycerol transporters evolved by functional co-option and gene replacement. *Nature Communications* **6**, 7814.

Garland, T., Bennett, A. F. and Rezende, E. L. (2005). Phylogenetic approaches in comparative physiology. *Journal of Experimental Biology* **208**, 3015-3035.

Gibbs, A. G. (1999). Laboratory selection for the comparative physiologist. *Journal of Experimental Biology* **202**, 2709-2718.

Gibbs, A. G. and Matzkin, L. M. (2001). Evolution of water balance in the genus *Drosophila*. *Journal of Experimental Biology* **204**, 2331-2338.

Goto, S. G., Philip, B. N., Teets, N. M., Kawarasaki, Y., Lee, R. E. and Denlinger, D. L. (2011). Functional characterization of an aquaporin in the Antarctic midge *Belgica antarctica*. *Journal of Insect Physiology* **57**, 1106-1114.

Gratz, S. J., Cummings, A. M., Nguyen, J. N., Hamm, D. C., Donohue, L. K., Harrison, M. M., Wildonger, J. and O'Connor-Giles, K. M. (2013). Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. *Genetics* **194**, 1029-1035.

Gulevsky, A. K., Relina, L. I. and Grishchenkova, Y. A. (2006). Variations of the antioxidant system during development of the cold-tolerant beetle, *Tenebrio molitor*. *CryoLetters* **27**, 283-290.

Hahn, D. A. and Denlinger, D. L. (2011). Energetics of insect diapause. *Annual Review of Entomology* **56**, 103-121.

Haji-Akbari, A. and Debenedetti, P. G. (2017). Computational investigation of surface freezing in a molecular model of water. *Proceedings of the National Academy of Sciences of the United States of America* **114**, 3316-3321.

Hamilton, R. L., Mullins, D. E. and Orcutt, D. M. (1985). Freezing-tolerance in the woodroach *Cryptocercus punctulatus* (Scudder). *Experientia* **41**, 1535-1537.

Harrison, J. F. (2001). Insect acid-base physiology. *Annual Review of Entomology* **46**, 221-250.

Hazel, J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annual Review of Physiology* **57**, 19-42.

Holmstrup, M. (2014). The ins and outs of water dynamics in cold tolerant soil invertebrates. *Journal of Thermal Biology* **45**, 117-123.

Horwath, K. L. and Duman, J. G. (1984). Yearly variations in the overwintering mechanisms of the cold-hardy beetle *Dendroides canadensis*. *Physiological Zoology* **57**, 40-45.

Horwath, K. L., Easton, C. M., Poggioli, G. J., Myers, K. and Schnorr, I. L. (1996). Tracking the profile of a specific antifreeze protein and its contribution to the thermal hysteresis activity in cold hardy insects. *European Journal of Entomology* **93**, 419-433.

Hub, J. S. and De Groot, B. L. (2008). Mechanism of selectivity in aquaporins and aquaglyceroporins. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 1198-1203.

Humble, L. M. (2006). Overwintering adaptations in Arctic sawflies (Hymenoptera: Tenthredinidae) and their parasitoids: cold tolerance. *The Canadian Entomologist* **138**, 59-71.

Irwin, J. T. and Lee, R. E. (2002). Energy and water conservation in frozen vs. supercooled larvae of the goldenrod gall fly, *Eurosta solidaginis* (Fitch) (Diptera: Tephritidae). *Journal of Experimental Zoology* **292**, 345-350.

Izumi, Y., Katagiri, C., Sonoda, S. and Tsumuki, H. (2009). Seasonal changes of phospholipids in last instar larvae of rice stem borer *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). *Entomological Science* **12**, 376-381.

Izumi, Y., Sonoda, S. and Tsumuki, H. (2007). Effects of diapause and cold-acclimation on the avoidance of freezing injury in fat body tissue of the rice stem borer, *Chilo suppressalis* Walker. *Journal of Insect Physiology* **53**, 685–690.

Izumi, Y., Sonoda, S., Yoshida, H., Danks, H. V. and Tsumuki, H. (2006). Role of membrane transport of water and glycerol in the freeze tolerance of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). *Journal of Insect Physiology* **52**, 215-220.

Izumi, Y., Sonoda, S., Yoshida, H. and Tsumuki, H. (2005). Identification of tissues showing the lowest tolerance to freezing in larvae of the rice stem borer, *Chilo suppressalis*. *Physiological Entomology* **30**, 324-331.

Joanisse, D. R. and Storey, K. B. (1994). Enzyme activity profiles in an overwintering population of freeze-tolerant larvae of the gall fly, *Eurosta solidaginis*. *Journal of Comparative Physiology B* **164**, 247-255.

Joanisse, D. R. and Storey, K. B. (1996). Oxidative stress and antioxidants in overwintering larvae of cold-hardy goldenrod gall insects. *Journal of Experimental Biology* **199**, 1483-1491.

Joanisse, D. R. and Storey, K. B. (1998). Oxidative stress and antioxidants in stress and recovery of cold-hardy insects. *Insect Biochemistry and Molecular Biology* **28**, 23-30.

Jung, E. S., Choi, H., Song, H., Hwang, Y. J., Kim, A., Ryu, H. and Mook-Jung, I. (2016). p53-dependent SIRT6 expression protects Aβ42-induced DNA damage. *Scientific Reports* **6**, 25628.

King, A. M. and MacRae, T. H. (2015). Insect heat shock proteins during stress and diapause. *Annual Review of Entomology* **60**, 59-75.

Klok, C. J. and Chown, S. L. (1997). Critical thermal limits, temperature tolerance and water balance of a sub-Antarctic caterpillar, *Pringleophaga marioni* (Lepidoptera: Tineidae). *Journal of Insect Physiology* **43**, 685-694.

Knight, C. A. and Duman, J. G. (1986). Inhibition of recrystallisation of ice by insect thermal hysteresis proteins: a possible cryoprotective role. *Cryobiology* **22**, 256-262.

Knight, C. A., Hallett, J. and DeVries, A. L. (1988). Solute effects on recrystallization: an assessment technique. *Cryobiology* **25**, 55-60.

Korsloot, A., van Gestel, C. A. and van Straalen, N. M. (2004). Environmental Stress and Cellular Response in Arthropods. Boca Raton: CRC Press.

Koštál, V. (2010). Cell structural modifications in insects at low temperatures. In *Low Temperature Biology of Insects* (eds. D. L. Denlinger and R. E. Lee), pp. 116-140. New York: Cambridge University Press.

Koštál, V., Berková, P. and Šimek, P. (2003). Remodelling of membrane phospholipids during transition to diapause and cold-acclimation in the larvae of *Chymomyza costata* (Drosophilidae). *Comparative Biochemistry and Physiology B* **135**, 407-419.

Koštál, V., Korbelová, J., Poupardin, R., Moos, M. and Šimek, P. (2016). Arginine and proline applied as food additives stimulate high freeze tolerance in larvae of *Drosophila melanogaster*. *Journal of Experimental Biology* **219**, 2358-2367.

Koštál, V., Renault, D., Mehrabianova, A. and Bastl, J. (2007). Insect cold tolerance and repair of chill-injury at fluctuating thermal regimes: role of ion homeostasis. *Comparative Biochemistry and Physiology A* **147**, 231-8.

Koštál, V., Šimek, P., Zahradníčková, H., Cimlová, J. and Štětina, T. (2012). Conversion of the chill susceptible fruit fly larva (*Drosophila melanogaster*) to a freeze tolerant organism. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 3270-3274.

Koštál, V., Urban, T., Řimnáčová, L., Berková, P. and Šimek, P. (2013). Seasonal changes in minor membrane phospholipid classes, sterols and tocopherols in overwintering insect, *Pyrrhocoris apterus*. *Journal of Insect Physiology* **59**, 934-941.

Koštál, V., Zahradníčková, H. and Šimek, P. (2011). Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata*, survive cryopreservation in liquid nitrogen. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 13041-13046.

Kristiansen, E. and Zachariassen, K. E. (2001). Effect of freezing on the transmembrane distribution of ions in freeze-tolerant larvae of the wood fly *Xylophagus cinctus* (Diptera, Xylophagidae). *Journal of Insect Physiology* **47**, 585-592.

Lalouette, L., Williams, C., Hervant, F., Sinclair, B. J. and Renault, D. (2011). Metabolic rate and oxidative stress in insects exposed to low temperature thermal fluctuations. *Comparative Biochemistry and Physiology A* **158**, 229-234.

Layne, J. R. and Blakeley, D. L. (2002). Effect of freeze temperature on ice formation and long-term survival of the woolly bear caterpillar (*Pyrrharctia isabella*). *Journal of Insect Physiology* **48**, 1133-1137.

Leader, J. P. and Bedford, J. J. (1978). Haemolymph composition of two species of New Zealand weta, *Hemideina* (Orthoptera; Stenopelmatidae). *Comparative Biochemistry and Physiology A* **61**, 173-176.

Lee, R. E. (2010). A primer on insect cold-tolerance. In *Low Temperature Biology of Insects* (eds. D. L. Denlinger and R. E. Lee), pp. 3-34. New York: Cambridge University Press.

Lee, R. E., Dommel, R. A., Joplin, K. H. and Denlinger, D. L. (1995). Cryobiology of the freeze-tolerant gall fly *Eurosta solidaginis*: overwintering energetics and heat shock proteins. *Climate Research* **5**, 61-67.

Lee, R. E., Elnitsky, M. A., Rinehart, J. P., Hayward, S. A. L., Sandro, L. H. and Denlinger, D. L. (2006). Rapid cold-hardening increases the freezing tolerance of the Antarctic midge *Belgica antarctica*. *Journal of Experimental Biology* **209**, 399-406.

Lee, R. E. and Lewis, E. A. (1985). Effect of temperature and duration of exposure on tissue ice formation in the gall fly, *Eurosta solidaginis* (Diptera, Tephritidae). *Cryo-Letters* **6**, 25-34.

Lee, R. E., McGrath, J. J., Morason, R. T. and Taddeo, R. M. (1993). Survival of intracellular freezing, lipid coalescence and osmotic fragility in fat body cells of the freeze-tolerant gall fly *Eurosta solidaginis*. *Journal of Insect Physiology* **39**, 445-450.

Li, A., Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. A., Lee, R. E. and Denlinger, D. L. (2009). Distinct contractile and cytoskeletal protein patterns in the Antarctic midge are elicited by desiccation and rehydration. *Proteomics* **9**, 2788-2798.

Li, F.-F. and Liu, J. (2010). Characterization of micro-/nano-scale ice crystal formation in cryo-biomedical engineering: a review. *Journal of Computational and Theoretical Nanoscience* **7**, 85-96.

Li, N. (2016). Strong tolerance to freezing is a major survival strategy in insects inhabiting central Yakutia (Sakha Republic, Russia), the coldest region on earth. *Cryobiology* **73**, 221-225.

Lopez-Martinez, G., Benoit, J. B., Rinehart, J. P., Elnitsky, M. A., Lee, R. E. and Denlinger, D. L. (2009). Dehydration, rehydration, and overhydration alter patterns of gene expression in the Antarctic midge, *Belgica antarctica*. *Journal of Comparative Physiology B* **179**, 481-491.

Lopez-Martinez, G., Elnitsky, M. A., Benoit, J. B., Lee, R. E. and Denlinger, D. L. (2008). High resistance to oxidative damage in the Antarctic midge *Belgica antarctica*, and developmentally linked expression of genes encoding superoxide dismutase, catalase and heat shock proteins. *Insect Biochemistry and Molecular Biology* **38**, 796-804.

Lu, M.-X., Liu, Z.-X., Cui, Y.-D. and Du, Y.-Z. (2014). Expression patterns of three heat shock proteins in *Chilo suppressalis* (Lepidoptera: Pyralidae). *Annals of the Entomological Society of America* **107**, 667-673.

Lundheim, R. (2002). Physiological and ecological significance of biological ice nucleators. *Philosophical Transactions of the Royal Society of London B* **357**, 937-943.

Lundheim, R. and Zachariassen, K. (1993). Water balance of over-wintering beetles in relation to strategies for cold tolerance. *Journal of Comparative Physiology B* **163**, 1-4.

MacMillan, H. A., Baatrup, E. and Overgaard, J. (2015). Concurrent effects of cold and hyperkalaemia cause insect chilling injury. *Proceedings of the Royal Society B* **282**, 20151483.

MacMillan, H. A., Findsen, A., Pedersen, T. H. and Overgaard, J. (2014). Cold-induced depolarization of insect muscle: differing roles of extracellular K^+ during acute and chronic chilling. *Journal of Experimental Biology* **217**, 2930-2938.

MacMillan, H. A., Knee, J. M., Dennis, A. B., Udaka, H., Marshall, K. E., Merritt, T. J. and Sinclair, B. J. (2016). Cold acclimation wholly reorganizes the *Drosophila melanogaster* transcriptome and metabolome. *Scientific Reports* **6**, 28999.

MacMillan, H. A., Williams, C. M., Staples, J. F. and Sinclair, B. J. (2012). Reestablishment of ion homeostasis during chill-coma recovery in the cricket *Gryllus pennsylvanicus*. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 20750-20755.

Marqués, M. I. (2006). Mechanism for proteins destabilization at low temperatures. *Physica Status Solidi (a)* **203**, 1487-1490.

Marshall, K. E. and Sinclair, B. J. (2011). The sub-lethal effects of repeated freezing in the woolly bear caterpillar *Pyrrharctia isabella*. *Journal of Experimental Biology* **214**, 1205-1212.

Marshall, K. E. and Sinclair, B. J. (2012). Threshold temperatures mediate the impact of reduced snow cover on overwintering freeze-tolerant caterpillars. *Naturwissenschaften* **99**, 33-41.

Marshall, K. E., Thomas, R. H., Roxin, Á., Chen, E. K. Y., Brown, J. C. L., Gillies, E. R. and Sinclair, B. J. (2014). Seasonal accumulation of acetylated

triacylglycerols by a freeze-tolerant insect. *Journal of Experimental Biology* **217**, 1580-1587.

Mazur, P. (2010). A biologist's view of the relevance of thermodynamics and physical chemistry to cryobiology. *Cryobiology* **60**, 4-10.

McKinnon, A. H (2015). Freeze tolerance in the spring field cricket, *Gryllus veletis*. MSc Thesis. London ON, Canada: University of Western Ontario.

Merksamer, P. I., Liu, Y., He, W., Hirschey, M. D., Chen, D. and Verdin, E. (2013). The sirtuins, oxidative stress and aging: an emerging link. *Aging* **5**, 144-150.

Meyering-Vos, M., Merz, S., Sertkol, M. and Hoffmann, K. H. (2006). Functional analysis of the allatostatin-A type gene in the cricket *Gryllus bimaculatus* and the armyworm *Spodoptera frugiperda*. *Insect Biochemistry and Molecular Biology* **36**, 492-504.

Michaud, M. R., Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. A., Lee, R. E. and Denlinger, D. L. (2008). Metabolomics reveals unique and shared metabolic changes in response to heat shock, freezing and desiccation in the Antarctic midge, *Belgica antarctica*. *Journal of Insect Physiology* **54**, 645-655.

Miguel, A., Montón, F., Li, T., Gómez-Herreros, F., Chávez, S., Alepuz, P. and Pérez-Ortín, J. E. (2013). External conditions inversely change the RNA polymerase II elongation rate and density in yeast. *Biochimica et Biophysica Acta* **1829**, 1248-1255.

Miller, L. K. (1969). Freezing tolerance in an adult insect. *Science* **166**, 105-106.

Miller, L. K. (1978). Freezing tolerance in relation to cooling rate in an adult insect. *Cryobiology* **15**, 345-349.

Miller, L. K. (1982). Cold-hardiness strategies of some adult and immature insects overwintering in interior Alaska. *Comparative Biochemistry and Physiology A* **73**, 595-604.

Miller, L. K. and Smith, J. S. (1975). Production of threitol and sorbitol by an adult insect: association with freezing tolerance. *Nature* **258**, 519-520.

Mitchell, D. E., Congdon, T., Rodger, A. and Gibson, M. I. (2015). Gold nanoparticle aggregation as a probe of antifreeze (glyco) protein-inspired ice recrystallization inhibition and identification of new IRI active macromolecules. *Scientific Reports* **5**, 15716.

Morey, A., Venette, R. and Hutchison, W. (2013). Could natural selection change the geographic range limits of light brown apple moth (Lepidoptera, Tortricidae) in North America? *NeoBiota* **18**, 151-156.

Morisaku, T., Arai, S. and Yui, H. (2014). Dehydration-induced initial conformational change of hydrated proteins detected by the changes in vibrational circular dichroism activity. *Analytical Sciences* **30**, 961-969.

Morrissey, R. E. and Baust, J. G. (1976). The ontogeny of cold tolerance in the gall fly, *Eurosta solidagensis*. *Journal of Insect Physiology* **22**, 431-437.

Mugnano, J. A., Lee, R. E. and Taylor, R. T. (1996). Fat body cells and calcium phosphate spherules induce ice nucleation in the freeze-tolerant larve of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). *Journal of Experimental Biology* **199**, 465-471.

Neufeld, D. S. and Leader, J. P. (1998). Freezing survival by isolated Malpighian tubules of the New Zealand alpine weta *Hemideina maori*. *Journal of Experimental Biology* **201**, 227-236.

Newman, C. E., Toxopeus, J., Udaka, H., Ahn, S., Martynowicz, D. M., Graether, S. P., Sinclair, B. J. and Percival-Smith, A. (2017). CRISPR-induced null alleles show that *Frost* protects *Drosophila melanogaster* reproduction after cold exposure. *Journal of Experimental Biology* **220**, 3344-3354.

Olsson, T. I. (1981). Overwintering of benthic macroinvertebrates in ice and frozen sediment in a North Swedish river. *Ecography* **4**, 161-166.

Orrenius, S., Zhivotovsky, B. and Nicotera, P. (2003). Regulation of cell death: the calcium-apoptosis link. *Nature Reviews: Molecular Cell Biology* **4**, 552-565.

Overgaard, J. and MacMillan, H. A. (2017). The integrative physiology of insect chill tolerance. *Annual Review of Physiology* **79**, 187-208.

Pedersen, P. and Holmstrup, M. (2003). Freeze or dehydrate: only two options for the survival of subzero temperatures in the Arctic enchytraeid *Fridericia ratzeli*. *Journal of Comparative Physiology B* **173**, 601-609.

Pegg, D. E. (2010). The relevance of ice crystal formation for the cryopreservation of tissues and organs. *Cryobiology* **60**, S36-S44.

Philip, B. N. and Lee, R. E. (2010). Changes in abundance of aquaporin-like proteins occurs concomitantly with seasonal acquisition of freeze tolerance in the goldenrod gall fly, *Eurosta solidaginis*. *Journal of Insect Physiology* **56**, 679–685.

Philip, B. N., Yi, S.-X., Elnitsky, M. A. and Lee, R. E. (2008). Aquaporins play a role in desiccation and freeze tolerance in larvae of the goldenrod gall fly, *Eurosta solidaginis*. *Journal of Experimental Biology* **211**, 1114-1119.

Poupardin, R., Riaz, M., Vontas, J., David, J. and Reynaud, S. (2010). Transcription profiling of eleven cytochrome P450s potentially involved in xenobiotic metabolism in the mosquito *Aedes aegypti*. *Insect Molecular Biology* **19**, 185-193.

Poupardin, R., Schöttner, K., Korbelová, J., Provazník, J., Doležel, D., Pavlinic, D., Beneš, V. and Koštál, V. (2015). Early transcriptional events linked to induction of diapause revealed by RNAseq in larvae of drosophilid fly, *Chymomyza costata*. *BMC Genomics* **16**, 720.

Prestrelski, S. J., Tedeschi, N., Arakawa, T. and Carpenter, J. F. (1993). Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. *Biophysical Journal* **65**, 661-671.

Preyat, N. and Leo, O. (2013). Sirtuin deacylases: a molecular link between metabolism and immunity. *Journal of Leukocyte Biology* **93**, 669-680.

Pruitt, N. L., Moqueet, N. and Shapiro, C. A. (2007). Evidence for a novel cryoprotective protein from freeze-tolerant larvae of the goldenrod gall fly *Eurosta solidaginis*. *Cryobiology* **54**, 125-128.

Rajpurohit, S., Parkash, R. and Ramniwas, S. (2008). Body melanization and its adaptive role in thermoregulation and tolerance against desiccating conditions in drosophilids. *Entomological Research* **38**, 49-60.

Ramløv, H. (1999). Microclimate and variations in haemolymph composition in the freezing-tolerant New Zealand alpine weta *Hemideina maori* Hutton (Orthoptera: Stenopelmatidae). *Journal of Comparative Physiology B* **169**, 224-235.

Ramløv, H. (2000). Aspects of natural cold tolerance in ectothermic animals. *Human Reproduction* **15**, 26-46.

Ramløv, H., Bedford, J. J. and Leader, J. P. (1992). Freezing tolerance of the New Zealand alpine weta, *Hemideina maori* Hutton [Orthoptera; Stenopelmatidae]. *Journal of Thermal Biology* **17**, 51-54.

Ramløv, H. and Westh, P. (1993). Ice formation in the freeze tolerant alpine weta *Hemideina maori* Hutton (Orthoptera, Stenopelmatidae). *Cryo-Letters* **14**, 169-176.

Ramløv, H., Wharton, D. A. and Wilson, P. W. (1996). Recrystallization in a freezing tolerant antarctic nematode, *Panagrolaimus davidi*, and an alpine weta, *Hemideina maori* (Orthoptera; Stenopelmatidae). *Cryobiology* **33**, 607-613.

Réaumur, R. (1736). Memoires Pour Servir à l'Histoire des Insectes. Paris: d'Imprimerie Royal.

Rinehart, J. P., Hayward, S. A. L., Elnitsky, M. A., Sandro, L. H., Lee, R. E. and Denlinger, D. L. (2006). Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 14223-14227.

Ring, R. A. (1982). Freezing-tolerant insects with low supercooling points. *Comparative Biochemistry and Physiology A* **73**, 605-612.

Ring, R. A. and Danks, H. V. (1994). Desiccation and cryoprotection: overlapping adaptations. *CryoLetters* **15**, 181-190.

Rojas, R. R. and Leopold, R. A. (1996). Chilling injury in the housefly: evidence for the role of oxidative stress between pupariation and emergence. *Cryobiology* **33**, 447-458.

Rosendale, A. J., Romick-Rosendale, L. E., Watanabe, M., Dunlevy, M. E. and Benoit, J. B. (2016). Mechanistic underpinnings of dehydration stress in the American dog tick revealed through RNA-Seq and metabolomics. *Journal of Experimental Biology* **219**, 1808-1819.

Rossolimo, T. (1997). Temperature adaptations of Siberian *Pterostichus* species (Coleoptera: Carabidae). *European Journal of Entomology* **94**, 235-242.

Rozsypal, J., Moos, M., Šimek, P. and Koštál, V. (2018). Thermal analysis of ice and glass transitions in insects that do and do not survive freezing. *Journal of Experimental Biology*, doi: 10.1242/jeb.170464.

Rudolph, A. S. and Crowe, J. H. (1985). Membrane stabilization during freezing: the role of two natural cryoprotectants, trehalose and proline. *Cryobiology* **22**, 367-377.

Rudolph, A. S. and Crowe, J. H. (1986). A calorimetric and infrared spectroscopic study of the stabilizing solute proline. *Biophysical Journal* **50**, 423-430.

Sakurai, M., Furuki, T., Akao, K.-i., Tanaka, D., Nakahara, Y., Kikawada, T., Watanabe, M. and Okuda, T. (2008). Vitrification is essential for anhydrobiosis in an African chironomid, *Polypedilum vanderplanki*. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 5093-5098.

Salt, R. W. (1957). Natural occurrence of glycerol in insects and its relation to their ability to survive freezing. *The Canadian Entomologist* **89**, 491-494.

Salt, R. W. (1961). A comparison of injury and survival of larvae of *Cephus cinctus* Nort. after intracellular and extracellular freezing. *Canadian Journal of Zoology* **39**, 349-357.

Sawchyn, W. and Gillott, C. (1975). The biology of two related species of coenagrionid dragonflies (Odonata: Zygoptera) in western Canada. *The Canadian Entomologist* **107**, 119-128.

Scholander, P. F., Flagg, W., Hock, R. J. and Irving, L. (1953). Studies on the physiology of frozen plants and animals in the Arctic. *Journal of Cellular Physiology* **42**, 1-56.

Scott, J. G., Michel, K., Bartholomay, L. C., Siegfried, B. D., Hunter, W. B., Smagghe, G., Zhu, K. Y. and Douglas, A. E. (2013). Towards the elements of successful insect RNAi. *Journal of Insect Physiology* **59**, 1212-1221.

Scott, J. G. and Wen, Z. (2001). Cytochromes P450 of insects: the tip of the iceberg. *Pest Management Science* **57**, 958-967.

Sformo, T., Kohl, F., McIntyre, J., Kerr, P., Duman, J. G. and Barnes, B. M. (2009). Simultaneous freeze tolerance and avoidance in individual fungus gnats, *Exechia nugatoria*. *Journal of Comparative Physiology B* **179**, 897-902.

Sformo, T., Walters, K. R., Jeannet, K., Wowk, B., Fahy, G. M., Barnes, B. M. and Duman, J. G. (2010). Deep supercooling, vitrification and limited survival to -100°C in the Alaskan beetle *Cucujus clavipes puniceus* (Coleoptera: Cucujidae) larvae. *Journal of Experimental Biology* **213**, 502-509.

Shapiro-Ilan, D. I., Brown, I. and Lewis, E. E. (2014). Freezing and desiccation tolerance in entomopathogenic nematodes: diversity and correlation of traits. *Journal of Nematology* **46**, 27-34.

Shimada, K. and Riihimaa, A. (1988). Cold acclimation, inoculative freezing and slow freezing: essential factors contributing to the freezing-tolerance in diapausing larvae of *Chymomyza costata* (Diptera, Drosophilidae). *Cryo-Letters* **9**, 5-10.

Sinclair, B. J. (1997). Seasonal variation in freezing tolerance of the New Zealand alpine cockroach *Celatoblatta quinquemaculata*. *Ecological Entomology* **22**, 462-467.

Sinclair, B. J. (1999). Insect cold tolerance: how many kinds of frozen? *European Journal of Entomology* **96**, 157-164.

Sinclair, B. J. (2001). Field ecology of freeze tolerance: interannual variation in cooling rates, freeze-thaw and thermal stress in the microhabitat of the alpine cockroach *Celatoblatta quinquemaculata*. *Oikos* **93**, 286-293.

Sinclair, B. J. (2015). Linking energetics and overwintering in temperate insects. *Journal of Thermal Biology* **54**, 5-11.

Sinclair, B. J., Addo-Beddiako, A. and Chown, S. L. (2003). Climatic variability and the evolution of insect freeze tolerance. *Biological Reviews* **78**, 181–195.

Sinclair, B. J. and Chown, S. L. (2005a). Climatic variability and hemispheric differences in insect cold tolerance: support from southern Africa. *Functional Ecology* **19**, 214-221.

Sinclair, B. J. and Chown, S. L. (2005b). Deleterious effects of repeated cold exposure in a freeze-tolerant sub-Antarctic caterpillar. *Journal of Experimental Biology* **208**, 869-879.

Sinclair, B. J. and Chown, S. L. (2010). The macrophysiology of insect cold-hardiness. In *Low Temperature Biology of Insects* (eds. D. L. Denlinger and R. E. Lee), pp. 191-223. New York: Cambridge University Press.

Sinclair, B. J., Coello Alvarado, L. E. and Ferguson, L. V. (2015). An invitation to measure insect cold tolerance: methods, approaches, and workflow. *Journal of Thermal Biology* **53**, 180-197.

Sinclair, B. J., Ferguson, L. V., Salehipour-shirazi, G. and MacMillan, H. A. (2013a). Cross-tolerance and cross-talk in the cold: relating low temperatures to desiccation and immune stress in insects. *Integrative and Comparative Biology* **53**, 545–556.

Sinclair, B. J., Gibbs, A. G., Lee, W.-K., Rajamohan, A., Roberts, S. P. and Socha, J. J. (2009). Synchrotron x-ray visualisation of ice formation in insects during lethal and non-lethal freezing. *PLoS One* **4**, e8259.

Sinclair, B. J., Klok, C. J. and Chown, S. L. (2004). Metabolism of the sub-Antarctic caterpillar *Pringleophaga marioni* during cooling, freezing and thawing. *Journal of Experimental Biology* **207**, 1287-1294.

Sinclair, B. J. and Marshall, K. E. (2018). The many roles of fats in overwintering insects. *Journal of Experimental Biology* **221**, doi: 10.1242/jeb.161836.

Sinclair, B. J. and Renault, D. (2010). Intracellular ice formation in insects: unresolved after 50 years? *Comparative Biochemistry and Physiology A* **155**, 14–18.

Sinclair, B. J. and Wharton, D. A. (1997). Avoidance of intracellular freezing by the freezing-avoidant New Zealand alpine weta *Hemideina maori* (Orthoptera: Stenopalmatidae). *Journal of Insect Physiology* **43**, 621-625.

Sinclair, B. J., Williams, C. M., MacMillan, H. A., Marshall, K. E. and Storey, K. B. (2013b). Real-time measurements of metabolic rate during freezing and thawing of the wood frog, *Rana sylvatica*: implications for overwinter energy use. *Journal of Experimental Biology* **216**, 292-302.

Sinclair, B. J., Worland, M. R. and Wharton, D. A. (1999). Ice nucleation and freezing tolerance in New Zealand alpine and lowland weta, *Hemideina* spp. (Orthoptera; Stenopelmatidae). *Physiological Entomology* **24**, 56-63.

Smith, P., Howes, E. and Treherne, J. (1990). Cell proliferation in the repairing adult insect central nervous system: incorporation of the thymidine analogue 5-bromo-2-deoxyuridine *in vivo*. *Journal of Cell Science* **95**, 599-604.

Somero, G. N., Lockwood, B. L. and Tomanek, L. (2017). Biochemical Adaptation: Response to Environmental Challenges from Life's Origins to the Anthropocene. Sunderland, USA: Sinauer Associates.

Sømme, L. (1999). The physiology of cold hardiness in terrestrial arthropods. *European Journal of Entomology* **96**, 1-10.

Sømme, L. (2012). Invertebrates in hot and cold arid environments. New York: Springer.

Sømme, L., Davidson, R. L. and Onore, G. (1996). Adaptations of insects at high altitudes of Chimborazo, Ecuador. *European Journal of Entomology* **93**, 313-318.

Sømme, L. and Zachariassen, K. E. (1981). Adaptations to low temperature in high altitude insects from Mount Kenya. *Ecological Entomology* **6**, 199-204.

Štětina, T., Hůla, P., Moos, M., Šimek, P., Šmilauer, P. and Koštál, V. (2018). Recovery from supercooling, freezing, and cryopreservation stress in larvae of the drosophilid fly, *Chymomyza costata*. *Scientific Reports* **8**, 4414.

Storey, J. M. and Storey, K. B. (1985). Freezing and cellular metabolism in the gall fly larva, *Eurosta solidaginis*. *Journal of Comparative Physiology B* **155**, 333-337.

Storey, K. B., Baust, J. G. and Storey, J. M. (1981). Intermediary metabolism during low temperature acclimation in the overwintering gall fly larva, *Eurosta solidaginis*. *Journal of Comparative Physiology B* **144**, 183-190.

Storey, K. B., Churchill, T. A. and Joanisse, D. R. (1993). Freeze tolerance in hermit flower beetle (*Osmoderma eremicola*) larvae. *Journal of Insect Physiology* **39**, 737-742.

Storey, K. B. and Storey, J. M. (1984). Biochemical adaptation for freezing in the wood frog, *Rana sylvatica*. *Journal of Comparative Physiology B* **155**, 29-36.

Storey, K. B. and Storey, J. M. (1988). Freeze tolerance in animals. *Physiological Reviews* **68**, 27-84.

Storey, K. B. and Storey, J. M. (2010). Oxygen: stress and adaptation in cold hardy insects. In *Low Temperature Biology of Insects* (eds. D. L. Denlinger and R. E. Lee), pp. 141-165. New York: Cambridge University Press.

Storey, K. B. and Storey, J. M. (2013). Molecular biology of freezing tolerance. *Comprehensive Physiology* **3**, 1283-1308.

Tattersall, G. J., Sinclair, B. J., Withers, P. C., Fields, P. A., Seebacher, F., Cooper, C. E. and Maloney, S. K. (2012). Coping with thermal challenges: physiological adaptations to environmental temperatures. *Comprehensive Physiology* **2**, 2151-2202.

Teets, N. M. and Denlinger, D. L. (2013). Physiological mechanisms of seasonal and rapid cold-hardening in insects. *Physiological Entomology* **38**, 105-116.

Teets, N. M., Elnitsky, M. A., Benoit, J. B., Lopez-Martinez, G., Denlinger, D. L. and Lee., R. E. (2008). Rapid cold-hardening in larvae of the Antarctic midge *Belgica antarctica*: cellular cold-sensing and a role for calcium. *American Journal of Physiology* **294**, R1938-R1946.

Teets, N. M., Yi, S.-X., Lee, R. E. and Denlinger, D. L. (2013). Calcium signaling mediates cold sensing in insect tissues. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 9154-9159.

Toxopeus, J., Lebenzon, J. E., McKinnon, A. H and Sinclair, B. J. (2016). Freeze tolerance of *Cyphoderris monstrosa* (Orthoptera: Prophalangopsidae). *The Canadian Entomologist* **148**, 668-672.

Toxopeus, J., Warner, A. H. and MacRae, T. H. (2014). Group 1 LEA proteins contribute to the desiccation and freeze tolerance of *Artemia franciscana* embryos during diapause. *Cell Stress and Chaperones* **19**, 939-948.

Tsumuki, H. and Konno, H. (1991). Tissue distribution of ice-nucleating agents in larve of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). *Cryobiology* **28**, 376-381.

Tsumuki, H., Konno, H., Maeda, T. and Okamoto, Y. (1992). An ice-nucleating active fungus isolated from the gut of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). *Journal of Insect Physiology* **38**, 119-125.

Tsvetkova, N., Koynova, R., Tsonev, L., Quinn, P. and Tenchov, B. (1991). Influence of proline on the stability of fully hydrated and freeze-dried lipid phases. *Chemistry and Physics of lipids* **60**, 51-59.

Turnock, W. J. and Fields, P. G. (2005). Winter climates and coldhardiness in terrestrial insects. *European Journal of Entomology* **102**, 561-576.

Tyrrell, C., Wharton, D. A., Ramløv, H. and Moller, H. (1994). Cold tolerance of an endoparasitic nematode within a freezing-tolerant orthopteran host. *Parasitology* **109**, 367-372.

Voituron, Y., Mouquet, N., de Mazancourt, C. and Clobert, J. (2002). To freeze or not to freeze? An evolutionary perspective on the cold-hardiness strategies of overwintering ectotherms. *The American Naturalist* **160**, 255-270.

Walters, K. R., Serianni, A. S., Sformo, T., Barnes, B. M. and Duman, J. G. (2009a). A nonprotein thermal hysteresis-producing xylomannan antifreeze in the freeze-tolerant Alaskan beetle *Upis ceramboides*. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 20210-20215.

Walters, K. R., Serianni, A. S., Voituron, Y., Sformo, T., Barnes, B. M. and Duman, J. G. (2011). A thermal hysteresis-producing xylomannan glycolipid antifreeze associated with cold tolerance is found in diverse taxa. *Journal of Comparative Physiology B* **181**, 631-640.

Walters, K. R., Sformo, T., Barnes, B. M. and Duman, J. G. (2009b). Freeze tolerance in an arctic Alaska stonefly. *Journal of Experimental Biology* **212**, 305-312.

Watson, P. F. and Morris, G. J. (1987). Cold shock injury in animal cells. *Symposia of the Society for Experimental Biology* **41**, 311-340.

Wen, X., Wang, S., Duman, J. G., Arifin, J. F., Juwita, V., Goddard, W. A., Rios, A., Liu, F., Kim, S.-K. and Abrol, R. (2016). Antifreeze proteins govern the precipitation of trehalose in a freezing-avoiding insect at low temperature. *Proceedings of the National Academy of Sciences of the United States of America* **113**, 6683-6688.

Wharton, D. A. (1995). Cold tolerance strategies in nematodes. *Biological Reviews* **70**, 161-185.

Wharton, D. A., Barrett, J., Goodall, G., Marshall, C. J. and Ramløv, H. (2005a). Ice-active proteins from the Antarctic nematode *Panagrolaimus davidi*. *Cryobiology* **51**, 198-207.

Wharton, D. A., Downes, M. F., Goodall, G. and Marshall, C. J. (2005b). Freezing and cryoprotective dehydration in an Antarctic nematode (*Panagrolaimus davidi*) visualised using a freeze substitution technique. *Cryobiology* **50**, 21-28.

Wharton, D. A., Pow, B., Kristensen, M., Ramløv, H. and Marshall, C. J. (2009). Ice-active proteins and cryoprotectants from the New Zealand alpine cockroach, *Celatoblatta quinquemaculata*. *Journal of Insect Physiology* **55**, 27-31.

Williams, C. M., Henry, H. A. and Sinclair, B. J. (2015). Cold truths: how winter drives responses of terrestrial organisms to climate change. *Biological Reviews* **90**, 214-35.

Williams, J. B. and Lee, R. E. (2011). Effect of freezing and dehydration on ion and cryoprotectant distribution and hemolymph volume in the goldenrod gall fly, *Eurosta solidaginis*. *Journal of Insect Physiology* **57**, 1163-1169.

Wilson, P. and Ramløv, H. (1995). Hemolymph ice nucleating proteins from the New Zealand alpine weta *Hemideina maori* (Orthoptera: Stenopelmatidae). *Comparative Biochemistry and Physiology B* **112**, 535-542.

Worland, M. R. and Block, W. (1999). Ice-nucleating bacteria from the guts of two sub-Antarctic beetles, *Hydromedion sparsutum* and *Perimylops antarcticus* (Perimylopidae). *Cryobiology* **38**, 60-67.

Worland, M. R., Block, W. and Grubor-Lajšić, G. (2000). Survival of *Heleomyza borealis* (Diptera, Heleomyzidae) larvae down to −60 °C. *Physiological Entomology* **25**, 1-5.

Worland, M. R. and Lukešová, A. (2000). The effect of feeding on specific soil algae on the cold-hardiness of two Antarctic micro-arthropods (*Alaskozetes antarcticu*s and *Cryptopygus antarcticus*). *Polar Biology* **23**, 766-774.

Worland, M. R., Wharton, D. A. and Byars, S. G. (2004). Intracellular freezing and survival in the freeze tolerant alpine cockroach *Celatoblatta quinquemaculata*. *Journal of Insect Physiology* **50**, 225-32.

Xu, L., Neven, L. G. and Duman, J. G. (1990). Hormonal control of hemolymph lipoprotein ice nucleators in overwintering freeze-susceptible larvae of the stag beetle *Ceruchus piceus*: adipokinetic hormone and juvenile hormone. *Journal of Comparative Physiology B* **160**, 51-59.

Yi, S.-X., Benoit, J. B., Elnitsky, M. A., Kaufmann, N., Brodsky, J. L., Zeidel, M. L., Denlinger, D. L. and Lee, R. E. (2011). Function and immuno-localization of aquaporins in the Antarctic midge *Belgica antarctica*. *Journal of Insect Physiology* **57**, 1096-1105.

Yi, S.-X. and Lee, R. E. (2003). Detecting freeze injury and seasonal cold-hardening of cells and tissues in the gall fly larvae, *Eurosta solidaginis* (Diptera: Tephritidae) using fluorescent vital dyes. *Journal of Insect Physiology* **49**, 999-1004.

Zachariassen, K. E. (1985). Physiology of cold tolerance in insects. *Physiological Reviews* **65**, 799-832.

Zachariassen, K. E. and Hammel, H. T. (1976). Nucleating agents in the haemolymph of insects tolerant to freezing. *Nature* **262**, 285-287.

Zachariassen, K. E., Hammel, H. T. and Schmidek, W. (1979a). Osmotically inactive water in relation to tolerance to freezing in *Eleodes blanchardi* beetles. *Comparative Biochemistry and Physiology A* **63**, 203-206.

Zachariassen, K. E., Hammel, H. T. and Schmidek, W. (1979b). Studies on freezing injuries in *Eleodes blanchardi* beetles. *Comparative Biochemistry and Physiology A* **63**, 199-202.

Zachariassen, K. E. and Kristiansen, E. (2000). Ice nucleation and antinucleation in nature. *Cryobiology* **41**, 257-279.

Zachariassen, K. E., Kristiansen, E. and Pedersen, S. A. (2004a). Inorganic ions in cold-hardiness. *Cryobiology* **48**, 126-133.

Zachariassen, K. E., Kristiansen, E., Pedersen, S. A. and Hammel, H. T. (2004b). Ice nucleation in solutions and freeze-avoiding insects-homogeneous or heterogeneous? *Cryobiology* **48**, 309-321.

Zhang, G., Storey, J. M. and Storey, K. B. (2011). Chaperone proteins and winter survival by a freeze tolerant insect. *Journal of Insect Physiology* **57**, 1115-1122.

Chapter 2

2 Cryoprotectant molecules differentially enhance freeze tolerance of the spring field cricket *Gryllus veletis*

I have prepared this chapter as a manuscript for submission.

2.1 Introduction

Ectotherms in temperate and polar regions are exposed to temperatures low enough to freeze their body fluids. Freezing is lethal in most cases (Sinclair et al., 2015), and many insects have evolved strategies to avoid freezing (Lee, 2010). Strikingly, some insects are freeze-tolerant, surviving internal ice formation (Lee, 2010), a strategy that has evolved multiple times in a diversity of insects (Dennis et al., 2015; Sinclair and Chown, 2010; Walters et al., 2009). Some of these freeze-tolerant insects survive in the frozen state for extended periods [e.g. the cockroach *Cryptocercus punctulatus* survives at least 205 days at -10 °C (Hamilton et al., 1985)] or to extremely low temperatures [e.g. larvae of the drosphilid fly *Chymomyza costata* can survive immersion in liquid nitrogen, -196 °C (Koštál et al., 2011b)]. Despite the prevalence of insect freeze tolerance, a lack of tractable laboratory model systems has limited investigations of the physiological mechanisms underlying this cold tolerance strategy (Chapter 1; Storey and Storey, 1988).

Freezing is usually harmful to biological systems (Chapter 1; Muldrew et al., 2004). Ice crystals can physically damage tissues, and intracellular ice formation (IIF) is often lethal (Lee, 2010; Zachariassen, 1985). Extracellular ice formation causes osmotic dehydration of cells, imposing challenges on structural integrity, macromolecule conformation, and function of diverse macromolecular structures [nucleoprotein complexes, enzymatic complexes, cytoskeleton, phospholipid bilayers, etc. (Chapter 1; Lee, 2010)]. In addition, ice crystals can grow over time *via* recrystallization (Ramløv et al., 1996), and metabolic waste products (e.g. lactate) can accumulate in the frozen state (Storey and Storey, 1985). Many insects suppress their metabolism over winter (diapause; Hahn and Denlinger, 2011) which may mitigate some of these metabolic impacts. For example, diapausing *Eurosta solidaginis* suppress their metabolic rate by c. 67 % (Irwin et al., 2001). Freeze-

tolerant insects will die if cooled to their lower lethal temperature (LLT) or held in the frozen state beyond a lethal time (Lt), suggesting that low temperatures and long freezing periods exacerbate these challenges (Chapter 1).

To minimize damage from ice, it is hypothesized that freeze-tolerant insects control the distribution and quality of ice crystals. Ice-nucleating agents (INAs) can control where and at what temperature ice begins to form (Zachariassen and Kristiansen, 2000). Aquaporins (AQPs) facilitate transmembrane water transport and may therefore facilitate effective osmotic dehydration of cells, preventing IIF (Chapter 1; Lee, 2010). Other ice-binding molecules, such as antifreeze proteins (AFPs), control the size and quality of ice crystals, and are hypothesized to prevent ice crystal growth (recrystallization) over time (Duman, 2015). Manipulating the conditions of ice formation (e.g. temperature and cooling rate) can have a strong impact on whether insects survive freezing. For example, larvae of *C. costata* are only freeze tolerant if ice formation is nucleated at a high subzero temperature (c. -1 °C) by contact with external ice, and cooling rate is slow (Koštál et al., 2011b).

Most freeze-tolerant insects accumulate low molecular weight cryoprotectants such as polyols (e.g. glycerol), sugars (e.g. trehalose), or amino acids (e.g. proline) (Chapter 1; Lee, 2010). There are two main hypotheses for the function of these cryoprotectants in freeze tolerance: 1) cryoprotectants minimize damage from ice by colligatively reducing ice content, and 2) cryoprotectants improve cell survival *via* non-colligative means by directly protecting macromolecules such as proteins and membranes (Lee, 2010). In support of hypothesis 1, high cryoprotectant concentrations (e.g. c. 300 mM glycerol in the woolly bear caterpillar *Pyrrharctia isabella*) are correlated with high hemolymph osmolality and low ice content at mild subzero temperatures (< 50 % of body water converted to ice; Chapter 1; Layne and Blakeley, 2002), and increased glycerol concentrations (up to 1.5 M) correlate with survival of freeze-tolerant *Pytho depressus* to lower temperatures (Zachariassen, 1979). Hypothesis 2 is supported by *in vitro* and *ex vivo* experiments. *In vitro*, trehalose and proline reduce membrane disruption during freezing (Rudolph and Crowe, 1985); and sugars (Arakawa and Timasheff, 1982), amino acids (Arakawa and Timasheff, 1983), and glycerol (Gekko and Timasheff, 1981) can

help maintain protein structure by stabilizing their hydration shell. Glycerol also improves survival of *Chilo suppressalis* fat body (Izumi et al., 2006) and *P. isabella* foregut cells (Yi and Lee, 2016) frozen *ex vivo*. There is currently limited support for either hypothesis *in vivo*. Elevated whole body proline concentrations improve survival of frozen *C. costata* larvae to lower temperatures (Koštál et al., 2011b), and confer freeze tolerance on *Drosophila melanogaster* larvae (Koštál et al., 2016), but not by substantially modifying ice content (Rozsypal et al., 2018). Injection of trehalose into *Belgica antarctica* larvae improves survival of cold shock (Benoit et al., 2009), but whether it does so by directly protecting macromolecules or modifying ice content is unclear.

No one cryoprotectant or group of cryoprotectants is accumulated by all freeze-tolerant insects, and many freeze-tolerant insects accumulate multiple cryoprotectants. For example, most orders of freeze-tolerant insects accumulate glycerol > 100 mM (Duman, 1984; Duman et al., 1985; Duman and Patterson, 1978; Layne and Blakeley, 2002; Morrissey and Baust, 1976; Walters et al., 2009; Wharton et al., 2009), but glycerol is absent or at low (< 20 mM) concentrations in *C. costata* (Koštál et al., 2011b) and freeze-tolerant orthopterans (Ramløv et al., 1992; Toxopeus et al., 2016). Some insects accumulate a diversity of cryoprotectants. For example, *E. solidaginis* accumulate glycerol, sorbitol and trehalose (Baust and Lee, 1981), and *Hemideina maori* accumulate proline and trehalose (Neufeld and Leader, 1998). Accumulating multiple cryoprotectants could reduce the toxicity of any one cryoprotectant (Zachariassen, 1985) or help manage energy reserves (Storey and Storey, 1988). Alternatively, each cryoprotectant may have a unique function, and different cryoprotectants contribute to freeze tolerance *via* distinct mechanisms (Storey and Storey, 1988). For example, while any cryoprotectant may act colligatively to reduce ice content, some cryoprotectants such as trehalose may protect cells from specific stresses, e.g. stabilizing macromolecules under dehydration stress (due to high ice content).

One way to investigate the mechanisms underlying freeze tolerance is to study the conditions under which it fails, i.e. what happens at the lethal limits (LLT and Lt). Several parameters should change as temperature decreases, including reduced stability

of macromolecules (Marqués, 2006), increased ice content (Lundheim, 2002), and formation of smaller ice crystals (Mazur, 2010). I therefore hypothesize that mortality at the LLT can be caused by: 1) macromolecule damage (protein denaturation, membrane phase transitions) due to low temperature itself (regardless of ice; Dias et al., 2010; Hazel, 1995); 2) increased mechanical damage and osmotic/dehydration stress due to high ice content (Pegg, 2010), and 3) propagation of ice crystals (Mazur, 2010) into cells or so-called "weak link" tissues, whose damage prevents recovery (Chapter 1). At a stable temperature, once equilibrium ice content is reached, ice content does not change over time, although ice crystals will grow *via* recrystallization (Knight and Duman, 1986; Ramløv et al., 1996), and continued metabolism in the frozen state will change metabolite concentration (Sinclair et al., 2004). Given these changes, I hypothesize that mortality at the Lt can be caused by: 1) physical damage from large ice crystals due to recrystallization; 2) cell damage from build-up of harmful metabolic end-products (Storey and Storey, 1985), and 3) failure to recover from freezing due to metabolic depletion of energy stores while frozen (Chapter 1; Irwin and Lee, 2002).

Here I develop the spring field cricket, *Gryllus veletis* (Alexander & Bigelow) (Orthoptera: Gryllidae), as a laboratory model system to study freeze tolerance. *Gryllus veletis* overwinters as a late-instar nymph in north eastern North America (Alexander and Bigelow, 1960). I demonstrate that the overwintering nymphs of *G. veletis* are freeze tolerant, characterize the physiological changes associated with laboratory-induced freeze tolerance, and test how control of ice nucleation and accumulation of multiple cryoprotectants contribute to survival of internal ice formation. The effects of each cryoprotectant on organismal and tissue freeze tolerance do not overlap, suggesting that a combination of mechanisms contribute to survival of internal ice formation.

2.2 Materials and methods

2.2.1 Rearing and acclimation conditions

The laboratory colony of *G. veletis* originated from individuals collected in 2010 from the University of Lethbridge campus, Alberta, Canada and was reared under constant summer-like conditions (25 ºC, 14:10 L:D photoperiod, 70 % RH), as described

previously (Coello Alvarado et al., 2015). I haphazardly assigned fifth-instar male *G. veletis* approximately eight weeks post-hatch to remain in rearing (control) conditions, or to undergo acclimatization or acclimation conditions, and tested if these conditions induced freeze tolerance. For subsequent experiments, I used the six week acclimation described in Fig. 2.1A.

For outdoor acclimatizations, I placed crickets in enclosed mesh cages (60 cm × 60 cm × 75 cm) in a shaded suburban garden (42°59'N, 81°17'W, 251 m elevation) in London, Ontario, Canada, along with soil, rabbit food pellets, chicory (*Cichorium endivia*) leaves, black locust (*Robinia pseudoacacia*) leaves, and grass from a cultivated lawn. The acclimitizations lasted for six to eight weeks, and included 'Outdoor 2013-a' (10 Oct – 3 Dec 2013), 'Outdoor 2013-b' (10 Oct 2013 – 9 Jan 2014), and 'Outdoor 2014' (1 Oct – 5 Dec 2014). I used a HOBO Pro v2 U23-003 data logger (Onset Computer Corporation, Bourne, MA, USA) to record surface soil temperature every 30 min, and obtained day length data for London from the National Research Council of Canada website (http://www.nrc-cnrc.gc.ca/eng/services/sunrise/). The temperature data for 'Outdoor 2013-a' and '-b' is available in Fig. 2.1B.

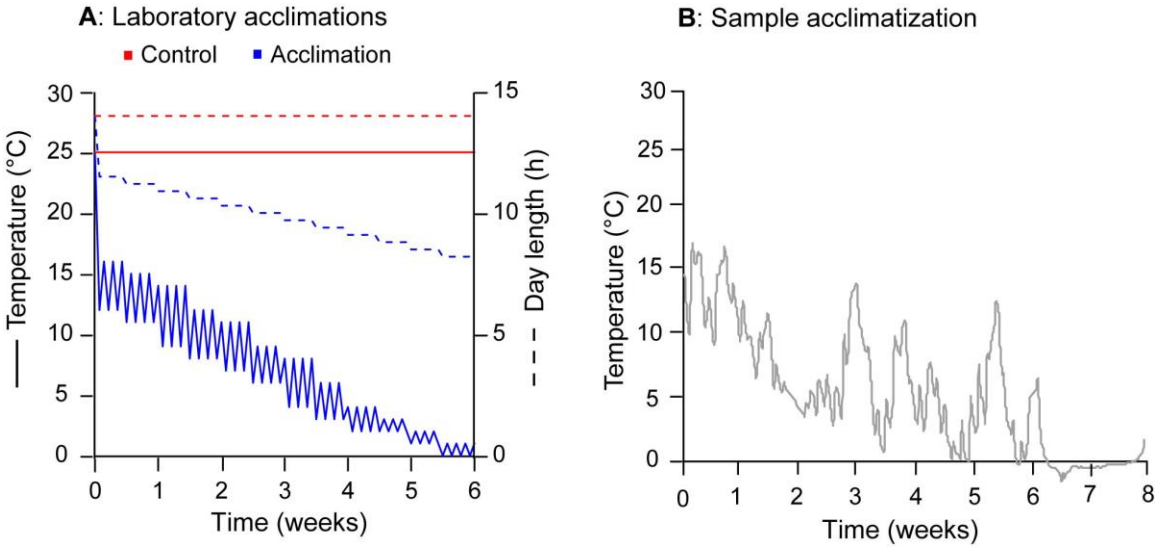

Figure 2.1. Acclimation and acclimatization regimes that induce freeze tolerance in *Gryllus veletis*. (A) Temperature (solid line) and photoperiod (dashed line) of laboratory control (red) and acclimation (blue) conditions. **(B)** Air temperature in London, Canada from 10 Oct 2013 – 9 Jan 2014 ('Outdoor-2013b'). The change in photoperiod over that time period was from 11.5:12.5 to 7.9:16.1 L:D). Data from McKinnon (2015).

For laboratory acclimations, I isolated crickets into individual 180 ml plastic cups (Polar Plastics, Summit Food Distributors, London, ON, Canada) with mesh covering and shelters made from egg cartons, and transferred them to a Sanyo MIR 154 incubator (Sanyo Scientific, Bensenville, IL, USA) or kept them in the rearing incubator. I provided rabbit food and water *ad libitum*. In the control conditions (Fig. 2.1A), I tested cricket freeze tolerance within 2 h of isolation ('zero weeks control'), and after three weeks ('three weeks control') and six weeks ('six weeks control'). Acclimation (Fig. 2.1A) was designed to mimic autumn conditions in London, Ontario, Canada: temperature fluctuated daily (12 h at daily high and low temperatures, based on the recorded upper- and lower-quartile values obtained from the 'Outdoor-2013' soil surface temperatures; Fig. 2.1B), and decreased over six weeks from 16/12 °C to 1/0 °C (high/low). Photoperiod decreased by 36 min per week (from 11.5:12.5 to 7.9:16.1 L:D). I tested cricket freeze tolerance after three weeks ('three weeks acclimation') and six weeks ('six weeks acclimation') of acclimation. In addition, I tested whether crickets were freeze tolerant after a six week acclimation that included only the decreases in temperature ('six weeks acclimation temperature only;' constant photoperiod 14:10 L:D) or photoperiod ('six weeks acclimation photoperiod only;' constant temperature 25 °C) used in the six week acclimation. To determine if freeze tolerance could be induced by short acclimations, I also exposed crickets to one week of low temperatures ('one week cold shock;' constant temperature -2°C, constant photoperiod 11:13 L:D), and a rapid cold exposure ('rapid cold hardening;' 15 min at -2 °C, 40 min at 22 °C).

2.2.2 Measuring metabolic rate and water loss rate

I measured CO_2 and H_2O emission of *G. veletis* nymphs that were acclimated or held under control conditions for six weeks using Sable Systems flow-through respirometry (Sable Systems International, Las Vegas, NV, USA) as described previously, with a flow rate of 80 ml min^{-1} (Lake et al., 2013). Crickets were starved for 24 h (Sinclair et al., 2011) at 25 °C (control) or on ice (acclimated) prior to respirometry measurements for 40 min each at 5 °C and 15 °C, with a 0.25 °C min^{-1} ramp rate between temperatures (example respirometry traces in Fig. A.1). The order of temperatures was randomized for each individual, and activity was recorded throughout data collection to ensure the

calculations were based on resting animals. Both CO_2 and H_2O production were corrected to 5 min baseline measurements. I calculated the rate of CO_2 production ($\dot{V}CO_2$) at both temperatures, and calculated the Q_{10} (the slope of log-transformed mass-specific $\dot{V}CO_2$ as a function of temperature) as per Lake et al. (2013). I determined the water loss rate (WLR), and cuticular and respiratory water loss as per Williams et al. (2010). I conducted all statistical analyses in R version 3.4.1 (R Core Team, 2017). I compared the effect of acclimation on $\dot{V}CO_2$ and WLR at both temperatures using ANCOVAs with mass as a covariate. I compared Q_{10} values from control and acclimated crickets using a two-tailed Welch's t-test.

2.2.3 Determining freeze tolerance

To freeze crickets, I placed them individually into 1.7 ml microcentrifuge tubes, which I placed in an aluminium block cooled by 50 % methanol circulated from a programmable refrigerated bath (Lauda Proline 3530, Würzburg, Germany). I equilibrated the crickets at 6 °C for 10 min, followed by cooling to the target temperature at 0.25 °C min^{-1}. Crickets were held at the target temperature for 1.5 h or more, followed by rewarming to 6 °C at 0.25 °C min^{-1}. To detect the supercooling point (SCP), each cricket was in contact with a 36-AWG type-T copper-constantan thermocouple (Omega, Laval, QC, Canada). Temperature was recorded at 0.5 s intervals by Picolog v5.24.1 software (Pico Technology, Cambridge, UK) *via* a Pico Technology TC-08 interface. The SCP was defined as the lowest temperature before the exotherm caused by the latent heat of crystallisation (Sinclair et al., 2015). After thawing, crickets were transferred to individual mesh-covered 180 ml transparent cups containing rabbit food, water, and shelters made from egg cartons for recovery at 15 °C. I assessed survival as the ability of crickets to move in response to gentle prodding within 48 h of recovery. Crickets were classified as freeze-tolerant if more than 75 % of individuals in a treatment group (control, acclimated, acclimatized) survived a freeze treatment of 1.5 h at -8 °C, and were otherwise classified as freeze-intolerant.

I estimated the acute LLT of 'six weeks acclimated' (hereafter referred to as freeze-tolerant) *G. veletis* by determining survival after freezing them to target temperatures between -6 °C and -15 °C for 1.5 h. I estimated the Lt of freeze-tolerant *G. veletis* by

determining survival after freezing them to -8 °C for between 1.5 h and 7 d. The range of temperatures/times encompassed 0 to 100 % mortality. I calculated the LLT_{80} (temperature at which 80 % of crickets will die after a 1.5 h exposure) and Lt_{80} (lethal time at which 80 % die when kept frozen at -8 °C) using a generalized linear model with a binomial distribution, and I tested the fit with Wald's χ^2 using the package MASS in R (Venables and Ripley, 2002).

2.2.4 *In vivo* cellular freeze tolerance

To test whether freeze-tolerant and control (hereafter referred to as freeze-intolerant) crickets protected cells during freezing, I conducted cell viability assays on fat body tissue dissected from crickets before or after they underwent a freeze treatment. These freeze treatments included -8 °C for 1.5 h (freeze-tolerant and freeze-intolerant crickets), as well as the -12 °C for 1.5 h (LLT) and -8 °C for 7 d (Lt) treatments (freeze-tolerant crickets only). I placed fat body tissue in a 0.6 ml microcentrifuge tube containing 10 µl *G. veletis* Ringer's solution (160 mM NaCl, 11 mM KCl, 8.4 mM $CaCl_2$, 5.9 mM $MgCl_2$, 5 mM HEPES, pH 7.6), to which I added 10 µl staining solution, containing 33 µg/ml DAPI (ThermoFisher Scientific, Missisauga, ON, Canada) and 33 µg/ml mM propidium iodide (ThermoFisher) in PBS (Sigma Aldrich, Oakville, ON, Canada; 0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, pH 7.4). DAPI stains the nuclei of all cells ('live' and 'dead'), whereas propidium iodide stains the nuclei of cells whose membranes are damaged ('dead'). I incubated the samples at room temperature for 5 min, washed fat body in PBS, and imaged samples with the Axio Imager Z1 upright compound fluorescence microscope (Carl Zeiss Canada, North York, ON, Canada), using excitation/emission wavelengths of 538/461 nm for DAPI, and 488/585 nm for propidium iodide. I imaged a single field of view of each fat body sample under 50× magnification, encompassing an area of c. 8.5 mm^2 (~300 cells; example micrographs in Fig. A.2). I measured cell survival by estimating the proportion of tissue area stained by DAPI only in ImageJ (Marshall and Sinclair, 2011). I calculated the average proportion of live cells from crickets in each treatment, and compared the effect of acclimation and time/temperature spent frozen on cell survival using a generalized linear model with a binomial distribution.

2.2.5 *Ex vivo* cellular freeze tolerance

To test whether cells from freeze-tolerant and freeze-intolerant crickets survived freezing *ex vivo*, I dissected fat body tissue from *G. veletis*, and performed cell viability assays on fat body tissue before or after a freeze treatment. To freeze fat body tissue *ex vivo*, I transferred the tissue into 0.6 ml tubes containing 10 µl Grace's Insect Medium and 2 µl 25 mg/ml silver iodide (AgI) slurry in water (Sigma Aldrich). Silver iodide is a potent INA, added to tubes to ensure the medium froze. Within 15 min of dissection, I placed these tubes in an aluminium block as described above. Thermocouples were attached to the outside of the tube, and temperature was recorded to detect the SCP when the medium froze. Samples were equilibrated for 1 min at 6 °C, cooled at 0.25 ° C min^{-1} to the target temperature, held at the target temperature for 10 min or more, and rewarmed at 0.25 ° C min^{-1} to 6 °C.

I determined cellular lethal limits by freezing fat body tissue from freeze-tolerant crickets in Grace's Insect Medium to a variety of target temperatures and times encompassing 0 to 100 % fat body cell mortality (Fig. A.3). After thawing, I determined cell viability by live-dead staining as described in Section 2.2.4, and I calculated the average proportion of live cells from three replicates of eight crickets from each treatment. I calculated the cellular LLT_{80} and Lt_{80} using generalized linear models with a binomial distribution, and I tested the fit with Wald's χ^2 using the package MASS in R. I then froze fat body from freeze-tolerant and freeze-intolerant crickets *ex vivo* at -8 °C for 10 min, and froze fat body from freeze-tolerant crickets to the cellular LLT (-16 °C for 10 min) and for the Lt (-8 °C for 24 h). I compared the effect of acclimation and time/temperature spent frozen on *ex vivo* cell survival using a generalized linear model with a binomial distribution.

2.2.6 Biochemical composition of hemolymph and tissues

I compared biochemical parameters of freeze-tolerant and freeze-intolerant *G. veletis* to identify potential mechanisms of freeze tolerance. To measure ice nucleator activity, I determined the SCP of hemolymph and tissues (gut, Malpighian tubules, fat body) extracted from live *G. veletis* at room temperature following previously described methods (Toxopeus et al., 2016). I blotted tissues with tissue paper to remove

hemolymph prior to transferring each tissue to a 0.2 ml microcentrifuge tube. I added 20 µl of anticoagulant (3 % ascorbic acid) to each sample, and included a control of Ringer's solution similarly diluted with 3 % ascorbic acid. I cooled the samples at 0.25 °C min^{-1} to -35 °C, and measured the SCP of each sample with a thermocouple attached to the outside of the tube. I compared the SCP of each sample to that of the Ringer's solution in 3 % ascorbic acid using a one-way ANOVA with planned contrasts. I compared the SCPs of samples between freeze-tolerant and freeze-intolerant crickets using a one-tailed Welch's t-test with a Bonferroni correction.

I measured osmolality and thermal hysteresis (TH) of hemolymph from freeze-tolerant and freeze-intolerant *G. veletis* using a nanolitre osmometer (Otago Osmometers, Dunedin, New Zealand) as described previously (Crosthwaite et al., 2011). Hemolymph was extracted from crickets, diluted 1:3 in an anticoagulant (3 % ascorbic acid), overlaid with type B immersion oil, flash frozen in liquid nitrogen and stored at -80 °C until analysis. I determined osmolality from the melting point (T_m) of hemolymph ice crystals (accounting for dilution in ascorbic acid), and TH from the difference between melting and freezing point (Crosthwaite et al., 2011). I determined differences in osmolality between freeze-tolerant and freeze-intolerant cricket hemolymph using a one-tailed Welch's t-test.

I measured hemolymph concentrations of sodium (Na^+) and potassium (K^+) in freeze-tolerant and freeze-intolerant crickets using an atomic absorption spectrometer (iCE 3000, ThermoScientific, Waltham, USA) as per MacMillan and Sinclair (2011). I incubated 4 µl hemolymph samples in 20 µl 3 % nitric acid, at room temperature (c. 22 °C) for 24 h, centrifuged the samples (600 × g for 1 minute), and diluted 20 µl of supernatant with 10 ml distilled deionized water (ddH_2O). I determined [Na^+] and [K^+] in each sample by comparing absorbance to Na^+ and K^+ standards diluted in 3 % nitric acid. I tested for differences in ion concentrations between freeze-tolerant and freeze-intolerant crickets (N = 8 per treatment) with two-tailed Welch's t-tests.

I used targeted GC/LC-MS (gas/liquid chromatography coupled to mass spectrometry) and GC-FID (gas chromatography coupled to flame ionization detection) to quantify

hemolymph and fat body concentrations of low molecular weight metabolites (sugars, polyhydric alcohols, and acidic metabolites) during six weeks of acclimation or control conditions (Li et al., 2015). I isolated fifth instar male nymphs into individual cups as described in Section 2.2.1, and haphazardly assigned them to remain in rearing conditions (control) or undergo acclimation (Fig. 2.1A). I extracted 4 µl of hemolymph and dissected fat body tissue from crickets at zero, three, and six weeks post-isolation. I briefly blotted fat body samples on tissue paper to remove hemolymph, and then flash froze samples in liquid nitrogen. Each biological replicate consisted of hemolymph or fat body tissue pooled from five crickets. I extracted metabolites from hemolymph and fat body samples in 70 % ethanol (Koštál et al., 2011b). Acidic metabolites (amino acids, organic acids, fatty acids) were derivatized by ethylchloroformate in pyridine/ethanol, extracted in chloroform (for GC-MS) or 30 % methanol (for LC-MS), and quantified as described in Li et al. (2015). Samples for sugar and polyol quantification were derivatized by oximation and methylsilylation, dissolved in iso-octane, and analyzed by GC-FID as per Li et al. (2015).

I centred and scaled hemolymph and fat body metabolite concentrations, and compared the metabolomes of control and acclimated crickets using Principal Components Analysis (PCA; Koštál et al., 2011b). I identified potential cryoprotectants as metabolites that were: 1) influential in the PCA (i.e. had the largest loadings), or 2) abundant (> 10 mM or nmol/mg tissue) in freeze-tolerant crickets. I compared concentrations of potential cryoprotectants between control and acclimated crickets using one-way ANOVAs. In addition, I validated hemolymph concentrations in freeze-tolerant and freeze-intolerant after six weeks of control or acclimation conditions (respectively) using spectrophotometric assays (see Appendix A for details), and compared these concentrations using one-tailed Welch’s t-tests. I used metabolic pathway analysis (Xia and Wishart, 2010) to identify potential pathways of cryoprotectant synthesis during acclimation by comparing fat body metabolite concentrations at zero and six weeks of acclimation.

2.2.7 Ice nucleation manipulations

To determine if manipulating the temperature and site of ice nucleation could confer freeze tolerance, I induced freezing of freeze-intolerant crickets with AgI, both externally (‘external AgI’), and in the gut (‘gut AgI’) and the hemolymph (‘hemolymph AgI’). To promote external ice formation, the crickets were briefly submerged in a 25 mg/ml AgI in water slurry prior to freezing. To initiate ice formation in the gut, I dusted their diet (rabbit food) with AgI for four weeks prior to freezing crickets. To manipulate hemolymph ice nucleation, I injected 4 µl of a 25 mg/ml AgI slurry under the cricket pronotum using a 5 µl gastight Hamilton syringe with a 25 gauge needle (Hamilton Company, Reno, NV, USA). Crickets were placed in 180 ml transparent plastic cups to recover at room temperature (c. 22 °C) for 40 min prior to a freeze treatment of -8 °C for 1.5 h.

2.2.8 *In vivo* manipulations of putative cryoprotectants

To elevate hemolymph concentrations of putative cryoprotectants, I injected 5 µl of cryoprotectant in Ringer’s solution under the cricket pronotum using a 10 µl gastight Hamilton syringe with a 30-gauge disposable needle (BD Canada, Mississauga, ON, Canada). Cryoprotectant solutions included: 0.5 M *myo*-inositol, 2.5 M proline, 1.3 M trehalose, 2.5 M glucose, 5 M glycerol, 15 % w/v PEG-8000 (polyethylene glycol), and a combination of 0.5 M *myo*-inositol, 2 M proline, and 1 M trehalose (Sigma Aldrich). To verify successful injection of cryoprotectants, I extracted 2 µl hemolymph samples 10 to 15 min post-injection, flash-froze these samples in liquid nitrogen, and stored them at -80 °C until analysis *via* spectrophotometric assays (see Appendix A for details). I transferred crickets to a freeze treatment 20 to 30 min post-injection, and pipetted 2 µl AgI slurry onto the dorsal abdomen of each cricket to ensure freezing at a uniform temperature (c. -4 °C) for all crickets. Freeze treatments included -8 °C for 1.5 h (freeze-tolerant and freeze-intolerant crickets), and the LLT and Lt treatments (freeze-tolerant crickets only). I compared survival of cryoprotectant-injected crickets to Ringer’s-injected (control) crickets from the same freeze treatment using a generalized linear model with a binomial distribution.

2.2.9 *Ex vivo* manipulations of putative cryoprotectants

To test the effect of putative cryoprotectants on *ex vivo* cellular freeze tolerance, I dissected fat body tissue from freeze-tolerant and freeze-intolerant *G. veletis*, and transferred the tissue into a 0.6 ml tube containing 2 µl AgI slurry and 10 µl Grace's Insect Medium or 10 µl cryoprotectant solution in Grace's Insect Medium. Physiologically-relevant cryoprotectant solutions included: 30 mM *myo*-inositol, 30 mM proline, 70 mM trehalose, 140 mM glucose, 30 mM glycerol, 0.3 mM PEG, and a combination of 30 mM *myo*-inositol, 30 mM proline, and 70 mM trehalose. Each cryoprotectant was also tested at a high concentration: 300 mM *myo*-inositol, proline, trehalose, glucose, glycerol, 3 mM PEG, and a combination of 300 mM each *myo*-inositol, proline and trehalose. The AgI was added to ensure extracellular freezing began at a uniform temperature (c. -4 °C) for all samples.

Freeze treatments began within 15 min of dissection, and included -8 °C for 10 min (fat body from freeze-tolerant and freeze-intolerant crickets), and the cellular LLT and Lt treatments (fat body from freeze-tolerant crickets only). I calculated the average proportion of live cells from three replicates of fat body samples from eight crickets for each freeze treatment × cryoprotectant combination. I compared survival of cells frozen in cryoprotectant solutions to those frozen in Grace's Insect Medium (control) from the same freeze treatment using a generalized linear model with a binomial distribution.

2.3 Results and discussion

2.3.1 Acclimation induces freeze tolerance in *G. veletis* nymphs

I induced freeze tolerance in a laboratory colony of *G. veletis*. Fifth-instar (juvenile) male *G. veletis* acclimated to decreasing, fluctuating, temperature and photoperiod for six weeks were freeze-tolerant: 92 ± 6 % survived being frozen for 1.5 h at -8°C (Table A.1). None of their counterparts maintained under control (rearing) conditions over the same period survived freezing, and were therefore freeze-intolerant (Table A.1). Lab-reared crickets acclimatized outside in autumn in London, Ontario, Canada for eight weeks were also freeze-tolerant (Fig. 2.1B; Table A.1). I could not induce freeze tolerance in crickets with short acclimation treatments (less than six weeks), nor with six-week acclimations in

which only temperature or photoperiod decreased (Table A.1). This suggests that acquiring freeze tolerance requires physiological changes that take many weeks to complete, and that these changes are induced by a combination of temperature and photoperiod.

Acclimated crickets reduced their metabolic rate by c. 33 %, as estimated from CO_2 emission using flow-through respirometry. Acclimation did not change thermal sensitivity of metabolic rate, expressed as Q_{10} (Fig. 2.2A, Table A.2). Freeze-tolerant *G. veletis* had c. 50 % lower water loss rates than freeze-intolerant crickets, most of which (>80 %) was accounted for by reduced cuticular water loss (Fig. 2.2B, Table A.2). The reduction in metabolic rate during acclimation is similar to other freeze-tolerant insects that overwinter in diapause (Irwin et al., 2001), and may partially reflect a change of fuel to solely lipids (Sinclair et al., 2011), although the crickets were all fasted for 24 h prior to measurement to control for this. Reduced metabolic rate likely conserves energy reserves during winter (Sinclair, 2015), while modifying cuticle composition to reduce water loss may mitigate dehydration stress (Bazinet et al., 2010; Stinziano et al., 2015).

Acclimated *G. veletis* were moderately freeze-tolerant (Sinclair, 1999): they died if frozen for 1.5 h below -12 °C or for 7 d at -8 °C, which set the bounds for the LLT (Fig. 2.3A) and Lt (Fig. 2.3B), respectively. These lethal limits are similar to other orthopterans that are freeze-tolerant as juveniles (Toxopeus et al., 2016) or adults (Ramløv et al., 1992; Sinclair et al., 1999). Winter air temperatures are likely to approach or exceed these lethal limits across much of the *G. veletis* geographical range (north eastern North America). However, *G. veletis* can likely still survive in these regions by overwintering in thermally-buffered microhabitats, such as beneath snow or leaf litter (Sinclair, 2015). Alternatively, *G. veletis* may further decrease their LLT or increase their Lt upon exposure to lower temperatures during winter, either *via* seasonal plasticity (cf. the alpine cockroach *Celattoblatta quinquemaculata*; Sinclair, 1997), or through other hardening responses, as shown by *B. antarctica* (Teets et al., 2008).

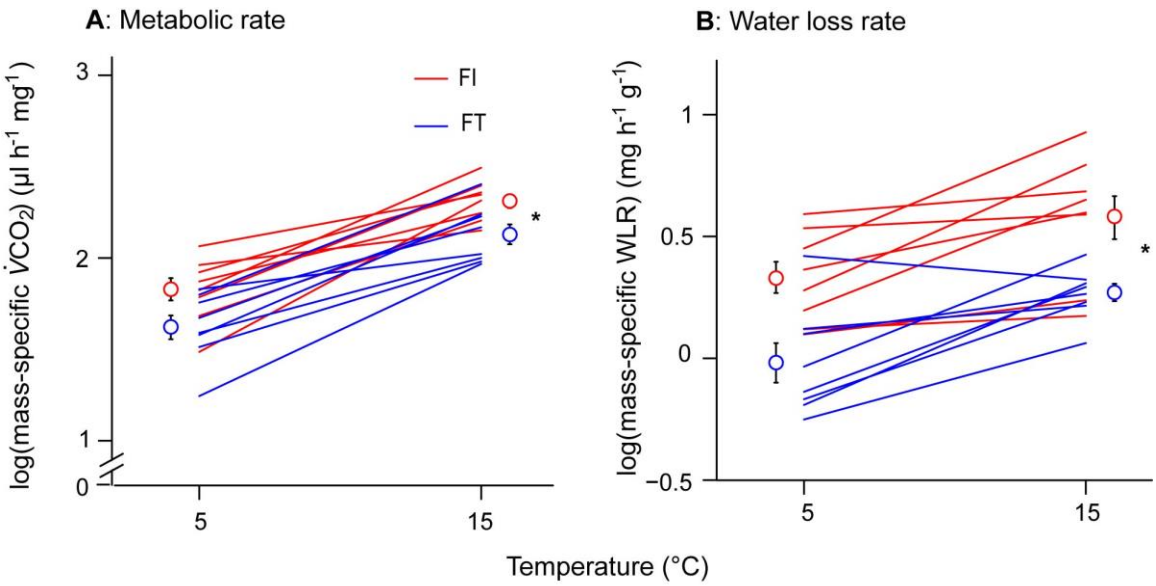

Figure 2.2. (**A) CO_2 emission ($\dot{V}CO_2$) and (B) water loss rate (WLR) of freeze-intolerant (FI) and freeze-tolerant (FT) *Gryllus veletis* at two temperatures.** Each line represents a single cricket (N = 8 per treatment), and open circles represent the mean ± SE for that group of crickets at 5 °C or 15 °C. Small error bars are obscured by symbols. Asterisks indicate a difference between FT and FI crickets at both temperatures ($\dot{V}_{CO2}$, ANCOVA: mass: $F_{1,31}$ = 12.01, P = 0.002; acclimation: $F_{1,31}$ = 9.67, P = 0.004; temperature: $F_{1,31}$ = 63.00, P < 0.001; acclimation × temperature: $F_{1,31}$ = 1.00, P = 0.327; WLR, ANCOVA: mass: $F_{1,31}$ = 8.35, P = 0.007; acclimation: $F_{1,31}$ = 16.45, P < 0.001; temperature: $F_{1,31}$ = 8.54, P < 0.001; acclimation × temperature: $F_{1,31}$ = 1.39, P = 0.248). Example respirometry traces are in Fig. A.1.

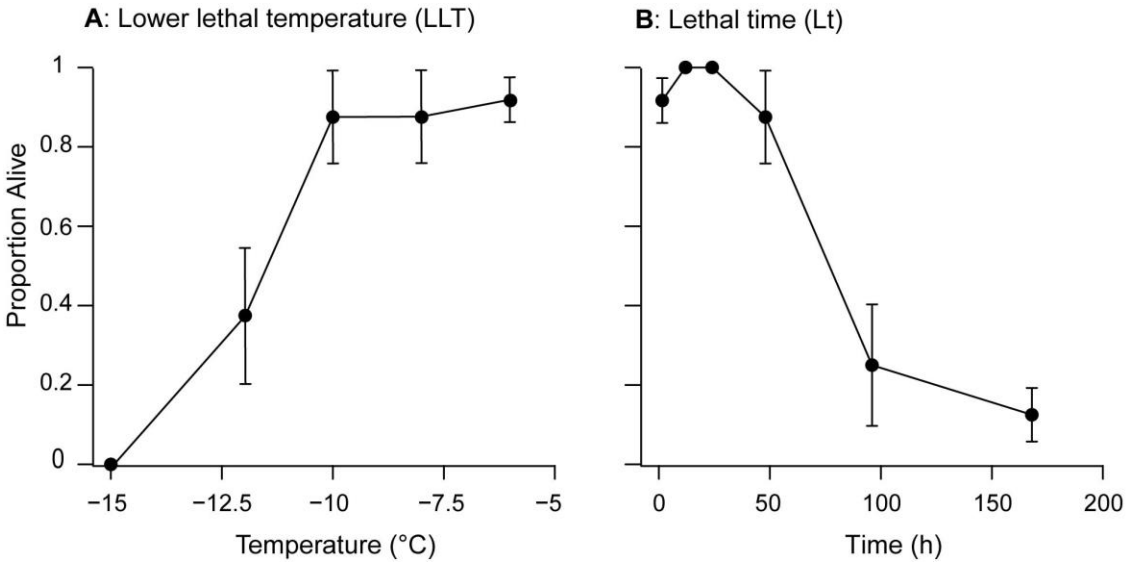

Figure 2.3. Lethal limits of freeze-tolerant (FT) *Gryllus veletis*. (A) Proportion of FT crickets that survived following 1.5 h exposures to temperatures ranging from -6 °C to -15 °C. **(B)** Proportion of FT crickets that survived following exposure to -8 °C for times ranging from 1.5 h to 7 d. Each point represents the proportion of 24 crickets ± SE. Low temperatures reduced survival (Wald χ^2 = 2.763, P = 0.006; LLT_{80} = -13.8 °C). Prolonged exposure to -8 °C reduced survival (Wald χ^2 = 3.956, P < 0.001; with Lt_{80} = 123 h). Data from McKinnon (2015).

Freeze-tolerant insects presumably protect their cells and tissues from freeze injury, but fail to do so at the lethal limits (LLT, Lt; Chapter 1). When I examined *in vivo* tissue damage, fat body cell survival in *G. veletis* was high prior to freezing, and in freeze-tolerant crickets frozen for 1.5 h at -8 °C (Fig. 2.4A). Conversely, freeze-intolerant crickets frozen for 1.5 h at -8 °C and freeze-tolerant crickets frozen to their LLT or for their Lt had low fat body cell survival (Fig. 2.4A). Thus, high fat body cell damage was associated with mortality in both freeze-tolerant and freeze-intolerant crickets. Fat body cells from freeze-tolerant crickets frozen *ex vivo* in Grace's medium for 10 min at -8 °C survived better than those frozen to the cellular LLT or Lt, or those from freeze-intolerant crickets (Figs. 2.4B). Therefore, whole animal freeze tolerance was correlated with cellular freeze tolerance. These results are similar to seasonal acquisition of freeze tolerance by *E. solidaginis*, which is associated with improved tissue freeze tolerance *ex vivo* (Yi and Lee, 2003). Thus, these *ex vivo* experiments can inform our understanding of *in vivo* freeze tolerance, and vice versa.

2.3.2 Freeze-tolerant *G. veletis* control ice nucleation

Freeze tolerance is often accompanied by elevated SCPs, which may facilitate freeze tolerance by slowing ice formation and minimizing IIF (Zachariassen and Kristiansen, 2000). Freeze-tolerant *G. veletis* had higher SCPs than freeze-intolerant crickets (Fig. 2.5). When frozen *ex vivo*, the SCP of the hemolymph and gut of freeze-tolerant crickets was significantly higher than those of freeze-intolerant individuals (Fig. 2.5), suggesting that freeze-tolerant *G. veletis* elevate their SCP by accumulating extracellular INAs in the hemolymph and gut during acclimation. Supercooling points of fat body and Malpighian tubules did not differ between freeze-tolerant and freeze-intolerant crickets (Fig. 2.5).

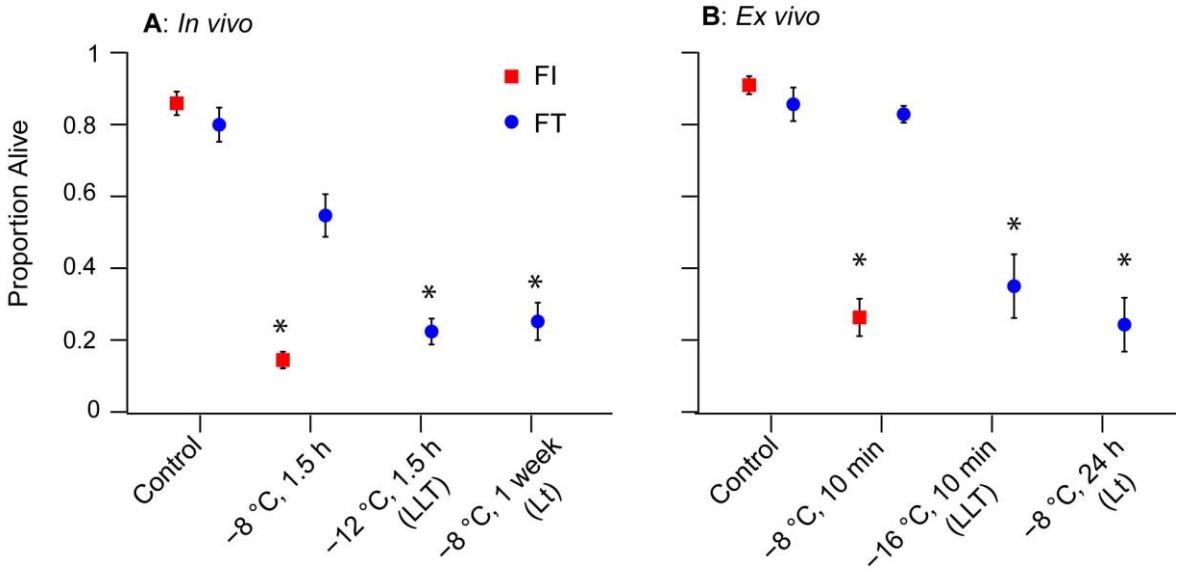

Figure 2.4. Fat body cell (FBC) survival following freezing (A) *in vivo* and (B) *ex vivo*. (A) The proportion of live FBCs dissected from freeze-tolerant (FT) and freeze-intolerant (FI) crickets that were never frozen (control), or frozen for 1.5 h at -8 °C, the LLT, or the Lt. **(B)** The proportion of live FBCs from FI and FT crickets after *ex vivo* freezing in Grace's Insect Medium to -8 °C for 10 min, or the cellular LLT or Lt. Control samples were dissected from crickets, and never frozen. The mean proportion of live FBCs was lower in all treatments relative to controls ($P < 0.05$, denoted by asterisks), except FBCs from FT crickets frozen *in vivo* for 1.5 h at -8 °C ($\chi^2 = 1.77$, $P = 0.077$) and FT FBCs frozen *ex vivo* for 10 min at -8 °C ($\chi^2 = 0.266$, $P = 0.790$). Each point represents the mean ± SE proportion of live FBCs from 18 (*in vivo*) or 24 (*ex vivo*) crickets. Example micrographs (from which survival was quantified) are in Fig. A.2.

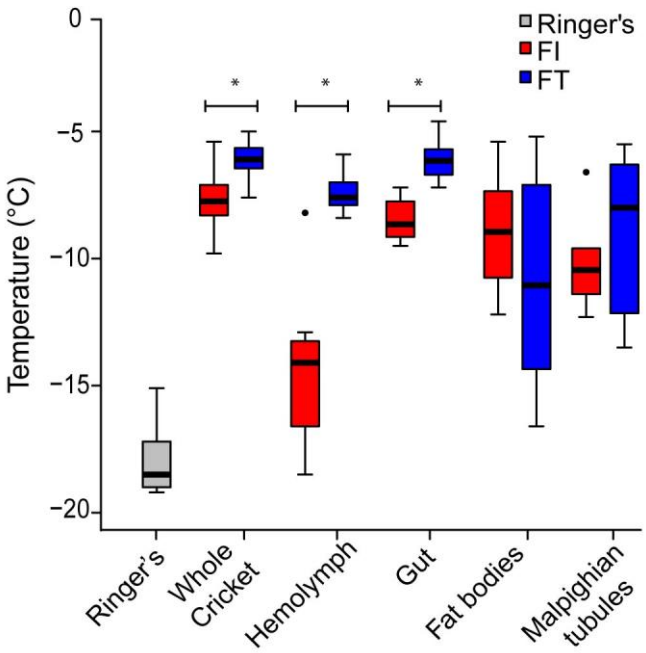

Figure 2.5. Supercooling points (SCPs) of *Gryllus veletis* and its tissues. The SCP was determined for freeze-tolerant (FT; $N = 24$) and freeze-intolerant (FI; $N = 64$) crickets, as well as samples of hemolymph and tissues (gut, fat bodies and Malpighian tubules) in anticoagulant ($N = 8$ per hemolymph/tissue type). The mean SCPs of hemolymph and all tissues differed from *G. veletis* Ringer's solution ($F_{9,70} = 27.18$, $P < 0.001$; all contrasts $P < 0.05$). The top and bottom of each box represents the upper and lower quartile, respectively; the horizontal line represents the median; the vertical lines extend to the minimum and maximum values within 1.5 times the inter-quartile range; and black dots indicate outliers. Mean SCPs that are significantly different between FT and FI crickets are denoted with an asterisk ($t_{56} = 8.36$, $P < 0.001$). Data from McKinnon (2015).

I tested the hypothesis that controlling the temperature and location of ice formation was sufficient to confer freeze tolerance on control crickets. I applied AgI to the cuticle, hemolymph or gut of freeze-intolerant crickets to increase the SCP to c. -4 °C. None of these treatments yielded any survival after 1.5 h at -8 °C (Table A.1), indicating that controlling the temperature and location of ice formation is not sufficient to confer freeze tolerance. These results are similar to observations of *Eleodes blanchardi* beetles, which lose their freeze tolerance if deacclimated despite retaining a high (c. -6 °C) SCP (Zachariassen et al., 1979). I conclude that controlling ice nucleation may be necessary, but is not sufficient, for freeze tolerance in *G. veletis*.

In addition to controlling the initiation of ice formation, freeze-tolerant insects may need to restrict the growth and recrystallization of ice once it has formed, for example by accumulating TH factors with recrystallization inhibition activity (Chapter 1; Knight and Duman, 1986). Nanolitre osmometry of *G. veletis* hemolymph did not reveal either TH or spicular/angular ice crystal growth (Fig. A.4, Table A.3), suggesting a lack of hemolymph proteins that limit ice growth. However, TH factors can be intracellular or bound to epithelia in other freeze-tolerant arthropods (Duman, 2015; Tursman and Duman, 1995; Wharton et al., 2009). In addition, recrystallization inhibitors do not always exhibit TH activity (Chapter 1; Wharton et al., 2005). Thus, ice-binding proteins may yet prove to play a more subtle role in freeze-tolerant *G. veletis*.

2.3.3 Acclimated *G. veletis* elevate hemolymph osmolality and alter metabolism to accumulate potential cryoprotectants

Acclimation altered the low molecular weight metabolite profile of both hemolymph and fat body cells (Fig. 2.6). I detected 49 metabolites in hemolymph and 48 in fat body tissue (all 48 hemolymph metabolites except sucrose; Tables A.4, A.5). *myo*-Inositol, proline and trehalose notably increased in concentration (>10 mM or 10 nmol/mg tissue) during acclimation (Fig. 2.7). The accumulation of *myo*-inositol, proline and trehalose accounted for c. 100 mOsm of the c. 250 mOsm increase in hemolymph osmolality during acclimation (Fig. 2.8). The concentrations of other hemolymph metabolites totalled c. 30 mM (Table A.4) and neither the total concentration of these metabolites nor [Na^+] and [K^+] differed between freeze-tolerant and freeze-intolerant crickets (Fig. 2.8).

Approximately 120 mOsm of the increased hemolymph osmolality in freeze-tolerant crickets is unidentified (Fig. 2.8), and may include additional cryoprotective molecules.

I focused on the most abundant metabolites (*myo*-inositol, proline, and trehalose), and explored their role as potential cryoprotectants in *G. veletis*. *myo*-Inositol has been reported in other cold-hardy insects (Purać et al., 2016) and at least one freeze-tolerant cockroach (Tanaka and Tanaka, 1997), but to my knowledge this is the first report in a freeze-tolerant orthopteran. *Gryllus veletis* accumulated c. 30 mM proline in the hemolymph, which is similar to other freeze-tolerant orthopterans [c. 40-80 mM in *H. maori*; c. 10 mM in *Cyphoderris monstrosa* (Neufeld and Leader, 1998; Ramløv, 1999; Toxopeus et al., 2016)], whereas *G. veletis* accumulated more hemolymph trehalose (c. 70 mM) than other freeze-tolerant orthopterans [c. 15 mM in *H. maori*; c. 20 mM in *C. monstrosa* (Ramløv et al., 1992; Toxopeus et al., 2016)]. Nevertheless, these concentrations of metabolites are likely too low to colligatively reduce ice content in a biologically meaningful fashion (except perhaps at high subzero temperatures above the SCP); for example, a c. 100 mOsm increase in osmolality of *D. melanogaster* hemolymph only reduces maximum ice fraction by 3 % (Rozsypal et al., 2018).

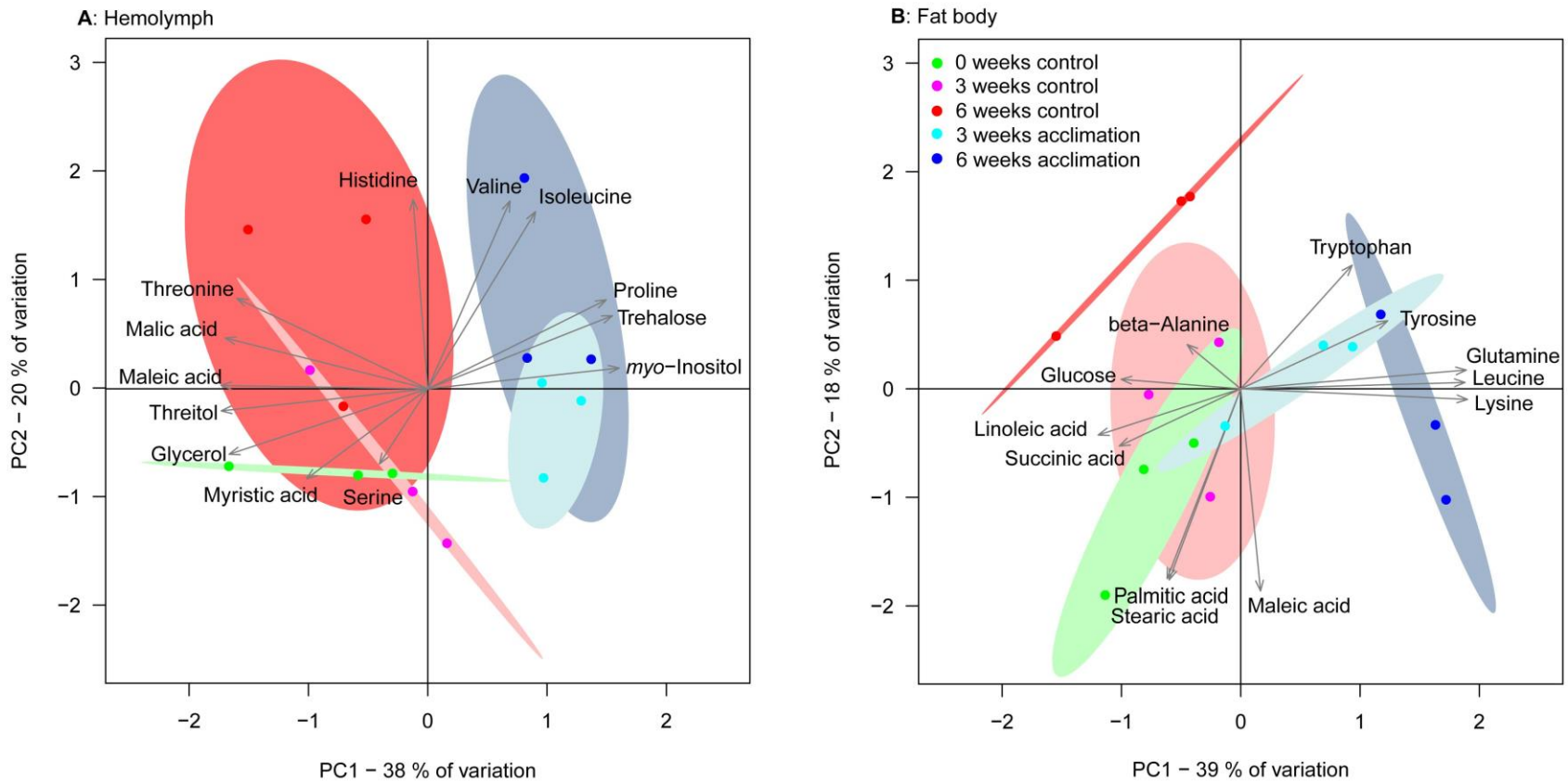

Figure 2.6. Hemolymph and fat body metabolite composition during acclimation. Principal components analysis (PCA) based on **(A)** hemolymph or **(B)** fat body concentrations of 49 or 48 low molecular weight metabolites (respectively) in fifth instar male *G. veletis* held under control or acclimation conditions for six weeks. Each point represents one sample of hemolymph or fat body pooled from five crickets. Ellipses represent the 95 % confidence interval of each group of points (coloured to match the points). Each arrow represents a metabolite, with longer arrows indicating a greater effect (loading) of that metabolite on the principal components (PCs). The 12 metabolites with the highest loadings along PC1 or PC2 are labelled.

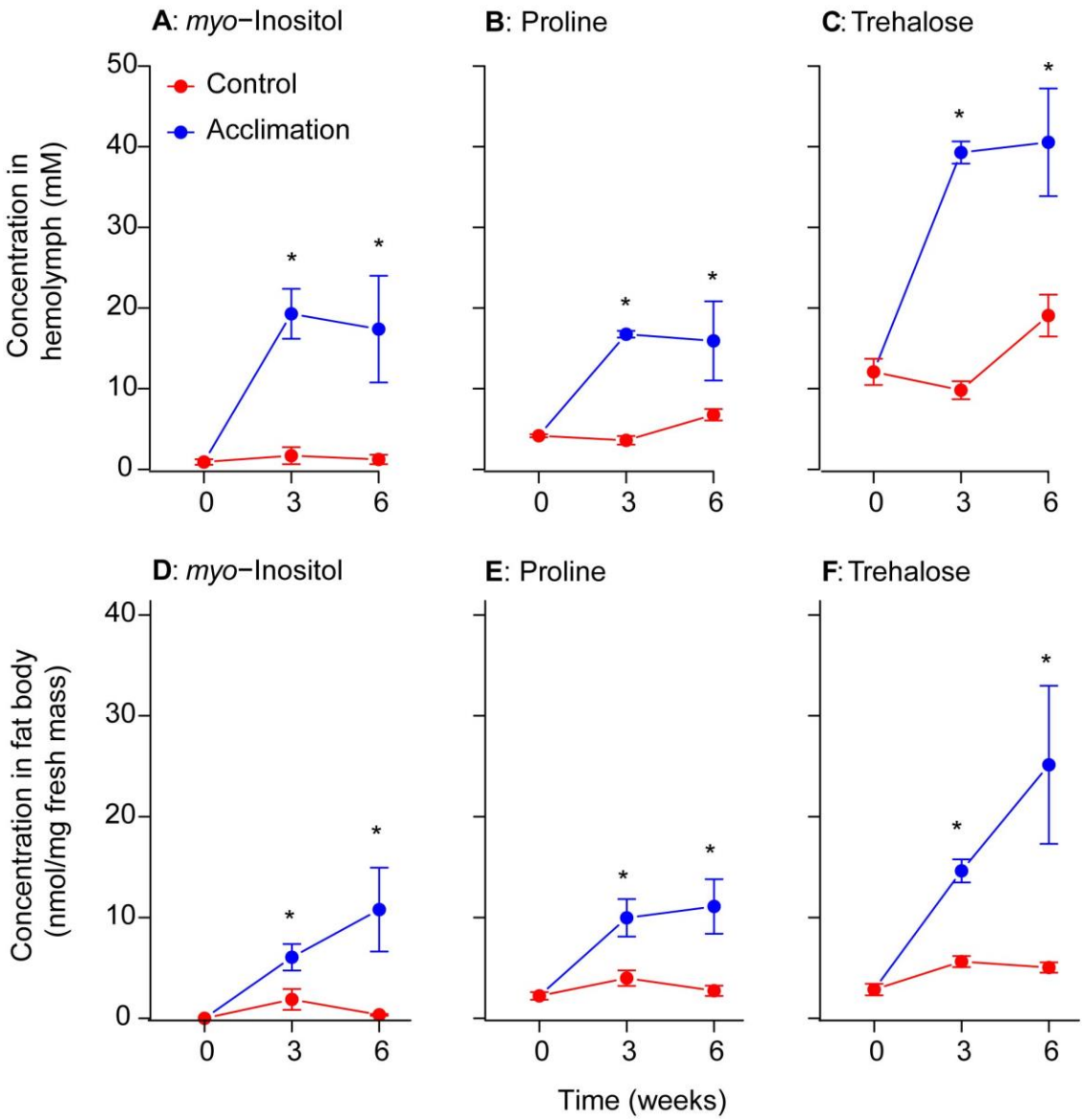

Figure 2.7. Putative cryoprotectant accumulation in hemolymph and fat body during acclimation. Concentrations (from metabolomics data) of the three most abundant metabolites **(A,D)** *myo*-inositol, **(B,E)** proline, and **(C,F)** trehalose in hemolymph or fat body of fifth instar male *G. veletis* held under control or acclimation conditions for six weeks. Each point represents the mean concentration ± SE of three samples, each containing hemolymph or fat body pooled from five individuals. Asterisks indicate that the mean concentration is different from zero week control. Hemolymph ANOVAs: inositol: $F_{4,10} = 8.00$, $P = 0.004$; proline: $F_{4,10} = 8.21$, $P = 0.003$; trehalose: $F_{4,10} = 19.18$, $P < 0.001$. Fat body ANOVAs: inositol: $F_{4,10} = 5.21$, $P = 0.016$; proline: $F_{4,10} = 7.53$, $P = 0.005$; trehalose: $F_{4,10} = 6.73$, $P = 0.007$.

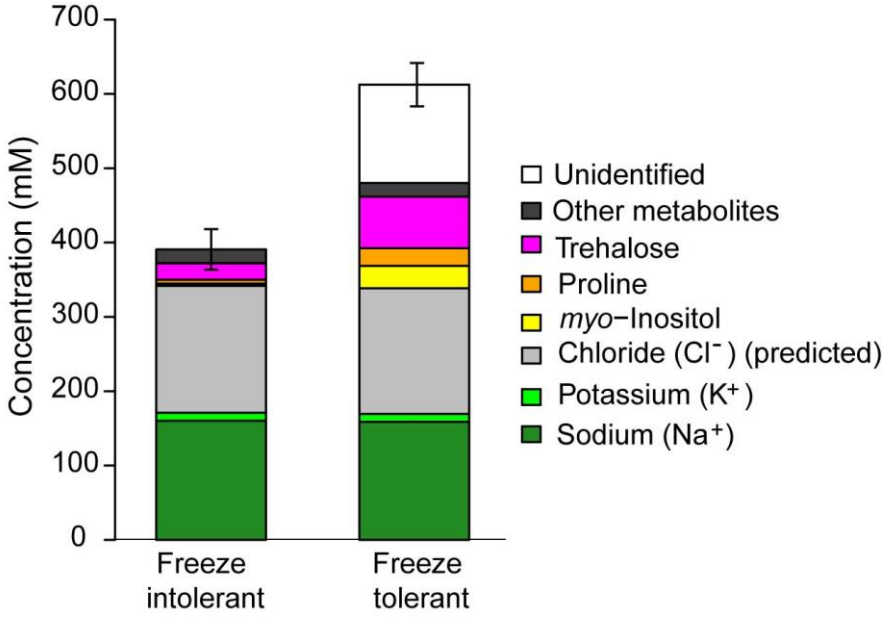

Figure 2.8. Hemolymph composition of freeze-tolerant (FT) and freeze-intolerant (FI) *Gryllus veletis*. Mean concentrations of ions (Na^+ and K^+) and putative cryoprotectants (*myo*-inositol, proline and trehalose) were determined *via* atomic absorption spectrometry and spectrophotometric assays, respectively, and are represented as a fraction of the total hemolymph osmolality in FT and FI *G. veletis* ($N = 8$). 'Other metabolites' is the sum concentration of 46 other molecules from the metabolomics screen (excluding *myo*-inositol, proline and trehalose). Cl^- concentrations were estimated to be equal to the sum of Na^+ and K^+ concentrations. Errors bars represent the SE of the mean osmolality. Osmolality, and concentrations of *myo*-inositol, proline, and trehalose were higher in FT than FI crickets (osmolality: $t_{14} = 5.64$; inositol: $t_{14} = 9.24$; proline: $t_{14} = 6.82$; trehalose: $t_{14} = 9.93$; $P < 0.001$ for all). Mean Na^+ and K^+ concentrations were similar between treatment groups (Na^+: $t_{14} = 0.10$, $P = 0.54$; K^+: $t_{14} = 0.12$, $P = 0.55$). Osmolality and ion concentration data from McKinnon (2015).

Metabolic pathway analysis (Xia and Wishart, 2010) of fat body metabolite concentrations suggested significant changes in carbohydrate and amino acid metabolism during acclimation (Table 2.1). I hypothesize these shifts in metabolic pathway activity promote cryoprotectant synthesis in fat body tissue (Arrese and Soulages, 2010). Increased [citrate] in freeze-tolerant *G. veletis* (Table A.5) indicates reduced flux through the 'TCA (tricarboxylic acid) cycle' (Table 2.1; Teets et al., 2012), which could promote increased use of glucose in synthesis of trehalose (Thompson, 2003) and *myo*-inositol (Hoshikawa, 1987; Loewus and Loewus, 1983), rather than use in catabolic pathways. Proline may be a product of protein degradation (Koštál et al., 2011a), or may be synthesized from other amino acids (e.g. alanine, glutamine, or glutamate) or TCA cycle intermediates (Bursell, 1977; Teets et al., 2012; Wan et al., 2014; Weeda et al., 1980), as indicated by altered activity of 'alanine, aspartate and glutamate metabolism,' and 'glutamine and glutamate metabolism' in freeze-tolerant *G. veletis* (Table 2.1). To test these hypotheses, I suggest manipulating the activity or abundance of enzymes in these metabolic pathways.

Table 2.1. Metabolic pathway analysis indicating which pathways appear to have altered activity in freeze-tolerant *G. veletis* fat body, based on metabolite concentrations measured at zero and six weeks of acclimation. High impact indicates high changes in pathway activity (Xia and Wishart, 2010) as a result of acclimation. Pathways with impact = 0 were excluded from the Table.

Pathway	Total Compounds[a]	Represented Compounds[b]	Impact	Corrected *P*-value[c]
Amino acid metabolism				
beta-Alanine metabolism	13	2	0.41	**0.07**
Alanine, aspartate and glutamate metabolism	23	10	0.80	**0.04**
Aminoacyl-tRNA biosynthesis	67	20	0.14	**0.08**
Arginine and proline metabolism	37	9	0.53	**0.08**
Cysteine and methionine metabolism	25	5	0.33	0.12
Glutamine and glutamate metabolism	5	3	1.00	**0.08**
Glutathione metabolism	26	6	0.49	**0.09**
Glycine, serine and threonine metabolism	25	6	0.57	**0.07**
Histidine metabolism	7	1	1.00	**0.05**
Phenylalanine metabolism	10	2	0.69	0.44
Phenylalanine, tyrosine and tryptophan biosynthesis	4	2	1.00	0.44
Tryptophan metabolism	23	1	0.38	0.27
Tyrosine metabolism	30	2	0.19	0.44
Valine, leucine and isoleucine biosynthesis	13	5	1.00	**0.04**
Carbohydrate metabolism				
Citrate cycle (TCA cycle)	20	7	0.33	**0.05**
Galactose metabolism	26	4	0.08	0.11
Glycolysis or gluconeogenesis	25	2	0.10	0.10
Glyoxylate and dicarboxylate metabolism	16	3	0.50	**0.05**
Pyruvate metabolism	24	3	0.17	0.10
Starch and sucrose metabolism	17	4	0.08	**0.07**
Lipid metabolism				
Butanoate metabolism	21	5	0.18	0.29
Glycerolipid metabolism	16	1	0.23	**0.04**
Inositol phosphate metabolism	24	1	0.20	0.11
Linoleic acid metabolism	6	1	1.00	0.25

[a]Number of compounds in metabolic pathway.
[b]Number of compounds in the metabolomics dataset that are represented in that pathway.
[c]Corrected for false discovery rate; *P*-values < 0.1 are bolded.

2.3.4 Exogenous cryoprotectants do not confer freeze tolerance

To determine the cryoprotective potential of *myo*-inositol, proline and trehalose, I tested the hypothesis that these metabolites were sufficient to confer freeze tolerance on freeze-intolerant crickets. I elevated hemolymph metabolite concentrations *in vivo* by injection, and determined the effect on whole cricket survival. Injection of *myo*-inositol, proline, or trehalose solutions (or a combination of all three) into freeze-intolerant crickets elevated concentrations to those measured in freeze-tolerant cricket hemolymph (Fig. A.5). However, no injected freeze-intolerant crickets survived freezing (Fig. 2.9A).

To determine which properties of metabolites (e.g. size, functional groups, permeability to cells) were important for cryoprotective function, I also injected other polyols (glycerol and PEG) and glucose (the monomer of trehalose). Glucose and PEG had no impact on freeze tolerance, but a small proportion (< 20 %) of freeze-intolerant crickets injected with glycerol survived freezing (Fig. 2.9A). High exogenous glycerol also increases freezing survival in < 10 % of *D. melanogaster* larvae (Koštál et al., 2012), and endogenous glycerol is a common cryoprotectant in many cold-hardy insects (Lee, 2010; Purać et al., 2016). Although *G. veletis* do not accumulate glycerol, my results support its potential as a cryoprotectant in freeze-intolerant animals *in vivo*.

I hypothesized that cryoprotectants facilitate whole animal survival by protecting cells, either indirectly (e.g. by reducing ice content) or directly (e.g. by protecting macromolecules). Fat body dissected from freeze-intolerant *G. veletis* had low cell survival when frozen *ex vivo* with exogenous cryoprotectants, whether those concentrations were similar to freeze-tolerant cricket hemolymph (30 mM *myo*-inositol, 30 mM proline, or 70 mM trehalose; Fig A.6), or higher (300 mM; Fig. 2.9B). Exogenous glycerol reduced freeze injury of freeze-intolerant cricket fat body frozen *ex vivo*, although only at high concentrations (300 mM; Fig. 2.9B). These results are similar to *ex vivo* experiments with other freeze-tolerant insects, including high survival of *P. isabella* foregut cell survival when frozen with 1 M glycerol (Yi and Lee, 2016), and of *C. suppressalis* fat body cells frozen in 250-750 mM glycerol (Izumi et al., 2006). In insects that accumulate it, high concentrations of glycerol may therefore contribute to whole animal freeze tolerance by improving cell survival.

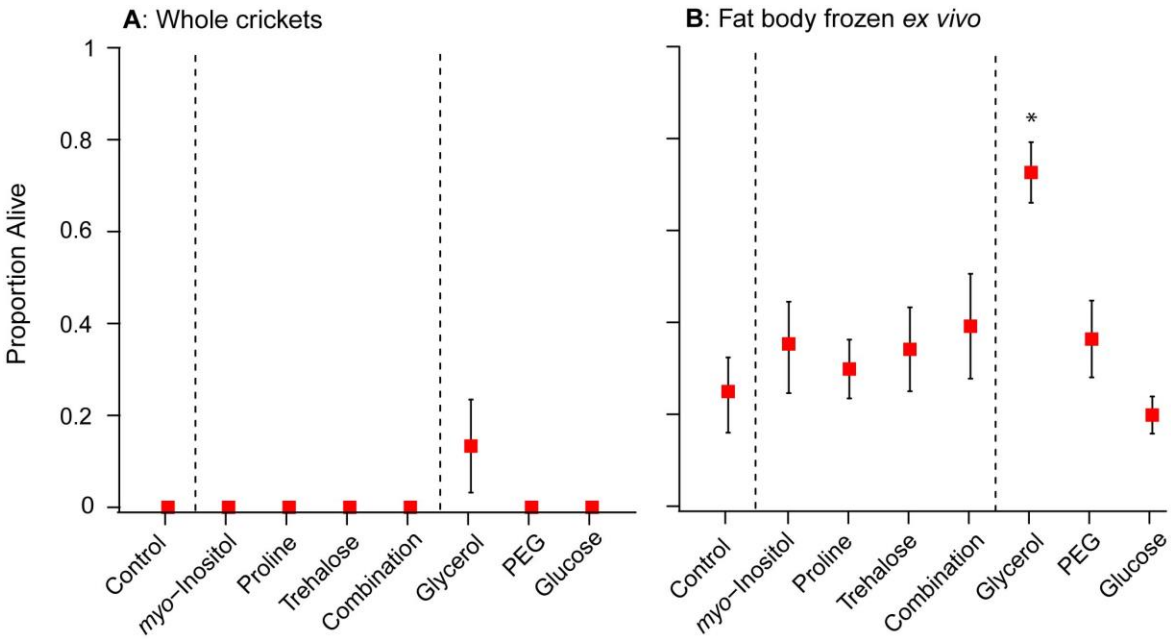

Figure 2.9. Effect of cryoprotectant injection on post-thaw survival of (A) freeze-intolerant (FI) *Gryllus veletis* and (B) their fat body cells (FBCs) frozen *ex vivo*. (A) Proportion of FI crickets that survived following injection with Ringer's solution (control) or the putative cryoprotectant in Ringer's solution, and being frozen to -8 °C for 1.5 h. **(B)** Proportion of live FBCs following freezing *ex vivo* to -8 °C for 10 min in Grace's Insect Medium (control) or the putative cryoprotectant (3 mM PEG, 300 mM for all others) in Grace's Insect Medium. *Ex vivo* FBC survival with lower cryoprotectant concentrations is presented in Fig. A.6. Each point represents the surviving proportion ± SE of 24 crickets or FBCs. Small error bars are obscured by symbols. Asterisks denote that survival is higher than the control sample in that group ($P < 0.05$, generalized linear models in Table A.6).

My experiments in freeze-intolerant crickets demonstrated that none of the endogenous *G. veletis* cryoprotectants (*myo*-inositol, proline, and trehalose) could confer freeze tolerance on control crickets or their tissues. This is congruent with experiments on *D. melanogaster*, where both elevated [proline] and cold acclimation are necessary to induce freeze tolerance (Koštál et al., 2016; Koštál et al., 2012). Acclimation may enhance cryoprotectant function, for example by inducing synthesis of cryoprotectant transporters that facilitate intracellular cryoprotectant accumulation (Wolkers et al., 2001). I note that glycerol can cross membranes through AQPs (Finn et al., 2015; Izumi et al., 2006) more easily than the larger and/or more charged trehalose, *myo*-inositol, or proline, which may account for its capacity to confer freeze tolerance on freeze-intolerant crickets. Acclimation likely also changes a range of non-cryoprotectant properties, such as membrane composition and cytoskeletal stability (Des Marteaux et al., 2018; Koštál et al., 2013). I hypothesize that cryoprotectants such as *myo*-inositol, proline and trehalose only facilitate *G. veletis* freeze tolerance in concert with other changes induced during acclimation.

2.3.5 Cryoprotectant-specific enhancement of freeze tolerance in acclimated *G. veletis*

To test the hypothesis that *myo*-inositol, proline, and trehalose facilitate freeze tolerance alongside other responses to acclimation, I injected cryoprotectant solutions into acclimated (freeze-tolerant) crickets and determined the effect on survival when frozen to their lethal limits (LLT and Lt). Elevated hemolymph concentrations of proline, trehalose, and glycerol increased the proportion of freeze-tolerant crickets that survived being frozen for the Lt, and both trehalose and glycerol injections improved survival at the LLT (Fig. 2.10A). Injection of the combination of all three cryoprotectants had similar effects on survival to proline (Lt) and trehalose (Lt and LLT) alone (Fig. 2.10A). Thus, increased concentrations of specific cryoprotectants enhance whole-organism freeze tolerance in a redundant fashion. When I froze freeze-tolerant cricket fat body tissue *ex vivo* to the cellular lethal limits, high concentrations of exogenous *myo*-inositol and glycerol improved cell survival at the cellular LLT, and *myo*-inositol and trehalose protected cells frozen for the cellular Lt (Fig. 2.10B). Survival of fat body cells frozen

with a combination of *myo*-inositol, proline and trehalose (300 mM each) did not differ from the survival of fat body cells frozen with 300 mM *myo*-inositol (LLT and Lt) or trehalose (Lt) alone (Fig. 2.10B). I did not see these effects of improved cell survival with cryoprotectant concentrations similar to those measured in freeze-tolerant cricket hemolymph (Fig. A.6).

Taken together, the *in vivo* and *ex vivo* experiments suggest that *myo*-inositol, proline, trehalose and glycerol are all cryoprotective in acclimated *G. veletis* or their tissues, but likely act *via* different mechanisms, supporting the hypothesis that freeze-tolerant insects accumulate multiple types of cryoprotectants to fulfil multiple functions. The evidence for this is two-fold. Firstly, each cryoprotectant improved survival under a unique set of conditions: *myo*-inositol only enhanced fat body cell survival *ex vivo*, proline only increased whole cricket survival at the Lt, and trehalose improved both whole animal and fat body cell survival. In contrast to my observations with glycerol in freeze-intolerant crickets, cryoprotectants that improved survival in freeze-tolerant crickets rarely improved cell survival *ex vivo,* suggesting that the causes of mortality differ at the cellular and organismal levels. Secondly, I observed no synergy or additive effects among cryoprotectants. For example, while both proline and trehalose improved survival of crickets frozen for the Lt, the combination of *myo*-inositol, proline and trehalose did not further increase the proportion of crickets that survived this treatment. Likewise, a similar proportion of freeze-tolerant cricket fat body cells survived at the cellular Lt with exogenous *myo*-inositol alone, trehalose alone, or a combination of *myo*-inositol, proline and trehalose.

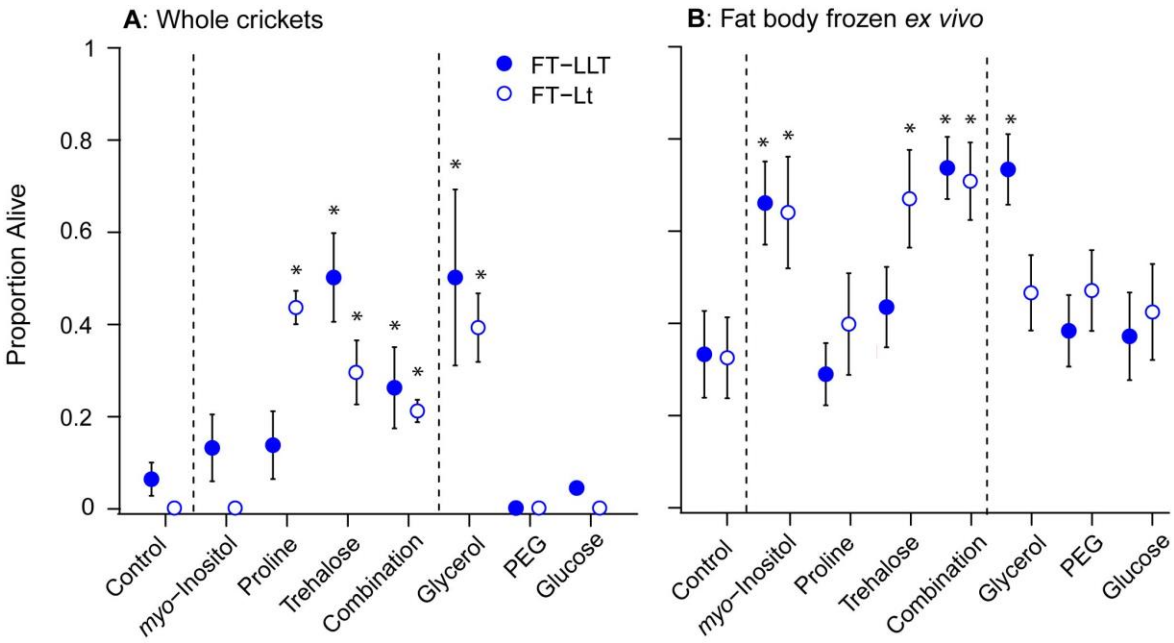

Figure 2.10. Effect of cryoprotectant injection on post-thaw survival of (A) freeze-tolerant (FT) *Gryllus veletis* and (B) their fat body cells (FBCs) frozen *ex vivo*. (A) Proportion of FT crickets that survived following injection with Ringer's solution (control) or the putative cryoprotectant in Ringer's solution, and being frozen to the lower lethal temperature (FT-LLT) or the lethal time (FT-Lt). **(B)** Proportion of live FBCs following freezing *ex vivo* in Grace's Insect Medium (control) or the putative cryoprotectant (3 mM PEG, 300 mM for all others) in Grace's Insect Medium. Fat body samples were frozen to the cellular LLT (FT-LLT) or Lt (FT-Lt). *Ex* vivo FBC survival with lower cryoprotectant concentrations is presented in Fig. A.6. Each point represents the surviving proportion ± SE of 24 crickets or FBCs. Small error bars are obscured by symbols. Asterisks denote that survival is higher than the control sample in that group ($P < 0.05$, generalized linear models in Table A.6).

My data do not support the hypothesis that low molecular weight cryoprotectants facilitate freeze tolerance in a solely colligative manner (e.g. by reducing ice content), either *in vivo* or *ex vivo*. The metabolites that improved survival at the whole animal LLT or Lt (trehalose and glycerol; Fig. 2.10A) were at similar (or lower) hemolymph concentrations to cryoprotectants that did not impact survival (*myo*-inositol, proline, and glucose; Fig. A.6). In addition, while a high concentration of cryoprotectants (e.g. 300 mM *myo*-inositol) was required to improve fat body cell survival *ex vivo*, there was limited benefit to increasing osmolality beyond this (e.g. 300 mM each of *myo*-inositol, proline and trehalose; Fig. 2.10B). This suggests that these cryoprotectants do not substantially contribute to freeze tolerance by colligatively reducing ice content. Rozsypal et al. (2018) also suggest that colligative reduction of ice content in *C. costata* (by proline accumulation) is not sufficient to explain the strong freeze tolerance of these insects. Thus, moderate accumulation of cryoprotectants by FT insects likely facilitates freeze tolerance *via* non-colligative mechanisms (Koštál et al., 2001), such as directly protecting macromolecules or cellular integrity from freezing stress.

I expect that cryoprotectants must be intracellular to protect most macromolecules, and I therefore hypothesize that cell membrane permeability is important for cryoprotectant function. My polyol experiments support for this hypothesis, because glycerol – which is small and highly permeable to cells (Izumi et al., 2006) – improved survival of both freeze-intolerant and freeze-tolerant crickets (and their cells), but the large, cell-impermeable, PEG did not (Figs. 2.9, 2.10). I speculate that intracellular accumulation of glycerol may protect cells by reducing the probability of IIF, and/or protecting macromolecules (Lee, 2010). Conversely, although PEG presents functional groups similar to glycerol (i.e. many hydroxyls), its inability to enter cells likely inhibits its effectiveness as a cryoprotectant. Because *myo*-inositol only improved freeze tolerance of fat body cells from acclimated crickets, I hypothesize that *myo*-inositol's ability to protect cells depends (in part) on accumulation of appropriate transmembrane transporters during acclimation, facilitating intracellular accumulation of the cryoprotectant. Similarly, proline and trehalose transporters are likely required to facilitate their cryoprotective function, and I predict an increase in the abundance or activity of these transporters during acclimation.

2.3.6 Mechanisms underlying mortality at the lethal limits

The differential effect of cryoprotectants on survival I observed at the lethal limits can inform our understanding of the mechanisms underlying mortality of frozen insects. Because cryoprotectants in this study appeared to provide non-colligative protection, I hypothesize that the mechanisms underlying mortality are linked to conformational stability and/or structural integrity of macromolecules. Because trehalose improves survival at the LLT, while proline does not, I suggest that the LLT is caused by increased dehydration stress imposed on cells as ice fraction increases with low temperature (Chapter 1). Trehalose and proline both stabilize soluble macromolecules *in vitro* (Arakawa and Timasheff, 1982; Arakawa and Timasheff, 1983), therefore, both trehalose and proline injections would improve survival at the LLT if it was determined by low temperature-induced macromolecule instability (e.g. cold denaturation of proteins; Privalov, 1990). However, trehalose also protects macromolecules (Crowe et al., 1987), cells (Wolkers et al., 2001), and whole insects (Benoit et al., 2009; Sakurai et al., 2008) at low water activity, and may therefore better protect against dehydration stress associated with high ice content at the LLT. Although high [proline] increase *C. costata* survival at liquid nitrogen temperatures (-196 °C), it likely does so by promoting vitrification rather than by directly mitigating the effects of ice and low temperatures (Koštál et al., 2011b). To test the hypothesis that trehalose (but not proline) facilitates survival by reducing dehydration stress, future experiments could test whether injecting these cryoprotectants into *G. veletis* improves survival under low water conditions.

Because both proline and trehalose improved survival at the Lt, I suggest that the mechanisms underlying the Lt are metabolic in nature (i.e. due to energy drain, or accumulation of harmful metabolites over time). Both trehalose and proline can be catabolized (Thompson, 2003; Weeda et al., 1980), and these cryoprotectants may therefore contribute indirectly to survival by fueling repair and recovery post-thaw, mitigating energy drain. Alternatively, proline and trehalose may protect macromolecules *in vivo* from damage over time by harmful metabolites, either by stabilizing macromolecules *via* preferential exclusion (as *in vitro*; Arakawa and Timasheff, 1983), or by scavenging reactive oxygen species, thereby reducing oxidative damage (Luo et al.,

2008; Rejeb et al., 2014). To test these hypotheses, future experiments should determine whether high cryoprotectant concentrations reduce the rate of macromolecule damage accumulation or energy drain over time in frozen *G. veletis*.

2.4 Conclusions

Here I present a new model for studying insect freeze tolerance. *Gryllus veletis* is easily laboratory-reared, facultatively acquires freeze tolerance with acclimation, and we can use injections to modify hemolymph composition and explore the impacts of cryoprotectants. I show that accumulation of cryoprotectants (*myo*-inositol, proline, and trehalose) and control of ice nucleation are associated with *G. veletis* freeze tolerance, but none of these factors is sufficient to confer freeze tolerance on freeze-intolerant crickets. I manipulate cryoprotectant concentrations to enhance survival of freeze-tolerant *G. veletis* and their cells at their lethal limits, revealing that cryoprotectants do function in freeze tolerance, but likely in concert with other as-yet-unidentified changes to cellular function. I show that there is limited functional convergence of cryoprotectants, and suggest that they contribute to freeze tolerance *via* different mechanisms. I expect that further development of the *G. veletis* model will continue to shed new light on the mechanisms underlying insect freeze tolerance.

2.5 References

Alexander, R. D. and Bigelow, R. S. (1960). Allochronic speciation in field crickets, and a new species, *Acheta veletis*. *Evolution* **14**, 334-346.

Arakawa, T. and Timasheff, S. N. (1982). Stabilization of protein structure by sugars. *Biochemistry* **21**, 6536-6544.

Arakawa, T. and Timasheff, S. N. (1983). Preferential interactions of proteins with solvent components in aqueous amino acid solutions. *Archives of Biochemistry and Biophysics* **224**, 169-177.

Arrese, E. L. and Soulages, J. L. (2010). Insect fat body: energy, metabolism, and regulation. *Annual Review of Entomology* **55**, 207-225.

Baust, J. G. and Lee, R. E. (1981). Divergent mechanisms of frost-hardiness in two populations of the gall fly, *Eurosta solidaginsis*. *Journal of Insect Physiology* **27**, 485-490.

Bazinet, A. L., Marshall, K. E., MacMillan, H. A., Williams, C. M. and Sinclair, B. J. (2010). Rapid changes in desiccation resistance in *Drosophila melanogaster* are facilitated by changes in cuticular permeability. *Journal of Insect Physiology* **56**, 2006-2012.

Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. A., Lee, R. E. and Denlinger, D. L. (2009). Dehydration-induced cross tolerance of *Belgica antarctica* larvae to cold and heat is facilitated by trehalose accumulation. *Comparative Biochemistry and Physiology A* **152**, 518-523.

Bursell, E. (1977). Synthesis of proline by fat body of the tsetse fly (*Glossina morsitans*): metabolic pathways. *Insect Biochemistry* **7**, 427-434.

Coello Alvarado, L. E., MacMillan, H. A. and Sinclair, B. J. (2015). Chill-tolerant *Gryllus* crickets maintain ion balance at low temperatures. *Journal of Insect Physiology* **77**, 15-25.

Crosthwaite, J. C., Sobek, S., Lyons, D. B., Bernards, M. A. and Sinclair, B. J. (2011). The overwintering physiology of the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). *Journal of Insect Physiology* **57**, 166-173.

Crowe, J. H., Crowe, L. M., Carpenter, J. F. and Wistrom, C. A. (1987). Stabilization of dry phospholipid bilayers and proteins by sugars. *Biochemical Journal* **242**, 1-10.

Dennis, A. B., Dunning, L. T., Sinclair, B. J. and Buckley, T. R. (2015). Parallel molecular routes to cold adaptation in eight genera of New Zealand stick insects. *Scientific Reports* **5**, 13965.

Des Marteaux, L. E., Stinziano, J. R. and Sinclair, B. J. (2018). Effects of cold acclimation on rectal macromorphology, ultrastructure, and cytoskeletal stability in *Gryllus pennsylvanicus* crickets. *Journal of Insect Physiology* **104**, 15-24.

Dias, C. L., Ala-Nissila, T., Wong-ekkabut, J., Vattulainen, I., Grant, M. and Karttunen, M. (2010). The hydrophobic effect and its role in cold denaturation. *Cryobiology* **60**, 91-99.

Duman, J. G. (1984). Change in overwintering mechanism of the Cucujid beetle, *Cucujus clavipes*. *Journal of Insect Physiology* **30**, 235-239.

Duman, J. G. (2015). Animal ice-binding (antifreeze) proteins and glycolipids: an overview with emphasis on physiological function. *Journal of Experimental Biology* **218**, 1846-1855.

Duman, J. G., Neven, L. G., Beals, J. M., Olson, K. R. and Castellino, F. J. (1985). Freeze-tolerance adaptations, including haemolymph protein and lipoprotein nucleators, in the larvae of the cranefly *Tipula trivittata*. *Journal of Insect Physiology* **31**, 1-8.

Duman, J. G. and Patterson, J. L. (1978). The role of ice nucleators in the frost tolerance of overwintering queens of the bald faced hornet. *Comparative Biochemistry and Physiology A* **59**, 69-72.

Finn, R. N., Chauvigné, F., Stavang, J. A., Belles, X. and Cerdà, J. (2015). Insect glycerol transporters evolved by functional co-option and gene replacement. *Nature Communications* **6**, 7814.

Gekko, K. and Timasheff, S. N. (1981). Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. *Biochemistry* **20**, 4667-4676.

Hahn, D. A. and Denlinger, D. L. (2011). Energetics of insect diapause. *Annual Review of Entomology* **56**, 103-121.

Hamilton, R. L., Mullins, D. E. and Orcutt, D. M. (1985). Freezing-tolerance in the woodroach *Cryptocercus punctulatus* (Scudder). *Experientia* **41**, 1535-1537.

Hazel, J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annual Review of Physiology* **57**, 19-42.

Hoshikawa, K. (1987). Interconversion between glycogen and inositol in hibernating adults of a phytophagous ladybeetle, *Epilachna vigintioctomaculata*. *Insect Biochemistry* **17**, 265-268.

Irwin, J. T., Bennett, V. A. and Lee, R. E. (2001). Diapause development in frozen larvae of the goldenrod gall fly, *Eurosta solidaginis* Fitch (Diptera: Tephritidae). *Journal of Comparative Physiology B* **171**, 181-188.

Irwin, J. T. and Lee, R. E. (2002). Energy and water conservation in frozen vs. supercooled larvae of the goldenrod gall fly, *Eurosta solidaginis* (Fitch) (Diptera: Tephritidae). *Journal of Experimental Zoology* **292**, 345-350.

Izumi, Y., Sonoda, S., Yoshida, H., Danks, H. V. and Tsumuki, H. (2006). Role of membrane transport of water and glycerol in the freeze tolerance of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). *Journal of Insect Physiology* **52**, 215-220.

Knight, C. A. and Duman, J. G. (1986). Inhibition of recrystallisation of ice by insect thermal hysteresis proteins: a possible cryoprotective role. *Cryobiology* **22**, 256-262.

Koštál, V., Korbelová, J., Poupardin, R., Moos, M. and Šimek, P. (2016). Arginine and proline applied as food additives stimulate high freeze tolerance in larvae of *Drosophila melanogaster*. *Journal of Experimental Biology* **219**, 2358-2367.

Koštál, V., Renault, D. and Rozsypal, J. (2011a). Seasonal changes of free amino acids and thermal hysteresis in overwintering heteropteran insect, *Pyrrhocoris apterus*. *Comparative Biochemistry and Physiology A* **160**, 245-251.

Koštál, V., Šimek, P., Zahradníčková, H., Cimlová, J. and Štětina, T. (2012). Conversion of the chill susceptible fruit fly larva (*Drosophila melanogaster*) to a freeze tolerant organism. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 3270-3274.

Koštál, V., Šlachta, M. and Šimek, P. (2001). Cryoprotective role of polyols independent of the increase in supercooling capacity in diapausing adults of *Pyrrhocoris*

apterus (Heteroptera: Insecta). *Comparative Biochemistry and Physiology B* **130**, 365-374.

Koštál, V., Urban, T., Řimnáčová, L., Berková, P. and Šimek, P. (2013). Seasonal changes in minor membrane phospholipid classes, sterols and tocopherols in overwintering insect, *Pyrrhocoris apterus*. *Journal of Insect Physiology* **59**, 934-941.

Koštál, V., Zahradníčková, H. and Šimek, P. (2011b). Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata*, survive cryopreservation in liquid nitrogen. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 13041-13046.

Lake, S. L., MacMillan, H. A., Williams, C. M. and Sinclair, B. J. (2013). Static and dynamic approaches yield similar estimates of the thermal sensitivity of insect metabolism. *Journal of Insect Physiology* **59**, 761-766.

Layne, J. R. and Blakeley, D. L. (2002). Effect of freeze temperature on ice formation and long-term survival of the woolly bear caterpillar (*Pyrrharctia isabella*). *Journal of Insect Physiology* **48**, 1133-1137.

Lee, R. E. (2010). A primer on insect cold-tolerance. In *Low Temperature Biology of Insects* (eds. D. L. Denlinger and R. E. Lee), pp. 3-34. New York: Cambridge University Press.

Li, Y., Zhang, L., Chen, H., Koštál, V., Šimek, P., Moos, M. and Denlinger, D. L. (2015). Shifts in metabolomic profiles of the parasitoid *Nasonia vitripennis* associated with elevated cold tolerance induced by the parasitoid's diapause, host diapause and host diet augmented with proline. *Insect Biochemistry and Molecular Biology* **63**, 34-46.

Loewus, F. A. and Loewus, M. W. (1983). *Myo*-inositol: its biosynthesis and metabolism. *Annual Review of Plant Physiology* **34**, 137-161.

Lundheim, R. (2002). Physiological and ecological significance of biological ice nucleators. *Philosophical Transactions of the Royal Society of London B* **357**, 937-943.

Luo, Y., Li, W.-M. and Wang, W. (2008). Trehalose: protector of antioxidant enzymes or reactive oxygen species scavenger under heat stress? *Environmental and Experimental Botany* **63**, 378-384.

MacMillan, H. A. and Sinclair, B. J. (2011). The role of gut in insect chilling injury: cold-induced disruption of osmoregulation in the field fall cricket, *Gryllus pennsylvanicus*. *Journal of Experimental Biology* **214**, 726-734.

Marqués, M. I. (2006). Mechanism for proteins destabilization at low temperatures. *Physica Status Solidi (a)* **203**, 1487-1490.

Marshall, K. E. and Sinclair, B. J. (2011). The sub-lethal effects of repeated freezing in the woolly bear caterpillar *Pyrrharctia isabella*. *Journal of Experimental Biology* **214**, 1205-1212.

Mazur, P. (2010). A biologist's view of the relevance of thermodynamics and physical chemistry to cryobiology. *Cryobiology* **60**, 4-10.

McKinnon, A. H (2015). Freeze tolerance in the spring field cricket, *Gryllus veletis*. MSc Thesis. London ON, Canada: University of Western Ontario.

Morrissey, R. E. and Baust, J. G. (1976). The ontogeny of cold tolerance in the gall fly, *Eurosta solidagensis*. *Journal of Insect Physiology* **22**, 431-437.

Muldrew, K., Acker, J. P., Elliott, J. A. and McGann, L. E. (2004). The water to ice transition: implications for living cells. In *Life in the Frozen State* (eds. B. Fuller N. Lane and E. E. Benson), pp. 93-134. Boca Raton: CRC Press.

Neufeld, D. S. and Leader, J. P. (1998). Freezing survival by isolated Malpighian tubules of the New Zealand alpine weta *Hemideina maori*. *Journal of Experimental Biology* **201**, 227-236.

Pegg, D. E. (2010). The relevance of ice crystal formation for the cryopreservation of tissues and organs. *Cryobiology* **60**, S36-S44.

Privalov, P. L. (1990). Cold denaturation of protein. *Critical Reviews in Biochemistry and Molecular Biology* **25**, 281-306.

Purać, J., Kojić, D., Petri, E., Popović, Ž. D., Grubor-Lajšić, G. and Blagojević, D. P. (2016). Cold adaptation responses in insects and other arthropods: an "Omics" approach. In *Short Views on Insect Genomics and Proteomics* (eds. C. Raman M. R. Goldsmith and T. A. Agunbiade), pp. 89-112. New York: Springer.

R Core Team. (2017). R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing.

Ramløv, H. (1999). Microclimate and variations in haemolymph composition in the freezing-tolerant New Zealand alpine weta *Hemideina maori* Hutton (Orthoptera: Stenopelmatidae). *Journal of Comparative Physiology B* **169**, 224-235.

Ramløv, H., Bedford, J. J. and Leader, J. P. (1992). Freezing tolerance of the New Zealand alpine weta, *Hemideina maori* Hutton [Orthoptera; Stenopelmatidae]. *Journal of Thermal Biology* **17**, 51-54.

Ramløv, H., Wharton, D. A. and Wilson, P. W. (1996). Recrystallization in a freezing tolerant antarctic nematode, *Panagrolaimus davidi*, and an alpine weta, *Hemideina maori* (Orthoptera; Stenopelmatidae). *Cryobiology* **33**, 607-613.

Rejeb, K. B., Abdelly, C. and Savouré, A. (2014). How reactive oxygen species and proline face stress together. *Plant Physiology and Biochemistry* **80**, 278-284.

Rozsypal, J., Moos, M., Šimek, P. and Koštál, V. (2018). Thermal analysis of ice and glass transitions in insects that do and do not survive freezing. *Journal of Experimental Biology*, doi: 10.1242/jeb.170464.

Rudolph, A. S. and Crowe, J. H. (1985). Membrane stabilization during freezing: the role of two natural cryoprotectants, trehalose and proline. *Cryobiology* **22**, 367-377.

Sakurai, M., Furuki, T., Akao, K.-i., Tanaka, D., Nakahara, Y., Kikawada, T., Watanabe, M. and Okuda, T. (2008). Vitrification is essential for anhydrobiosis in an African chironomid, *Polypedilum vanderplanki*. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 5093-5098.

Sinclair, B. J. (1997). Seasonal variation in freezing tolerance of the New Zealand alpine cockroach *Celatoblatta quinquemaculata*. *Ecological Entomology* **22**, 462-467.

Sinclair, B. J. (1999). Insect cold tolerance: how many kinds of frozen? *European Journal of Entomology* **96**, 157-164.

Sinclair, B. J. (2015). Linking energetics and overwintering in temperate insects. *Journal of Thermal Biology* **54**, 5-11.

Sinclair, B. J., Bretman, A., Tregenza, T., Tomkins, J. L. and Hosken, D. J. (2011). Metabolic rate does not decrease with starvation in *Gryllus bimaculatus* when changing fuel use is taken into account. *Physiological Entomology* **36**, 84-89.

Sinclair, B. J. and Chown, S. L. (2010). The macrophysiology of insect cold-hardiness. In *Low Temperature Biology of Insects* (eds. D. L. Denlinger and R. E. Lee), pp. 191-223. New York: Cambridge University Press.

Sinclair, B. J., Coello Alvarado, L. E. and Ferguson, L. V. (2015). An invitation to measure insect cold tolerance: methods, approaches, and workflow. *Journal of Thermal Biology* **53**, 180-197.

Sinclair, B. J., Klok, C. J. and Chown, S. L. (2004). Metabolism of the sub-Antarctic caterpillar *Pringleophaga marioni* during cooling, freezing and thawing. *Journal of Experimental Biology* **207**, 1287-1294.

Sinclair, B. J., Worland, M. R. and Wharton, D. A. (1999). Ice nucleation and freezing tolerance in New Zealand alpine and lowland weta, *Hemideina* spp. (Orthoptera; Stenopelmatidae). *Physiological Entomology* **24**, 56-63.

Stinziano, J. R., Sové, R. J., Rundle, H. D. and Sinclair, B. J. (2015). Rapid desiccation hardening changes the cuticular hydrocarbon profile of *Drosophila melanogaster*. *Comparative Biochemistry and Physiology A* **180**, 38-42.

Storey, J. M. and Storey, K. B. (1985). Freezing and cellular metabolism in the gall fly larva, *Eurosta solidaginis*. *Journal of Comparative Physiology B* **155**, 333-337.

Storey, K. B. and Storey, J. M. (1988). Freeze tolerance in animals. *Physiological Reviews* **68**, 27-84.

Tanaka, K. and Tanaka, S. (1997). Winter survival and freeze tolerance in a northern cockroach, *Periplaneta japonica* (Blattidae: Dictyoptera). *Zoological Science* **14**, 849-853.

Teets, N. M., Elnitsky, M. A., Benoit, J. B., Lopez-Martinez, G., Denlinger, D. L. and Lee., R. E. (2008). Rapid cold-hardening in larvae of the Antarctic midge *Belgica antarctica*: cellular cold-sensing and a role for calcium. *American Journal of Physiology* **294**, R1938-R1946.

Teets, N. M., Peyton, J. T., Ragland, G. J., Colinet, H., Renault, D., Hahn, D. A. and Denlinger, D. L. (2012). Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. *Physiological Genomics* **44**, 764-777.

Thompson, S. N. (2003). Trehalose - the insect 'blood'sugar. In *Advances in Insect Physiology*, vol. 31 (ed. S. J. Simpson), pp. 205-287. Oxford: Elsevier Ltd.

Toxopeus, J., Lebenzon, J. E., McKinnon, A. H and Sinclair, B. J. (2016). Freeze tolerance of *Cyphoderris monstrosa* (Orthoptera: Prophalangopsidae). *The Canadian Entomologist* **148**, 668-672.

Tursman, D. and Duman, J. G. (1995). Cryoprotective effects of thermal hysteresis protein on survivorship of frozen gut cells from the freeze-tolerant centipede *Lithobius forficatus*. *Journal of Experimental Zoology A* **272**, 249-257.

Venables, W. N. and Ripley, B. D. (2002). Modern Applied Statistics with S. New York: Springer.

Walters, K. R., Sformo, T., Barnes, B. M. and Duman, J. G. (2009). Freeze tolerance in an arctic Alaska stonefly. *Journal of Experimental Biology* **212**, 305-312.

Wan, P.-J., Fu, K.-Y., Lü, F.-G., Guo, W.-C. and Li, G.-Q. (2014). A putative Δ^1-pyrroline-5-carboxylate synthetase involved in the biosynthesis of proline and arginine in *Leptinotarsa decemlineata*. *Journal of Insect Physiology* **71**, 105-113.

Weeda, E., Koopmanschap, A., De Kort, C. and Beenakkers, A. T. (1980). Proline synthesis in fat body of *Leptinotarsa decemlineata*. *Insect Biochemistry* **10**, 631-636.

Wharton, D. A., Barrett, J., Goodall, G., Marshall, C. J. and Ramløv, H. (2005). Ice-active proteins from the Antarctic nematode *Panagrolaimus davidi*. *Cryobiology* **51**, 198-207.

Wharton, D. A., Pow, B., Kristensen, M., Ramløv, H. and Marshall, C. J. (2009). Ice-active proteins and cryoprotectants from the New Zealand alpine cockroach, *Celatoblatta quinquemaculata*. *Journal of Insect Physiology* **55**, 27-31.

Williams, C. M., Pelini, S. L., Hellmann, J. J. and Sinclair, B. J. (2010). Intra-individual variation allows an explicit test of the hygric hypothesis for discontinuous gas exchange in insects. *Biology Letters* **6**, 274-277.

Wolkers, W. F., Walker, N. J., Tablin, F. and Crowe, J. H. (2001). Human platelets loaded with trehalose survive freeze-drying. *Cryobiology* **42**, 79-87.

Xia, J. and Wishart, D. S. (2010). MetPA: a web-based metabolomics tool for pathway analysis and visualization. *Bioinformatics* **26**, 2342-2344.

Yi, S.-X. and Lee, R. E. (2003). Detecting freeze injury and seasonal cold-hardening of cells and tissues in the gall fly larvae, *Eurosta solidaginis* (Diptera: Tephritidae) using fluorescent vital dyes. *Journal of Insect Physiology* **49**, 999-1004.

Yi, S.-X. and Lee, R. E. (2016). Cold-hardening during long-term acclimation in a freeze-tolerant woolly bear caterpillar, *Pyrrharctia isabella*. *Journal of Experimental Biology* **219**, 17-25.

Zachariassen, K. E. (1979). The mechanism of the cryoprotective effect of glycerol in beetles tolerant to freezing. *Journal of Insect Physiology* **25**, 29-32.

Zachariassen, K. E. (1985). Physiology of cold tolerance in insects. *Physiological Reviews* **65**, 799-832.

Zachariassen, K. E., Hammel, H. T. and Schmidek, W. (1979). Studies on freezing injuries in *Eleodes blanchardi* beetles. *Comparative Biochemistry and Physiology A* **63**, 199-202.

Zachariassen, K. E. and Kristiansen, E. (2000). Ice nucleation and antinucleation in nature. *Cryobiology* **41**, 257-279.

Chapter 3

3 How crickets become freeze tolerant: the transcriptomic underpinnings of acclimation in *Gryllus veletis*

I have prepared this chapter as a manuscript for submission.

3.1 Introduction

Many insects that overwinter in temperate regions risk freezing of their body fluids. Insects survive these low temperatures using a range of physiological strategies, including freeze avoidance (depressing the temperature at which body fluids freeze), cryoprotective dehydration (decreasing the amount of freezable water in the body), vitrification (preventing ice crystallization by transitioning body fluids to a "glass" state), and freeze tolerance (surviving internal ice formation; Lee, 2010). Freeze-tolerant insects must survive a combination of challenges, including those imposed by low temperatures, internal ice, and metabolic limitations (Chapter 1). Low temperatures and freezing are hypothesized to damage cellular macromolecules *via* cold- or dehydration-induced protein denaturation or membrane phase transitions (Dias et al., 2010; Hazel, 1995; Rinehart et al., 2006), accumulation of oxidative damage (Doelling et al., 2014; Lalouette et al., 2011), and build-up of toxic metabolites (e.g. lactate) over time (Storey and Storey, 1985). In addition, ice formation and recrystallization (ice crystal growth at equilibrium ice content) may mechanically damage cells and tissues (Pegg, 2010). The mechanisms by which insects mitigate these challenges and survive internal ice formation are not well-understood (Chapter 1).

Many temperate insects become freeze-tolerant as winter approaches, and many studies have identified biochemical and molecular correlates of this cold tolerance strategy (Lee, 2010). For example, freeze-tolerant insects can seasonally alter macromolecule composition, and accumulate low molecular weight cryoprotectants, cytoprotective proteins, ice-binding molecules, and aquaporins (AQPs; Chapter 1). These changes are thought to contribute to freeze tolerance *via* several mechanisms. Altering macromolecule composition (e.g. membranes; Koštál et al., 2003) may reduce

macromolecule damage due to low temperatures and ice. Low molecular weight cryoprotectants such as sugars, polyols, and amino acids (Lee, 2010), and potentially cyto- and cryo-protective proteins such as heat shock proteins (HSPs; Lu et al., 2014; Zhang et al., 2011) can protect cells and macromolecules at low temperatures. Ice-binding molecules may reduce mechanical damage from ice: ice-nucleating agents (INAs) can control where and when ice begins to form, and many antifreeze proteins (AFPs) can inhibit ice recrystallization (Duman, 2015; Zachariassen et al., 2004). Similarly, AQPs may help control ice location, by facilitating osmotic dehydration of cells during freezing, thereby preventing intracellular ice formation (IIF; Philip and Lee, 2010; Yi et al., 2011). Freeze-tolerant insects may also suppress their metabolic rate (e.g. in diapause; Irwin and Lee, 2002), which could reduce metabolic dysregulation in the frozen state.

Despite the large range of molecules that may contribute to freeze tolerance, we have limited understanding of the pathways that regulate seasonal changes, and have explored only a narrow range of other cellular and physiological processes during acclimation that may contribute to freeze tolerance. Many seasonally-induced changes (e.g. entry into diapause) are regulated by hormones, including juvenile hormone (JH; Sim and Denlinger, 2013), 20-hydroxyecdysone (20HE; Koštál et al., 2017; Poupardin et al., 2015) and insulin signalling (Koštál et al., 2017; Sim and Denlinger, 2008; Sinclair and Marshall, 2018), but we have limited understanding of whether these initiate freeze tolerance. Seasonal changes in enzyme activity may account for cryoprotectant accumulation in *Eurosta solidaginis* (Joanisse and Storey, 1994; Storey and Storey, 1981), but the mechanisms underpinning other changes (e.g. membrane composition) are less clear. Untargeted '-omics' (e.g. metabolomics, transcriptomics) studies of freeze-tolerant insects to date have identified acute responses to cooling, freezing, or dehydration (Courteau et al., 2012; Dennis et al., 2015; Štětina et al., 2018; Teets et al., 2013; Teets et al., 2012a), but not the changes associated with seasonal acquisition of freeze tolerance. Some genes that are seasonally upregulated in freeze-tolerant insects (e.g. HSPs, AQPs) have been identified *via* targeted studies (Lu et al., 2014; Philip and Lee, 2010; Yi et al., 2011; Zhang et al., 2011), but very few other genes (e.g. those

encoding ice-binding proteins; Duman, 2015) have been linked to the acquisition of freeze tolerance.

The spring field cricket, *Gryllus veletis* (Orthoptera: Gryllidae), is an emerging model for mechanistic studies of insect freeze tolerance (Chapter 2). A laboratory acclimation that mimics autumn (six weeks of decreasing temperature and photoperiod) induces freeze tolerance in late instar juveniles, which are freeze tolerant when overwintering in nature (Chapter 2). Like many other freeze-tolerant insects, this acquisition of freeze tolerance is accompanied by accumulation of low molecular weight cryoprotectants (proline, trehalose and *myo*-inositol), increased control of ice nucleation, and reduced metabolic rate (Chapter 2). However, neither these low molecular weight cryoprotectants nor control of ice nucleation is sufficient for freeze tolerance (Chapter 2). Here I assembled a transcriptome for *G. veletis* and compared gene expression in the fat body tissue of fifth instar males during six weeks of acclimation or control conditions. I chose to examine the fat body because of its role in producing cryoprotectants and regulating energetics (Arrese and Soulages, 2010). I aimed to determine which pathways may regulate the physiological changes associated with freeze tolerance, and to identify previously unexplored cellular processes that may contribute to freeze tolerance.

3.2 Materials and methods

3.2.1 Study animals

I reared the laboratory colony of *G. veletis* as described in Section 2.2.1. Approximately eight weeks post-hatch, I isolated fifth instar male nymphs into individual mesh-covered 180 ml transparent cups (Polar Plastics, Summit Food Distributors, London, ON, Canada) containing rabbit food, water, and shelters made from egg cartons. I then haphazardly assigned crickets to either remain in rearing (control) conditions, or to undergo a six week acclimation. I acclimated crickets in a Sanyo MIR 154 incubator (Sanyo Scientific, Bensenville, IL, USA), with photoperiod decreasing from 11.5:12.5 L:D to 7.9:16.1 L:D and fluctuating temperatures decreasing from 16/12 ºC (12 h at each high/low temperature) to 1/0 ºC over six weeks (Fig. 2.1A). This regime induces freeze tolerance

in *G. veletis* nymphs, while nymphs maintained under control conditions are freeze-intolerant (Chapter 2).

3.2.2 RNA extraction, cDNA library preparation, and sequencing

I dissected fat body tissue for RNA extraction after zero, three, and six weeks of control or acclimation conditions. At zero weeks, I collected tissue from control crickets only. At three and six weeks, I collected tissue from control and acclimated crickets. I briefly blotted fat body on tissue paper to remove hemolymph, transferred them to 1.7 ml microcentrifuge tubes, and flash froze the samples in liquid nitrogen. Each biological replicate was comprised of fat body tissue pooled from five individuals from the same cohort. I generated mRNA libraries for three biological replicates of each treatment, representing three cohorts of crickets (15 libraries total). To maximize the breadth of transcript representation for the *de novo* assembly, I extracted RNA from an additional *G. veletis* sample including tissues pooled from various developmental stages: whole male and female adult crickets, first through fifth instar nymphs, fifth instar nymphs that had undergone chilling (0 °C for 1, 4, and 24 h), freezing (-8 °C for 1.5 h), thawing, dehydration (incubation at room temperature with silica gel for 1, 4, and 24 h), and an immune challenge (injection with heat-killed bacteria, recovery for 1, 6, and 24 h). All samples were stored at -80 ºC until RNA extraction.

I homogenized each of the 16 tissue samples with a plastic micropestle in TRIzol (ThermoFisher Scientific, Missisauga, ON, Canada), and extracted RNA according to manufacturer's instructions. I purified RNA extracts using the GeneJet RNA Cleanup & Concentration Micro Kit (ThermoFisher Scientific) according to manufacturer's instructions, and measured absorbance at 260 nm to determine the RNA concentration. Génome Québec (Montréal, QC, Canada) conducted quality analysis on each sample with an Agilent Bioanalyzer, prepared KAPA/NEB stranded cDNA libraries from mRNA transcripts, and performed paired-end, 125 bp sequencing on the Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA).

3.2.3 *De novo* transcriptome assembly and annotation

I assembled the *G. veletis* transcriptome using a pipeline similar to that of Des Marteaux et al. (2017; code available in Appendix B). Briefly, I removed Illumina adapter sequences and discarded sequences shorter than 15 nucleotides or containing unknown bases using Cutadapt software (Martin, 2011). These trimmed reads are available in the NCBI Sequence Read Archive (accession ID: SRP151981). I grouped trimmed sequences from all 16 libraries, and assembled *de novo* an initial transcriptome with a minimum contig (putative transcript) length of 200 nucleotides using Trinity v2.2.0 (Grabherr et al., 2011; Haas et al., 2013) on the SHARCNET computing cluster (https://www.sharcnet.ca). I compared transcriptome assembly 'completeness' to a database (October 2016) of arthropod Benchmark Universal Single Copy Orthologs (BUSCO) using BUSCO v1.22 (Simão et al., 2015). I used Trinotate v3.0.1 on the SHARCNET computing cluster to assign putative identities to each contig from the Trinity assembly using BLASTx and BLASTp with an e-value threshold = 1×10^{-3} (Altschul et al., 1990) against the UniProt database (September 2016). I also used Trinotate to identify GO (Gene Ontology) terms (Ashburner et al., 2000), KEGG (Kyoto Encyclopedia of Genes and Genomes) terms (Kanehisa et al., 2011), and Pfam (protein family) domains (Punta et al., 2011) associated with each contig. The transcriptome is available in the NCBI Transcriptome Shotgun Assembly sequence database (accession ID: GGSD00000000, first version: GGSD01000000).

3.2.4 Differential gene expression analysis

To determine contig read counts in each library, I first mapped the original cleaned sequence reads back onto the Trinity-assembly using Bowtie2 v2.2.9 (Langmead and Salzberg, 2012; Li et al., 2009b) and reassembled them with the Cufflinks package v2.2.1 (Trapnell et al., 2012) to filter out transcriptional artifacts, misassembled transcripts, and poorly supported transcripts. I then quantified contig abundance using HTSeq v0.6.1p1 (Anders et al., 2015), and normalized read counts for differential gene expression analysis using the edgeR Bioconductor package (Robinson et al., 2010) in R v3.2.2 (R Core Team, 2017; Risso et al., 2014).

To compare gene expression over time between control and acclimated crickets, I used the maSigPro Bioconductor package (Conesa et al., 2006) in R to identify differentially expressed genes (with at least one read count in half of the libraries) and cluster them into nine expression patterns using a stepwise regression function. This approach revealed that gene expression changed over six weeks in both control and acclimation conditions. As a result, I approached any age-matched pairwise comparisons indirectly in subsequent analyses, by comparing each group to the zero week control. I avoided direct age-matched pairwise comparisons because of their potential to be misleading. If (for example) control crickets upregulated a gene but acclimated crickets did not differentially express that same gene, comparing the control and acclimated groups at the six week time point would (incorrectly) suggest that acclimated crickets actively downregulated that gene.

To identify processes that were up- or down-regulated during acclimation, I conducted GO enrichment analysis using the goseq Bioconductor package (Young et al., 2010) in R on contigs identified as differentially regulated by maSigPro. I did pairwise comparisons of each treatment group relative to the zero week control, and accepted GO terms as over- or under-represented if the FDR-adjusted *P*-value was < 0.1, and if there was more than three genes representing that GO term. Redundant GO terms were removed with REViGO (Supek et al., 2011), using the SimRel algorithm, allowing medium similarity. I also identified differentially-regulated KEGG pathways in each treatment group relative to the zero week control using the Generally Applicable Gene-set Enrichment (GAGE) and Pathview Bioconductor packages (Luo and Brouwer, 2013; Luo et al., 2009) in R. These packages identify coordinated differential expression in gene sets (pre-defined, functionally-related groups of genes; Luo et al., 2009). I accepted pathways as differentially-expressed if the FDR-adjusted *P*-value was < 0.1.

3.3 Results and discussion

3.3.1 Transcriptome summary

I assembled 672 million 125-bp paired-end reads from 16 libraries into a reference transcriptome (see Tables 3.1, B.1 for detailed description). The transcriptome included

77.6 % complete arthropod BUSCOs, which is similar to or better than other recent arthropod *de novo* transcriptome assemblies (Des Marteaux et al., 2017; Tassone et al., 2016; Theissinger et al., 2016). Approximately 28,000 contigs (putative transcripts) in the reference transcriptome were annotated, of which 97 % were assigned identities based on BLAST matches to the UniProt database, 88 % had GO terms, 47 % had KEGG IDs, and 65 % had identifiable Pfam domain(s) (Table 3.1). My analysis focuses primarily on these annotated transcripts, although I speculate that subsequent exploration of unannotated transcripts may reveal novel factors associated with freeze tolerance.

3.3.2 Differential gene expression early and late in acclimation may contribute to freeze tolerance

A total of 3,306 putative genes were differentially-regulated in juvenile *G. veletis* fat body tissue during six weeks of control or acclimation conditions, each clustering into one of nine differential expression patterns (Fig. 3.1). The quantity of differentially-regulated genes is similar to that of cold-acclimated *Gryllus pennsylvanicus* tissues (Des Marteaux et al., 2017). Approximately one third (1,054) of the differentially-regulated genes had putative identities. Most (2,508) of the differentially-regulated genes exhibited different expression patterns between control and acclimated crickets (Fig. 3.1A, B, D, E, G, and H), and the remaining (800) genes changed similarly over time in both conditions (Fig. 3.1C, F, and I). The latter group of genes likely represents changes due to aging, and I therefore focus on the former group to identify transcriptional changes in the fat body that may be important for freeze tolerance. Acclimation likely also initiates differential gene expression in other tissues that may contribute to freeze tolerance, which I have not captured in this experiment.

Gryllus veletis differentially expressed 63 GO terms (Fig. 3.2) and 29 KEGG pathways (Fig. 3.3), suggesting altered activity of many biochemical and physiological processes in fat body during acclimation. Some of these transcriptional changes appeared to support physiological changes I have previously identified in acclimated *G. veletis* (altered biochemical pathway activity and metabolic rate suppression; Chapter 2), as indicated by altered expression of carbohydrate and amino acid metabolism enzymes (Tables 3.2, 3.3). In addition, *G. veletis* differentially regulated genes involved in membrane lipid

biochemistry, cytoskeletal regulation, cryoprotectant transport, general cytoprotection and detoxification, and regulatory (e.g. cell cycle/signalling) processes during acclimation (Tables 3.2, 3.3). These changes are consistent with the hypothesized need for cytoprotection against damage caused by internal ice formation (Chapter 1). Many of the transcriptional changes parallel those during cold acclimation of chill-susceptible insects, such as differential expression of genes encoding cytoskeletal regulators and antioxidant enzymes in *G. pennsylvanicus* (Des Marteaux et al., 2017) and *Drosophila melanogaster* (MacMillan et al., 2016). I hypothesize that these changes in gene expression increase chill tolerance of *G. veletis*, a requisite for freeze tolerance (Chapter 1). Freeze-tolerant *G. veletis* also accumulate low molecular weight cryoprotectants, reduce cuticular permeability, and elevate supercooling point (SCP; temperature at which ice formation begins; Chapter 2). However, acclimated *G. veletis* did not differentially regulate genes required for cryoprotectant synthesis or altered cuticle composition, and I did not detect any putative ice binding proteins in the transcriptome, suggesting that these processes are not transcriptionally regulated in *G. veletis* fat body.

Gryllus veletis differentially expressed most (2,559) of the putative genes in the first three weeks of acclimation, after which expression did not change (Fig. 3.1A, B, D, E), whereas the remaining genes (747) were differentially regulated throughout acclimation (Fig. 3.1G, H). However, *G. veletis* only becomes freeze tolerant after six weeks of acclimation (Chapter 2). Therefore, I hypothesize that early differential gene expression (Fig. 3.1A, B, D, E) contributes to chill tolerance or long-term changes that are necessary for freeze tolerance, e.g. changes to cell structure/function that take several weeks to complete. In addition, I hypothesize that differential gene expression late in acclimation (Fig. 3.1G, H) may be associated with short-term responses to the freezing process, e.g. mitigating the damaging effects of ice formation. Broadly, I expect both early and late processes to act in synergy to confer freeze tolerance, as I expand on in subsequent sections.

Table 3.1. Summary of the *Gryllus veletis* transcriptome *de novo* assembly. bp, base pairs; GC %, percentage of transcriptome comprised of guanines and cytosines; N50, weighted median statistic.

Method Parameter	Value of Parameter
Sequencing & Quality Control	
Libraries[a]	16
125-bp paired-end reads (raw)	672,647,607
125-bp paired-end reads (trimmed/cleaned)	666,449,419
Trinity Assembly	
Assembly length (bp)	108,582,884
Contigs	136,332
Mean contig length (bp)	796
Median contig length (bp)	396
N50	1429
GC %	40
BUSCO Analysis[b]	
Complete BUSCOs (%)	77.6
Fragmented BUSCOs (%)	5.5
Missing BUSCOs (%)	16.9
Trinotate Annotation	
Contigs with BLAST hit	27,311
Contigs with GO description	24,688
Contigs with KEGG IDs	13,308
Contigs with Pfam domain(s)	18,331

[a]Details for each library in Table B.1.
[b]Percentage of the 2,675 Arthropod BUSCOs (Benchmark Universal Single Copy Orthologs) in the transcriptome assembly.

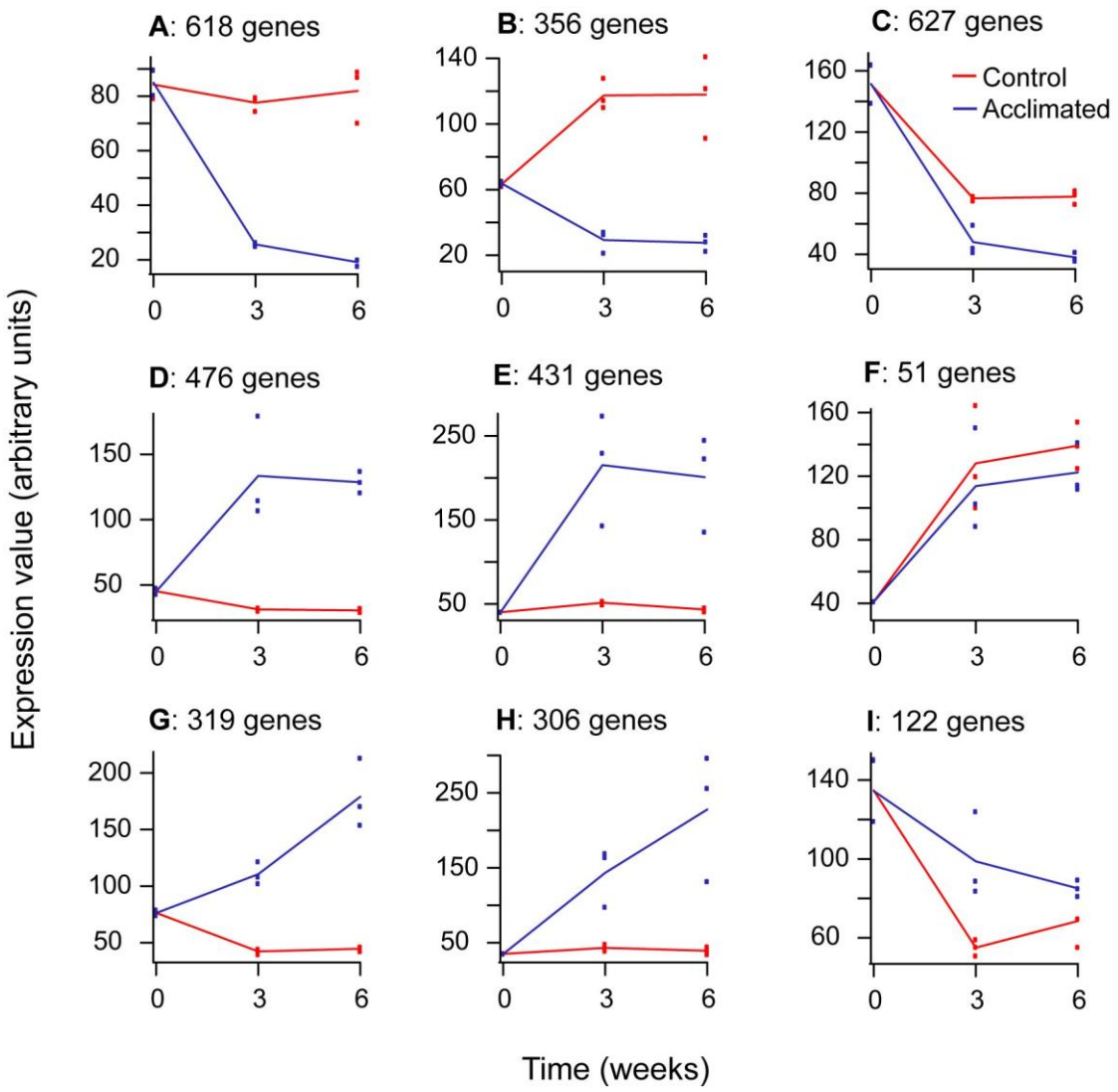

Figure 3.1. Acclimation alters patterns of gene expression in *Gryllus veletis* fat body. Genes were clustered into expression profiles using maSigPro. Each panel shows the relative median gene expression (arbitrary units) for one cluster over six weeks of control (red) or acclimation (blue) conditions, with the number of genes in that cluster indicated above the panel. Dots represent outliers, with each dot representing one putative gene.

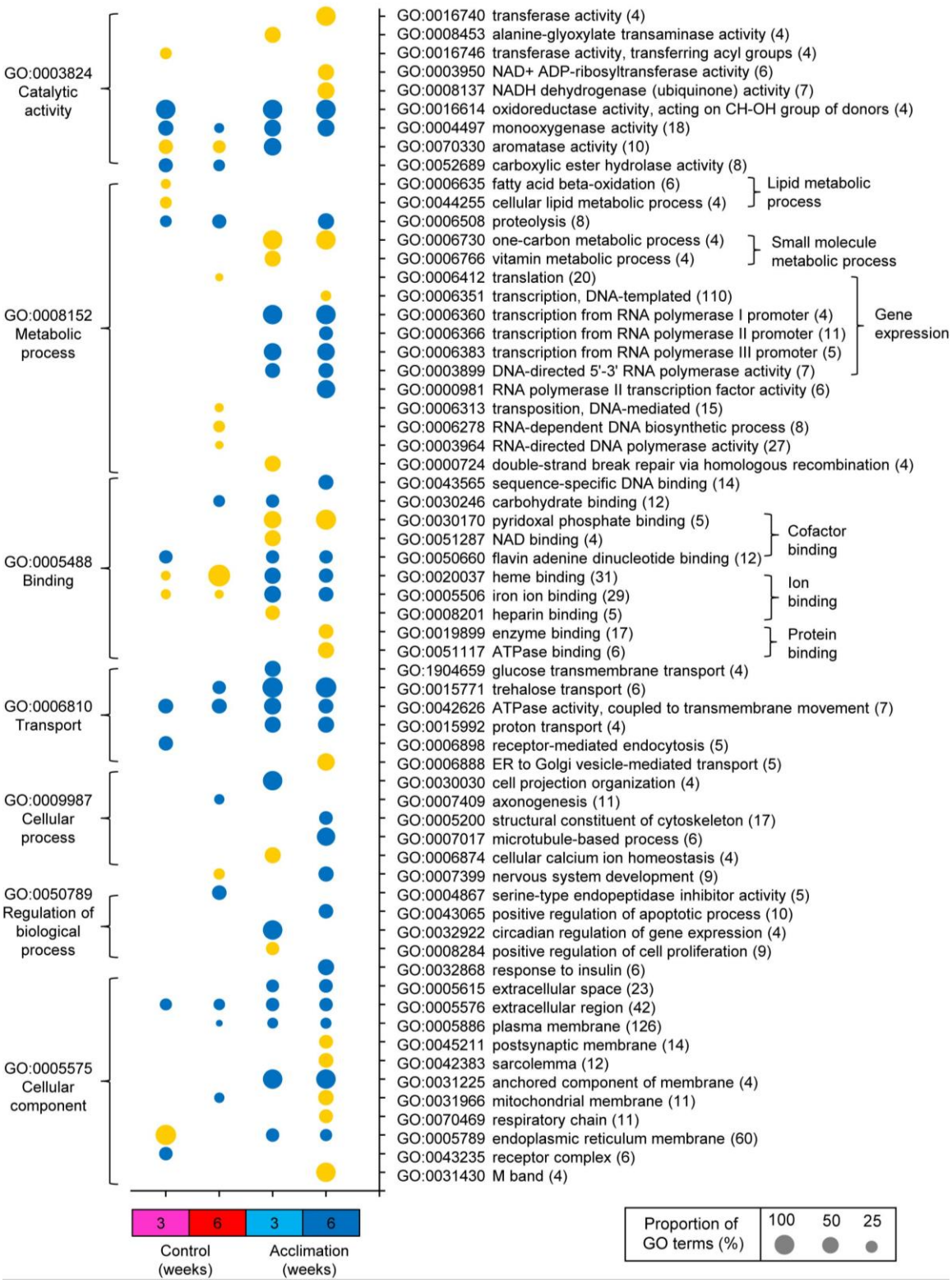
GO:0003824 Catalytic activity
GO:0008152 Metabolic process
GO:0005488 Binding
GO:0006810 Transport
GO:0009987 Cellular process
GO:0050789 Regulation of biological process
GO:0005575 Cellular component
GO:0016740 transferase activity (4)
GO:0008453 alanine-glyoxylate transaminase activity (4)
GO:0016746 transferase activity, transferring acyl groups (4)
GO:0003950 NAD+ ADP-ribosyltransferase activity (6)
GO:0008137 NADH dehydrogenase (ubiquinone) activity (7)
GO:0016614 oxidoreductase activity, acting on CH-OH group of donors (4)
GO:0004497 monooxygenase activity (18)
GO:0070330 aromatase activity (10)
GO:0052689 carboxylic ester hydrolase activity (8)
GO:0006635 fatty acid beta-oxidation (6)
GO:0044255 cellular lipid metabolic process (4)
Lipid metabolic process
GO:0006508 proteolysis (8)
GO:0006730 one-carbon metabolic process (4)
GO:0006766 vitamin metabolic process (4)
Small molecule metabolic process
GO:0006412 translation (20)
GO:0006351 transcription, DNA-templated (110)
GO:0006360 transcription from RNA polymerase I promoter (4)
GO:0006366 transcription from RNA polymerase II promoter (11)
GO:0006383 transcription from RNA polymerase III promoter (5)
GO:0003899 DNA-directed 5'-3' RNA polymerase activity (7)
Gene expression
GO:0000981 RNA polymerase II transcription factor activity (6)
GO:0006313 transposition, DNA-mediated (15)
GO:0006278 RNA-dependent DNA biosynthetic process (8)
GO:0003964 RNA-directed DNA polymerase activity (27)
GO:0000724 double-strand break repair via homologous recombination (4)
GO:0043565 sequence-specific DNA binding (14)
GO:0030246 carbohydrate binding (12)
GO:0030170 pyridoxal phosphate binding (5)
GO:0051287 NAD binding (4)
GO:0050660 flavin adenine dinucleotide binding (12)
Cofactor binding
GO:0020037 heme binding (31)
GO:0005506 iron ion binding (29)
GO:0008201 heparin binding (5)
Ion binding
GO:0019899 enzyme binding (17)
GO:0051117 ATPase binding (6)
Protein binding
GO:1904659 glucose transmembrane transport (4)
GO:0015771 trehalose transport (6)
GO:0042626 ATPase activity, coupled to transmembrane movement (7)
GO:0015992 proton transport (4)
GO:0006898 receptor-mediated endocytosis (5)
GO:0006888 ER to Golgi vesicle-mediated transport (5)
GO:0030030 cell projection organization (4)
GO:0007409 axonogenesis (11)
GO:0005200 structural constituent of cytoskeleton (17)
GO:0007017 microtubule-based process (6)
GO:0006874 cellular calcium ion homeostasis (4)
GO:0007399 nervous system development (9)
GO:0004867 serine-type endopeptidase inhibitor activity (5)
GO:0043065 positive regulation of apoptotic process (10)
GO:0032922 circadian regulation of gene expression (4)
GO:0008284 positive regulation of cell proliferation (9)
GO:0032868 response to insulin (6)
GO:0005615 extracellular space (23)
GO:0005576 extracellular region (42)
GO:0005886 plasma membrane (126)
GO:0045211 postsynaptic membrane (14)
GO:0042383 sarcolemma (12)
GO:0031225 anchored component of membrane (4)
GO:0031966 mitochondrial membrane (11)
GO:0070469 respiratory chain (11)
GO:0005789 endoplasmic reticulum membrane (60)
GO:0043235 receptor complex (6)
GO:0031430 M band (4)
3
6
3
6
Control (weeks)
Acclimation (weeks)
Proportion of GO terms (%)
100
50
25

Figure 3.2. (above) Relative enrichment of GO terms differentially expressed in *Gryllus veletis* fat body during control and acclimation conditions. Differentially-expressed GO categories in each group of crickets (three and six weeks of control or acclimation conditions) relative to the zero week control (FDR-corrected $P < 0.1$). The number of genes in each GO category that are represented in the *G. veletis* transcriptome is indicated in parentheses. The circle area represents the relative proportion (0 to 100 %; indicated in legend at bottom right) of genes within GO category that were upregulated (blue) or downregulated (yellow) in the treatment group.

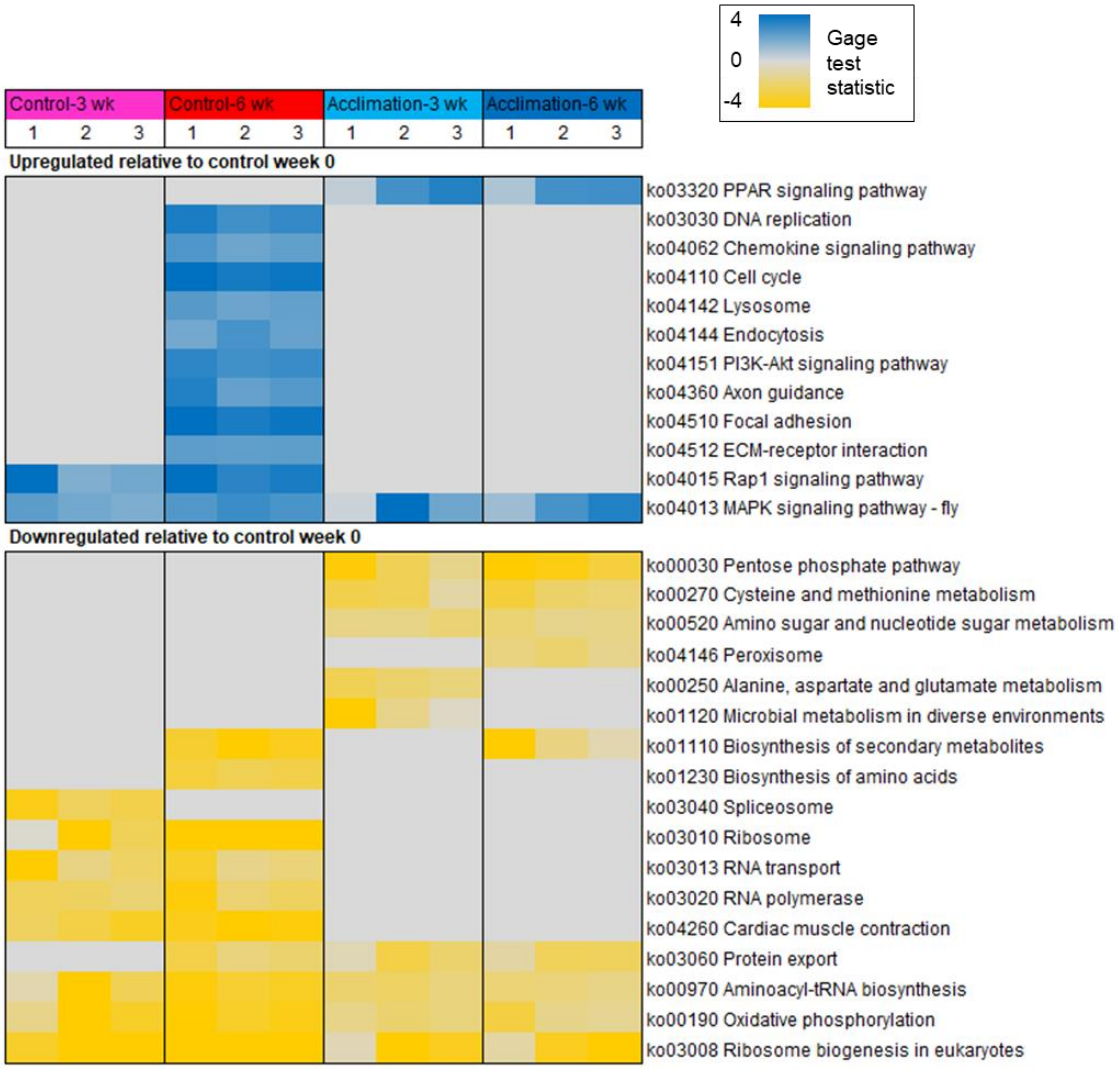

Figure 3.3. Summary of KEGG pathways differentially regulated in *Gryllus veletis* fat body during control or acclimation conditions. Each box represents the differential regulation of the KEGG pathway in one biological replicate (1, 2, or 3) within a treatment group (three and six weeks of control or acclimation conditions) relative to the average expression in zero week controls (FDR-corrected $P < 0.1$). Blue indicates upregulation (positive Gage statistic); yellow indicates downregulation (negative Gage statistic); solid grey areas indicate that pathways were not differentially regulated in that treatment group. Selected KEGG pathway diagrams are available in Appendix B.

Table 3.2. Selected genes upregulated in *Gryllus veletis* during acclimation whose putative function in freeze tolerance is discussed in text. Pattern refers to the maSigPro clusters; i.e. the panels in Fig. 3.1. Select KEGG pathways are illustrated in Appendix B. Fold change indicates the $\log_2$(fold change), calculated in edgeR relative to zero week control crickets.

					Fold change	
Function	Description	Contig ID	Pattern	KEGG	3 wk	6 wk
Metabolism						
Gluconeogenesis	Glycerol kinase	Gvel_56057_c0_g1_i2	D	ko03320	2.19	2.41
	Phosphoenolpyruvate carboxykinase	Gvel_47855_c0_g2_i1		ko03320	2.37	2.41
	6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase	Gvel_54367_c0_g1_i1	E		2.72	2.34
Lipid metabolism	Medium chain acyl-CoA dehydrogenase	Gvel_18079_c0_g1_i1		ko03320	2.73	1.96
	Stearoyl CoA desaturase	Gvel_67809_c0_g1_i1	E	ko03320	1.79	2.05
	1-acyl-sn-glycerol-3-phosphate acyltransferase	Gvel_62590_c2_g1_i1	E		2.53	2.40
Transport						
Trehalose transport	Facilitated trehalose transporter Tret-1	Gvel_80068_c0_g1_i1	E		3.28	3.57
		Gvel_51736_c0_g2_i2	H		3.79	5.12
Cytoskeleton proteins and regulators						
Microtubules	Tubulin (alpha)	Gvel_16545_c0_g1_i1	H		1.33	1.58
		Gvel_8000_c0_g1_i1	E		1.98	1.99
	Tubulin (beta)	Gvel_43107_c1_g1_i1	H		2.31	2.76
Myosin regulators	Microtubule-associated protein Jupiter	Gvel_79158_c0_g1_i2	G		1.75	3.67
Actin regulators	Supervillin	Gvel_64991_c0_g1_i1	E		2.51	2.36
Cell protection						
Detoxification	Cytochrome P450 4C1	Gvel_10729_c0_g1_i1	E		4.86	4.62
	Cytochrome P450 6a23	Gvel_69868_c0_g1_i1	E		4.61	3.74
	Cytochrome P450 6k1	Gvel_10560_c0_g3_i5	E		4.81	3.72
	Cytochrome P450 6j1	Gvel_39812_c2_g1_i1	E		4.19	4.69
Chaperones	Heat shock protein 70	Gvel_60996_c0_g1_i2	D		1.36	1.16
Antioxidants	Catalase	Gvel_66513_c3_g1_i1	H	ko04146	1.02	2.11
	Ferritin heavy chain	Gvel_62685_c1_g2_i1	E		2.79	3.16
Cellular processes						
Transcription	RNA polymerases I, II, and III subunit RPACBC3	Gvel_16630_c0_g2_i1	E		3.74	3.61
Cell cycle & division	Death-associated inhibitor of apoptosis 1	Gvel_56478_c0_g1_i1	D		0.96	0.62
Signal transduction						
AMP kinase pathway	5'-AMP-activated protein kinase subunit gamma-2	Gvel_61474_c0_g1_i1	E		1.40	1.65
cAMP pathways	Adenylate cyclase type 5	Gvel_28980_c0_g4_i4	D		3.00	2.60
	G-protein coupled receptor Mth	Gvel_35842_c0_g1_i1	H		1.82	2.38
	G-protein coupled receptor Mth2	Gvel_27887_c0_g1_i1	E		2.91	2.23
Endocrine						
Insulin signalling	Insulin-like peptide receptor	Gvel_33468_c0_g1_i1	H		2.23	2.34

Table 3.3. Selected genes downregulated in *Gryllus veletis* during acclimation whose putative function in freeze tolerance is discussed in text. Pattern refers to the maSigPro clusters; i.e. the panels in Fig. 3.1. Select KEGG pathways are illustrated in Appendix B. Fold change indicates the $\log_2$(fold change), calculated in edgeR relative to zero week control crickets.

					Fold change	
Function	Description	Contig ID	Pattern	KEGG	3 wk	6 wk
Metabolism						
Pentose phosphate pathway	Gluconolactonase	Gvel_9398_c0_g2_i1	A	ko00030	2.00	2.38
Tricarboxylic acid (TCA) cycle	Isocitrate dehydrogenase subunit gamma	Gvel_54615_c0_g1_i1	A		1.99	0.72
Electron transport system	NADH-ubiquinone oxidoreductase chain 1	Gvel_44854_c1_g1_i1	C		0.39	1.31
	NADH-ubiquinone oxidoreductase chain 3	Gvel_46034_c0_g1_i1	C		0.59	1.99
	NADH-ubiquinone oxidoreductase chain 5	Gvel_41420_c0_g1_i1	C		1.34	2.65
Amino acid metabolism	Alanine-glyoxylate transaminase	Gvel_56575_c0_g1_i1	A	ko04146	2.68	2.79
	Alanine aminotransferase	Gvel_31546_c0_g1_i1	A		1.20	1.66
Polyol metabolism	Inositol oxygenase	Gvel_26533_c0_g3_i4			2.65	1.51
Cytoskeleton proteins and regulators						
Microfilament	Actin	Gvel_18303_c0_g1_i1			0.89	1.56
Actin regulators	Integrin	Gvel_32105_c2_g1_i1	A		1.40	1.38
Cellular processes						
DNA replication	DNA polymerase alpha catalytic subunit	Gvel_31138_c0_g1_i1		ko03030	1.48	1.47
	DNA polymerase delta catalytic subunit	Gvel_11665_c0_g1_i1		ko03030	1.65	1.62
Cell cycle & division	Serine/threonine-protein kinase Chk2	Gvel_15060_c0_g1_i1	B		2.67	3.08
	Caspase-1	Gvel_79011_c0_g1_i2	A		1.55	1.77
Signal transduction						
MAPK pathway	Ras GTPase activating protein 1	Gvel_17341_c0_g1_i2	B	ko04013	1.03	1.22
Phosphatidylinositol pathway	Inositol 1,4,5-trisphosphate receptor	Gvel_41178_c0_g4_i1	B		1.62	1.76
	Phosphatidylinositol 5-phosphate 4-kinase	Gvel_53190_c0_g1_i1	B		1.08	1.09
	Phosphatidylinositol phosphatase PTPRQ	Gvel_80174_c5_g1_i1	A		1.43	1.59
Endocrine						
Juvenile hormone signalling	Juvenile hormone epoxide hydrolase 1	Gvel_46923_c0_g1_i1	B		0.86	1.33

3.3.3 Transcriptional regulation of metabolism and cell cycle activity

Acclimated *G. veletis* suppresses its metabolic rate and has slow development (Chapter 2), which I expected to see reflected in differential gene expression. Dehydrated *Belgica antarctica* downregulates several genes involved in electron transport and the TCA (tricarboxylic acid) cycle, which coincides with metabolic rate suppression (Teets et al., 2012a). *Gryllus veletis* downregulated several metabolic pathways in its fat body tissue early in acclimation, including the KEGG pathways 'pentose phosphate pathway,' 'cysteine and methionine metabolism,' 'amino sugar and nucleotide sugar metabolism,' and 'alanine, aspartate and glutamate metabolism' (Figs. 3.3, B.1, Table 3.3). In addition, acclimated crickets downregulated genes with the GO identifiers 'NADH dehydrogenase (ubiquinone) activity,' 'mitochondrial membrane,' and 'respiratory chain' (Fig. 3.2, Table 3.3). I hypothesize that these latter changes contribute to downregulation of electron transport activity late in acclimation. However, control crickets downregulated the KEGG pathway 'oxidative phosphorylation' more than acclimated crickets (Fig. 3.3), suggesting that the mechanisms underlying metabolic rate suppression in freeze-tolerant *G. veletis* should be investigated further, particularly given the tentative link between transcription and metabolism (Suarez and Moyes, 2012). In addition, I hypothesize that transcriptional and post-transcriptional changes are required in multiple tissue types to drive whole-animal metabolic rate suppression by c. 30 % in acclimated crickets.

Insects modify metabolism in response to various stressors. For example, *B. antarctica* upregulates the lipid metabolism enzyme *Acyl-CoA dehydrogenase* (Lopez-Martinez et al., 2009) and the rate-limiting enzyme in gluconeogenesis *Phophoenolpyruvate kinase* (*PEPCK*; Teets et al., 2012a) in response to dehydration, while both *B. antarctica* (Teets et al., 2013) and *Sarcophaga bullata* (Teets et al., 2012b) upregulate *PEPCK* following a cold shock. Aphids starved for 36 h also upregulate both *Acyl-CoA dehydrogenase* and *PEPCK* (Enders et al., 2015). *Gryllus veletis* upregulated the 'PPAR signaling pathway' throughout acclimation (Figs. 3.3, B.2), which suggests transcriptional upregulation of lipid catabolism and carbohydrate metabolism. For example, upregulation of *Medium chain acyl-CoA dehydrogenase* may increase lipid catabolism *via* β-oxidation, and upregulation of *PEPCK* may increase glucose synthesis through the gluconeogenesis

pathway (Fig. B.2, Table 3.2). *Gryllus veletis* stops eating late in acclimation and therefore must rely on stored energy reserves. I therefore hypothesize that these transcriptional changes reflect an increase in lipid consumption (rather than carbohydrates) in response to starvation (Enders et al., 2015; Sinclair et al., 2011). This transcriptional restructuring of metabolism may also improve stress tolerance, which I discuss in Section 3.3.4.

Developmental arrest often accompanies metabolic suppression (Irwin and Lee, 2002): for example, diapausing *C. costata* downregulate genes that promote cell cycle activity and DNA replication (Koštál et al., 2009; Poupardin et al., 2015). I observed some evidence for developmental arrest in the transcriptome. Early in acclimation, *G. veletis* downregulated genes with the GO identifier 'positive regulation of cell proliferation' (Fig. 3.2), and subunits of *DNA polymerase* (Table 3.3). I therefore hypothesize that *G. veletis* transcriptionally inhibits developmental progression *via* cell cycle arrest during acclimation. Conversely, control *G. veletis* upregulated the KEGG pathways 'DNA replication' and 'cell cycle' after six weeks of control conditions (Fig. 3.3). These changes likely promote cell cycle activity, and may be an indicator of continued developmental progression under control conditions.

3.3.4 Low molecular weight cryoprotectants and their transporters

Cold-hardy arthropods that accumulate low molecular weight cryoprotectants often upregulate genes encoding cryoprotectant synthesis enzymes, yet I did not find evidence of direct transcriptional control of cryoprotectant synthesis in *G. veletis* fat body tissue. *Belgica antarctica* (Teets et al., 2013), *Polypedilum vanderplanki* (Mitsumasu et al., 2010), and *Megaphorura arctica* (Clark et al., 2009) promote trehalose accumulation by upregulating *Trehalose-6-synthase* and/or *Trehalose phosphatase*. Similarly, *D. melanogaster* (MacMillan et al., 2016) and *B. antarctica* (Teets et al., 2012a) upregulate *Pyrroline-5-carboxylate synthase* and/or *reductase* in association with proline accumulation. *Sarcophaga bullata* accumulates *myo*-inositol following cold exposure, concurrent with transcriptional enrichment of the pathways 'inositol phosphate metabolism' and 'glycerolipid metabolism' (Teets et al., 2012b). During acclimation, *G. veletis* did not alter transcript abundance of any cryoprotectant synthesis enzymes (Table

B.2), and I therefore hypothesize that they post-translationally increase activity of these enzymes (Joanisse and Storey, 1994) to promote cryoprotectant accumulation during acclimation (Chapter 2). In addition, I hypothesize that the following transcriptional changes indirectly support cryoprotectant accumulation: 1) *G. veletis* downregulated *Inositol oxygenase* early in acclimation (Table 3.3), which may facilitate inositol accumulation by reducing its degradation (Torabinejad and Gillaspy, 2006); 2) transcriptional changes in the 'alanine, aspartate and glutamate metabolism' pathway (Fig. B.1) could facilitate the accumulation of glutamate and glutamine (Chapter 2), which are precursors for proline synthesis (Weeda et al., 1980); 3) increased gluconeogenesis activity (*via* upregulation of *PEPCK*; Table 3.2) could increase abundance of glucose, a precursor for the low molecular weight cryoprotectants trehalose (Teets et al., 2013; Teets et al., 2012a) and *myo*-inositol (Loewus and Loewus, 1983).

I expect low molecular weight cryoprotectants to most effectively protect cells when they accumulate intracellularly (Wolkers et al., 2001), a process that likely requires cryoprotectant transmembrane transporters. For example, *P. vanderplanki* (Kikawada et al., 2007) upregulates the *Facilitated trehalose transporter Tret-1* during dehydration, facilitating both trehalose export from fat body, and trehalose import into tissues throughout the insect (Kikawada et al., 2007; Sakurai et al., 2008). This trehalose transporter is not, however, upregulated by chill-susceptible insects during cold acclimation (Des Marteaux et al., 2017; MacMillan et al., 2016) or by *B. antarctica* in response to cooling/freezing (Teets et al., 2013). *Gryllus veletis* upregulated the GO term 'trehalose transport' in fat body tissue throughout acclimation (Fig. 3.2), driven by increased transcript abundance of *Tret-1* (Table 3.2). This study is the first (to my knowledge) to document a correlation between freeze tolerance and the upregulation of a cryoprotectant transporter. I hypothesize that Tret-1 in *G. veletis* facilitates trehalose export from fat body during acclimation, resulting in hemolymph trehalose accumulation (Chapter 2). If *Tret-1* is upregulated in other tissues, I hypothesize that this transporter imports trehalose into tissues (Kikawada et al., 2007), facilitating intracellular trehalose accumulation (and therefore cryoprotection) during acclimation and/or freezing (Wolkers et al., 2001). While I detected other putative cryoprotectant transporter genes (i.e. transcripts with GO identifiers 'proline transport' or '*myo*-inositol transport') in the

transcriptome (Table B.2), *G. veletis* did not differentially express them in fat body tissue. These transporters may instead be post-transcriptionally regulated, or differentially expressed in other tissues.

3.3.5 Upregulation of cytoprotective genes during acclimation

In Chapter 1, I hypothesized that several families of cytoprotective proteins contribute to macromolecule protection during freezing and thawing, including antioxidants, ion chelators, molecular chaperones, cytochrome P450s, disordered proteins, and sirtuins (Table 1.3). I identified putative genes representing most of these families in the *G. veletis* transcriptome, except disordered proteins (no annotations containing the words 'disordered' or 'unstructured'). *Gryllus veletis* upregulated several of these genes encoding during acclimation, which may improve chill and freeze tolerance.

Cold-hardy arthropods increase antioxidant capacity to mitigate oxidative stress, and *G. veletis* differentially regulated several genes during acclimation that may likewise reduce oxidative damage of macromolecules. For example, many insects upregulate transcription or activity of antioxidant enzymes (e.g. catalase, superoxide dismutase, glutathione S transferase, peroxiredoxins) and cytochrome P450s (CYPs) during cold acclimation (Des Marteaux et al., 2017; Torson et al., 2015) or in association with freeze tolerance (Joanisse and Storey, 1996; Poupardin et al., 2015), in response to cold shock or freezing (Dunning et al., 2014; Joanisse and Storey, 1998; Štětina et al., 2018), and during or following dehydration (Clark et al., 2009; Lopez-Martinez et al., 2009; Sørensen and Holmstrup, 2013). Freeze-tolerant *E. solidaginis* (Storey and Storey, 2010) and other cold-hardy arthropods (Clark et al., 2009; Rinehart et al., 2010) also upregulate *Ferritin*, which encodes an iron ion chelator that is hypothesized to reduce ROS (reactive oxygen species) production *via* the iron-catalyzed Fenton reaction (Theil, 1987). *Gryllus veletis* upregulated genes with GO identifiers related to detoxification (i.e. 'iron ion binding' and 'aromatase activity;' Fig. 3.2), including *Ferritin* and several putative *CYP* genes early in acclimation, and *Catalase* throughout acclimation (Table 3.2). I hypothesize that the genes upregulated early in acclimation mitigate oxidative stress (e.g. by reducing ROS abundance) during acclimation itself – i.e. upregulation of *Ferritin* and *CYPs* improve chill tolerance. Because *Catalase* is highly expressed late in acclimation, I hypothesize

that accumulation of this antioxidant enzyme protects specifically against oxidative stress associated with freezing and thawing (Doelling et al., 2014).

Many insects upregulate molecular chaperones such as HSPs to mitigate the challenges of thermal stress (King and MacRae, 2015), but I observed minimal transcriptional regulation of HSPs during *G. veletis* acclimation. Freeze-tolerant *C. costata* (Poupardin et al., 2015) and *E. solidaginis* (Zhang et al., 2011) upregulate multiple HSP family members (e.g. small HSPs, HSP40, HSP70, HSP83), which I hypothesize facilitates freeze tolerance by decreasing protein denaturation and aggregation induced by low temperatures and ice (Chapter 1; Rinehart et al., 2006). *Gryllus veletis* upregulated one HSP family member (*HSP70*) early in acclimation (Table 3.2), but over 30 other putative heat shock genes were not differentially expressed (Table B.2). However, this study could not detect transient changes in *HSP* expression due the relatively long (three week) intervals between sampling. For example, *C. costata* upregulates *HSP* genes a few hours after transfer to acclimation conditions, but the differential regulation is no longer detectable at the transcript level at later time points (Poupardin et al., 2015). In addition, *HSP* expression varies by tissue in freeze-tolerant *Chilo supressalis* larvae: the hindgut, midgut, and Malpighian tubules have higher transcript abundance of *HSP60*, *HSP70*, and *HSP90* than other tissues (Lu et al., 2014). I hypothesize that *G. veletis* may upregulate additional HSPs, but at different times during acclimation, or in different tissues.

Many insects differentially regulate cytoprotective genes in response to acute thermal stress or freezing (i.e. after the acclimation process), and I speculate that *G. veletis* similarly upregulates genes that facilitate cytoprotection during or following freezing. For example, *E. solidaginis* accumulates HSP40 and HSP70 proteins during freezing (Zhang et al., 2011) and upregulates catalase activity after a freeze treatment (Joanisse and Storey, 1998); *C. suppressalis* increases expression of *HSP60*, *HSP70*, and *HSP90* following cold shock (Lu et al., 2014); and *C. costata* upregulates a suite of antioxidant enzymes after a cold shock or freeze treatment (Štětina et al., 2018). In addition, *C. costata* (Štětina et al., 2018) and *B. antarctica* (Teets and Denlinger, 2013) upregulate proteolysis and autophagy pathways post-thaw. I hypothesize that these changes facilitate recovery following a thermal/freezing stress, and speculate that *G. veletis* differentially

regulates similar genes after a freeze treatment to mitigate oxidative damage, re-fold partially denatured proteins, and degrade damaged macromolecules.

3.3.6 Potential cellular remodelling during acclimation

Cold-acclimated insects alter cell membrane composition to retain membrane fluidity at low temperatures (Bennett et al., 1997; Koštál et al., 2011; Koštál et al., 2013), and I observed some transcriptional support for this process in *G. veletis*. Early in acclimation, *G. veletis* upregulated *Stearoyl CoA desaturase* (*SCD*, or *Δ9 Fatty acid desaturase*) (Fig. B.2, Table 3.2), a rate-limiting enzyme in synthesis of monounsaturated fatty acids (Stanley-Samuelson et al., 1988). Increased expression of *SCD* is hypothesized to facilitate homeoviscous adaptation of membranes (Clark and Worland, 2008), and is upregulated in cold-tolerant arthropods such as *B. antarctica* (Lopez-Martinez et al., 2009), *C. costata* (Poupardin et al., 2015), *M. arctica* (Sørensen and Holmstrup, 2013), and *Sarcophaga crassipalpis* (Rinehart et al., 2000), but downregulated in hindgut and Malpighian tubules of *G. pennsylvanicus* (Des Marteaux et al., 2017). In addition, *G. veletis* upregulated putative *Acyl transferase* genes (Table 3.2), which could facilitate modification of membrane phospholipid composition (Hazel, 1984). Based on these changes in gene expression, I suggest additional characterization of *G. veletis* to determine whether membrane lipid composition (e.g. Koštál et al., 2003; MacMillan et al., 2009) and/or fluidity (e.g. Lee et al., 2006) change during acclimation, and the extent to which those changes protect cells at low temperatures and when frozen.

Cold-acclimated or -acclimatized insects can differentially regulate cytoskeletal genes (Carrasco et al., 2011; Des Marteaux et al., 2017; MacMillan et al., 2016), which may improve cytoskeleton stability at low temperatures (Des Marteaux et al., 2018; Kim et al., 2006). *Gryllus veletis* differentially-regulated cytoskeletal genes, including those with the GO terms ‘structural constituent of cytoskeleton’ and ‘microtubule-based process’ during acclimation (Fig. 3.2). I hypothesize that early differential expression of cytoskeletal genes (e.g. actin-regulators *Supervillin* and *Integrin*; Tables 3.2, 3.3) reduces cytoskeletal depolymerization in the cold (see Des Marteaux et al., 2018), maintaining cell integrity during acclimation. In addition, I hypothesize that differential expression of cytoskeleton genes later in acclimation (e.g. *Actin*, *alpha-* and *beta-Tubulin*, and *Microtubule-*

associated protein Jupiter; Tables 3.2, 3.3) are necessary specifically for preserving cell integrity during freezing and thawing.

3.3.7 Regulation of acclimation

I drew inferences from the transcriptome data to identify potential regulation of acclimation at the local (subcellular) or central (neuroendocrine) level. Studies of whole-body transcriptomes have not identified differential expression of many transcription factors that might be expected to coordinate acclimation or acclimatization (MacMillan et al., 2016; Poupardin et al., 2015; Torson et al., 2015). Tissue-level transcriptomics has been more successful at detecting transcription factors (Amaral et al., 2017; Esquivel et al., 2016; Peng et al., 2017). For example, Des Marteaux et al. (2017) identified upregulation of several circadian transcription factors, as well as genes in the KEGG pathway 'basal transcription factors' when examining the response of hingdut and Malpighian tubules to cold acclimation. Acclimated *G. veletis* differentially regulated more than 40 transcription factors (Table B.3) in fat body tissue, and several GO terms related to transcription throughout acclimation (e.g. 'transcription from RNA polymerase II promoter;' Fig. 3.2). Similar to acclimated *G. pennsylvanicus*, these transcription factors included circadian rhythm regulators such as *Protein cycle* ('circadian regulation of gene expression'; Fig. 3.2), a transcriptional regulator of the circadian genes *Period* and *Timeless* (Tomioka and Matsumoto, 2010). However, the downstream effects of most of the differentially-regulated transcription factors are uncharacterized (Table B.3). To identify putative downstream targets, I suggest high resolution co-expression or gene network analysis (Alok et al., 2017; Wang et al., 2017; Wang and Chen, 2017; Xing et al., 2017): I predict that transcriptional activators will exhibit similar expression patterns to their targets, while transcriptional repressors will have opposite expression patterns to their targets.

Intracellular signalling is likely an important regulator of gene expression, but I observed few transcriptional changes in cell signalling pathways in *G. veletis* fat body tissue. Cold-acclimated *G. pennsylvanicus* differentially expresses more than a dozen KEGG cell signalling pathways in hindgut and Malpighian tubule tissues (Des Marteaux et al., 2017). Conversely, acclimated *G. veletis* upregulated only two KEGG cell signalling

pathways in the fat body: the ‘PPAR signaling pathway’ and the ‘MAPK signaling pathway,’ the latter of which was also upregulated under control conditions (Figs. 3.3, B.3). I discussed the metabolic implications of altered PPAR signalling in Section 3.3.3. I hypothesize that slight differences in MAPK signalling between control and acclimated crickets (e.g. downregulation of *Ras GTPase activating protein* in acclimated crickets only; Fig. B.3, Table 3.3) contribute to differences in cell cycle activity and cytoskeletal remodelling (Pearson et al., 2001). I identified several other differentially-regulated signal transduction genes (Tables 3.2, 3.3, B.3), but it is challenging to predict the role of these transcriptional changes in cold or freeze tolerance.

Neuroendocrine signalling *via* insulin, JH, and 20HE can coordinate processes such as metabolic regulation and developmental arrest (Denlinger, 2002; Hahn and Denlinger, 2011), and likely influences the acclimation process. For example, several arthropod species differentially regulate genes involved in JH signalling during cold acclimation (Des Marteaux et al., 2017; MacMillan et al., 2016; Torson et al., 2015), and in response to dehydration (Clark et al., 2009; Lopez-Martinez et al., 2009). In addition, freeze-tolerant *C. costata* (Poupardin et al., 2015) and cold-shocked *S. crassipalpis* (Teets et al., 2012b) upregulate ecdysteroid signalling genes. *Gryllus veletis* downregulated an inhibitor of JH signalling, *Juvenile hormone epoxide hydrolase 1* early in acclimation (Table 3.3), and upregulated genes with the GO identifier ‘response to insulin’ late in acclimation (Fig. 3.2; Table 3.2). I did not detect any differential regulation of genes involved in ecdysteroid signalling. Fat body tissue is responsive to both insulin and JH (Sim and Denlinger, 2013), and I hypothesize that these hormones coordinate expression of genes that drive changes in metabolism and developmental progression in *G. veletis* during acclimation (Sim and Denlinger, 2008; Sim and Denlinger, 2013).

3.3.8 Acclimation processes lacking transcriptional support

Freeze-tolerant *G. veletis* appears to accumulate INAs in the hemolymph and gut tissue (Chapter 2), but I did not detect any genes encoding putative ice-binding proteins in the transcriptome (which represents RNA from all tissues; Table B.3).That is, the annotation did not identify any putative genes with the GO identifier ‘ice binding,’ or with Pfam domains ‘ice nucleation,’ ‘AFP,’ or ‘CfAFP.’ However, no insect INAs have been

sequenced, and ice binding proteins have evolved multiple times (Duman, 2015), limiting my ability to identify putative INAs in freeze-tolerant insects based on homology to known sequences. I therefore suggest two approaches to identifying INAs in *G. veletis*: 1) investigating non-proteinaceous INAs (e.g. Mugnano et al., 1996); and 2) determining if highly expressed but unannotated genes (Table B.4) produce INAs [e.g. *via* expression in bacterial or yeast systems, followed by INA detection outlined by Zachariassen et al., (1982)].

Aquaporins are hypothesized to contribute to freeze tolerance by facilitating osmotic dehydration of cells during ice formation. Freeze-tolerant *E. solidaginis* accumulates AQPs seasonally (Philip and Lee, 2010) and during cold acclimation (Philip et al., 2008), while other stress-tolerant arthropod upregulate *AQPs* in response to dehydration (Kikawada et al., 2008; Li et al., 2009a; Sørensen and Holmstrup, 2013). Although I identified four putative AQPs in the *G. veletis* transcriptome (Table B.2), none of them were differentially regulated in fat body tissue during acclimation. I propose three hypotheses for this lack of *AQP* differential expression: 1) *G. veletis* may post-transcriptionally regulate AQP abundance or activity, 2) constitutive expression of AQPs may be sufficient for water redistribution during freezing and thawing, and 3) AQPs in *G. veletis* fat body cells may not be necessary for freeze tolerance. For example, AQP function is important for *B. antarctica* gut and Malpighian tubule viability under dehydrating conditions, but has no bearing on fat body survival (Yi et al., 2011).

Freeze-tolerant *C. costata* (Poupardin et al., 2015) and stick insects (Dennis et al., 2015; Dunning et al., 2013; Dunning et al., 2014) upregulate cuticular proteins during acclimation and after cold shock (respectively), but I found no evidence for transcriptional regulation of cuticular proteins in acclimated *G. veletis*. However, I expect the epidermis (not fat body tissue) to regulate cuticle composition (Charles, 2010; Moussian, 2010). I therefore speculate that *G. veletis* differentially regulates genes encoding cuticle proteins in epidermal layers during acclimation. In addition, because acclimation reduces cuticular water loss in *G. veletis* (Chapter 2), I hypothesize that these crickets alter expression or activity of enzymes that regulate cuticular hydrocarbon composition in the epidermis.

3.3.9 How does differential gene expression during acclimation confer freeze tolerance?

In Chapter 1, I identified five broad mechanisms that are hypothesized to contribute to freeze tolerance: 1) controlling the process of ice formation and propagation, 2) reducing ice content, 3) stabilizing cells and macromolecules, 4) preventing accumulation of harmful metabolites, and 5) coordinating repair and recovery post-thaw. The transcriptional changes in *G. veletis* fat body during acclimation appear to support mechanisms 3 and 4 (Fig. 3.4).

Freeze-tolerant *G. veletis* upregulated molecular chaperones and cryoprotectant transporters, both of which could promote macromolecule stability (Fig. 3.4). For example, I hypothesize that accumulation of HSP70 reduces protein denaturation and aggregation at low temperatures and while frozen. However, *G. veletis* did not upregulate expression of other molecular chaperones or disordered proteins in fat body tissue, and I therefore suggest broadening the characterization of cytoprotective proteins to other tissues (e.g. Lu et al., 2014). This experiment is the first to identify upregulation of the trehalose transporter *Tret-1* in association with freeze tolerance. I hypothesize that this transporter protects macromolecules by promoting extra- and intracellular accumulation of the cryoprotectant trehalose (i.e. facilitating export of fat body trehalose to the hemolymph, and subsequent import into other cell types). Transcriptional changes in carbohydrate and amino acid metabolism may indirectly support synthesis of these low molecular weight cryoprotectants during acclimation (Chapter 2), but *G. veletis* did not differentially express enzymes directly involved in synthesis of trehalose, proline or *myo*-inositol.

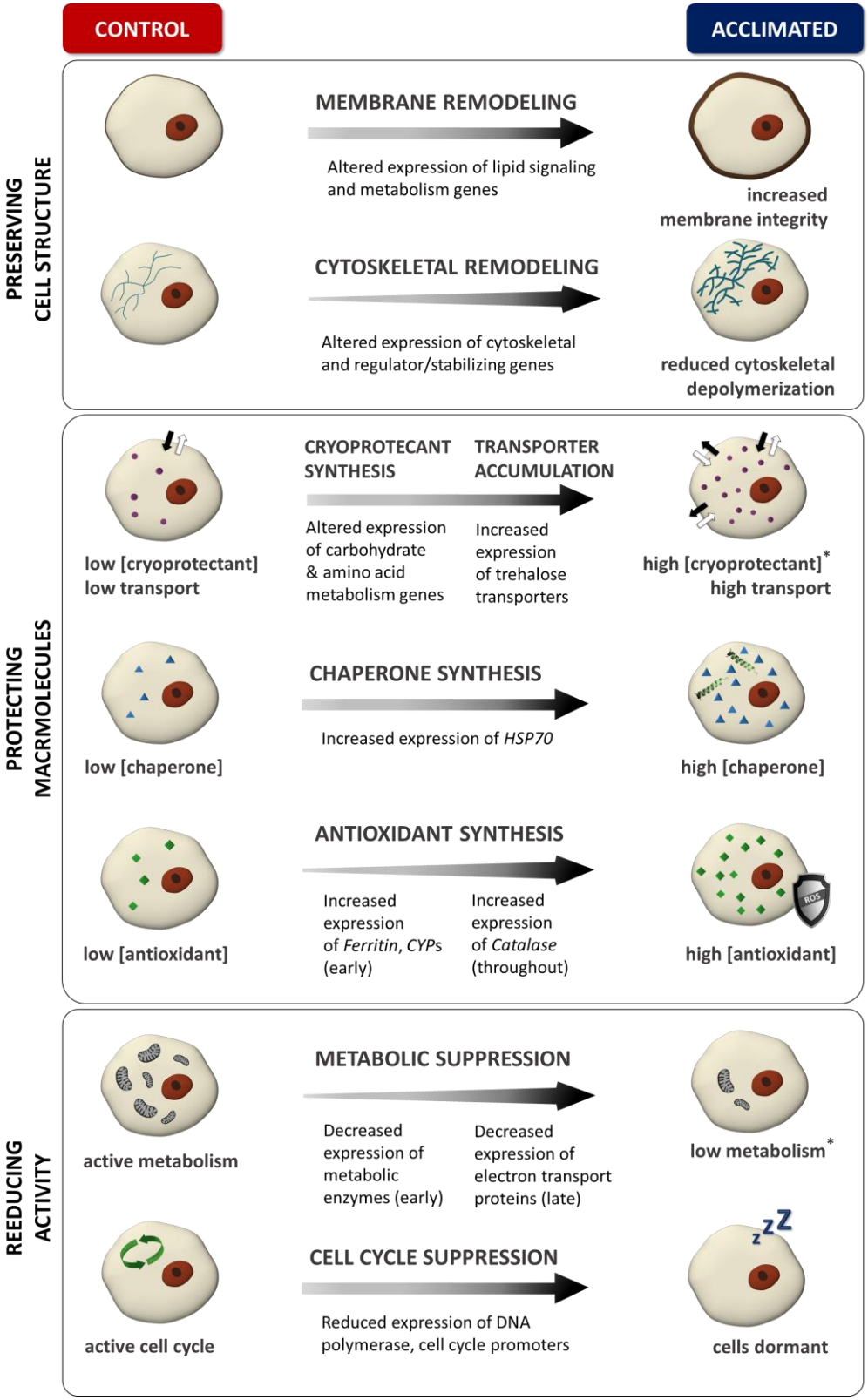
CONTROL
ACCLIMATED
PRESERVING CELL STRUCTURE
MEMBRANE REMODELING
Altered expression of lipid signaling and metabolism genes
increased membrane integrity
CYTOSKELETAL REMODELING
Altered expression of cytoskeletal and regulator/stabilizing genes
reduced cytoskeletal depolymerization
PROTECTING MACRMOLECULES
CRYOPROTECANT SYNTHESIS
TRANSPORTER ACCUMULATION
Altered expression of carbohydrate & amino acid metabolism genes
Increased expression of trehalose transporters
low [cryoprotectant]
low transport
high [cryoprotectant]*
high transport
CHAPERONE SYNTHESIS
Increased expression of HSP70
low [chaperone]
high [chaperone]
ANTIOXIDANT SYNTHESIS
Increased expression of Ferritin, CYPs (early)
Increased expression of Catalase (throughout)
ROS
low [antioxidant]
high [antioxidant]
REEDUCING ACTIVITY
METABOLIC SUPPRESSION
Decreased expression of metabolic enzymes (early)
Decreased expression of electron transport proteins (late)
active metabolism
low metabolism*
CELL CYCLE SUPPRESSION
Reduced expression of DNA polymerase, cell cycle promoters
active cell cycle
cells dormant

Figure 3.4. (above) Candidate mechanisms of freeze tolerance acclimation in *Gryllus veletis* fat body tissue. Processes associated with acclimation that are hypothesized to preserve cell structure, protect macromolecules, and reduce activity. Changes in gene expression that support each process are listed under the arrows. Straight arrows indicate gene expression that changes early and then plateaus; widening arrows indicate gene expression that changes throughout acclimation. The predicted effect of each process is described under each cell icon. Asterisks indicate physiological processes that have previously been verified (Chapter 2). CYP, cytochrome P450; HSP70, heat shock protein 70.

I hypothesize that *G. veletis* remodels its cytoskeleton and membrane composition to preserve cell integrity at low temperatures and when frozen (Fig. 3.4). Much of the differential gene expression that could support this remodelling (e.g. upregulation of cytoskeleton regulators, lipid metabolism enzymes) occurs early in acclimation, and I hypothesize that these changes protect cells at low temperatures (e.g. Des Marteaux et al., 2017; Des Marteaux et al., 2018). Later changes in gene expression (e.g. upregulation of *Microtubule-associated protein Jupiter*) may specifically improve cell survival under freezing conditions. I also speculate that timing of gene expression is important in facilitating freeze tolerance. For example, ‘early genes’ may need to be expressed for several weeks to facilitate sufficient changes to cell structure, or to facilitate late changes in gene expression that are necessary for freeze tolerance.

During acclimation, *G. veletis* upregulated several genes encoding proteins that could reduce accumulation of harmful metabolites, including antioxidant enzymes, iron chelators, and cytochrome P450s (Fig. 3.4). *Gryllus veletis* also downregulated enzymes in several metabolic pathways, and I hypothesize that this reduces general metabolism and build-up of unfavourable metabolic end products. I speculate that cell cycle suppression contributes to metabolic suppression by reducing energy demand. Most of these metabolism-related changes in gene expression occurred early in acclimation, and may therefore mitigate damage from harmful metabolites (e.g. ROS) during acclimation. I speculate that late changes in acclimation (e.g. continued upregulation of *Catalase*) further improve capacity to manage the accumulation of harmful metabolites, facilitating freeze tolerance.

3.4 Conclusions

Freeze tolerance likely requires coordination of many systems, yet we know little about how insects regulate the changes that promote survival of internal ice formation. By characterizing the transcriptome of the laboratory model *G. veletis*, I generated novel hypotheses about how acclimation promotes freeze tolerance. While low molecular weight cryoprotectants and control of ice formation are likely important for freeze tolerance (Chapter 2), I hypothesize that *G. veletis* also: 1) preserves cell integrity at low temperatures and when frozen by remodelling the cell membrane and cytoskeleton; 2) protects macromolecules by accumulating cryoprotectant transporters, cytoprotective proteins, and antioxidants; and 3) transcriptionally suppresses metabolic and developmental activity (Fig. 3.4). This broadens our understanding of potential mechanisms that contribute to freeze tolerance, and I encourage further investigation (e.g. functional genetics experiments) in *G. veletis* and other organisms to test the hypotheses that these mechanisms facilitate survival of low temperatures and internal ice formation.

3.5 References

Alok, A. K., Saha, S. and Ekbal, A. (2017). Semi-supervised clustering for gene-expression data in multiobjective optimization framework. *International Journal of Machine Learning and Cybernetics* **8**, 421-439.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410.

Amaral, D. T., Silva, J. R. and Viviani, V. R. (2017). Transcriptomes from the photogenic and non-photogenetic tissues and life stages of the *Aspisoma lineatum* firefly (Coleoptera: Lampyridae): Implications for the evolutionary origins of bioluminescence and its associated light organs. *Gene Reports* **8**, 150-159.

Anders, S., Pyl, P. T. and Huber, W. (2015). HTSeq - a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166-169.

Arrese, E. L. and Soulages, J. L. (2010). Insect fat body: energy, metabolism, and regulation. *Annual Review of Entomology* **55**, 207-225.

Bennett, V. A., Pruitt, N. L. and Lee, R. E. (1997). Seasonal changes in fatty acid composition associated with cold-hardening in third instar larvae of *Eurosta solidaginis*. *Journal of Comparative Physiology B* **167**, 249-255.

Carrasco, M. A., Buechler, S. A., Arnold, R. J., Sformo, T., Barnes, B. M. and Duman, J. G. (2011). Elucidating the biochemical overwintering adaptations of larval *Cucujus clavipes puniceus*, a nonmodel organism, via high throughput proteomics. *Journal of Proteome Research* **10**, 4634-4646.

Charles, J.-P. (2010). The regulation of expression of insect cuticle protein genes. *Insect Biochemistry and Molecular Biology* **40**, 205-213.

Clark, M. S., Thorne, M. A. S., Purać, J., Burns, G., Hillyard, G., Popović, Ž. D., Grubor-Lajšić, G. and Worland, M. R. (2009). Surviving the cold: molecular analyses of insect cryoprotective dehydration in the Arctic springtail *Megaphorura arctica* (Tullberg). *BMC Genomics* **10**, 328.

Clark, M. S. and Worland, M. R. (2008). How insects survive the cold: molecular mechanisms - a review. *Journal of Comparative Physiology B* **178**, 917-933.

Coello Alvarado, L. E., MacMillan, H. A. and Sinclair, B. J. (2015). Chill-tolerant *Gryllus* crickets maintain ion balance at low temperatures. *Journal of Insect Physiology* **77**, 15-25.

Conesa, A., Nueda, M. J., Ferrer, A. and Talón, M. (2006). maSigPro: a method to identify significantly differential expression profiles in time-course microarray experiments. *Bioinformatics* **22**, 1096-1102.

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S. et al. (2000). Gene Ontology: tool for the unification of biology. *Nature Genetics* **25**, 25-29.

Courteau, L. A., Storey, K. B. and Morin, P. (2012). Differential expression of microRNA species in a freeze tolerant insect, *Eurosta solidaginis*. *Cryobiology* **65**, 210-214.

Denlinger, D. L. (2002). Regulation of diapause. *Annual Review of Entomology* **47**, 93-122.

Dennis, A. B., Dunning, L. T., Sinclair, B. J. and Buckley, T. R. (2015). Parallel molecular routes to cold adaptation in eight genera of New Zealand stick insects. *Scientific Reports* **5**, 13965.

Des Marteaux, L. E., McKinnon, A. H, Udaka, H., Toxopeus, J. and Sinclair, B. J. (2017). Effects of cold-acclimation on gene expression in Fall field cricket (*Gryllus pennsylvanicus*) ionoregulatory tissues. *BMC Genomics* **18**, 357.

Des Marteaux, L. E., Stinziano, J. R. and Sinclair, B. J. (2018). Effects of cold acclimation on rectal macromorphology, ultrastructure, and cytoskeletal stability in *Gryllus pennsylvanicus* crickets. *Journal of Insect Physiology* **104**, 15-24.

Dias, C. L., Ala-Nissila, T., Wong-ekkabut, J., Vattulainen, I., Grant, M. and Karttunen, M. (2010). The hydrophobic effect and its role in cold denaturation. *Cryobiology* **60**, 91-99.

Doelling, A. R., Griffis, N. and Williams, J. B. (2014). Repeated freezing induces oxidative stress and reduces survival in the freeze-tolerant goldenrod gall fly, *Eurosta solidaginis*. *Journal of Insect Physiology* **67**, 20-27.

Duman, J. G. (2015). Animal ice-binding (antifreeze) proteins and glycolipids: an overview with emphasis on physiological function. *Journal of Experimental Biology* **218**, 1846-1855.

Dunning, L. T., Dennis, A. B., Park, D., Sinclair, B. J., Newcomb, R. D. and Buckley, T. R. (2013). Identification of cold-responsive genes in a New Zealand alpine stick insect using RNA-Seq. *Comparative Biochemistry and Physiology D* **8**, 24-31.

Dunning, L. T., Dennis, A. B., Sinclair, B. J., Newcomb, R. D. and Buckley, T. R. (2014). Divergent transcriptional responses to low temperature among populations of alpine and lowland species of New Zealand stick insects (*Micrarchus*). *Molecular Ecology* **23**, 2712-2726.

Enders, L. S., Bickel, R. D., Brisson, J. A., Heng-Moss, T. M., Siegfried, B. D., Zera, A. J. and Miller, N. J. (2015). Abiotic and biotic stressors causing equivalent mortality induce highly variable transcriptional responses in the soybean aphid. *Genes, Genomes, Genetics* **5**, 261-270.

Esquivel, C. J., Cassone, B. J. and Piermarini, P. M. (2016). A *de novo* transcriptome of the Malpighian tubules in non-blood-fed and blood-fed Asian tiger mosquitoes *Aedes albopictus*: insights into diuresis, detoxification, and blood meal processing. *PeerJ* **4**, e1784.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q. et al. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology* **29**, 644-652.

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., Couger, M. B., Eccles, D., Li, B., Lieber, M. et al. (2013). De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nature Protocols* **8**, 1494-1512.

Hahn, D. A. and Denlinger, D. L. (2011). Energetics of insect diapause. *Annual Review of Entomology* **56**, 103-121.

Hazel, J. R. (1984). Effects of temperature on the structure and metabolism of cell membranes in fish. *American Journal of Physiology* **246**, R460-R470.

Hazel, J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annual Review of Physiology* **57**, 19-42.

Irwin, J. T. and Lee, R. E. (2002). Energy and water conservation in frozen vs. supercooled larvae of the goldenrod gall fly, *Eurosta solidaginis* (Fitch) (Diptera: Tephritidae). *Journal of Experimental Zoology* **292**, 345-350.

Joanisse, D. R. and Storey, K. B. (1994). Enzyme activity profiles in an overwintering population of freeze-tolerant larvae of the gall fly, *Eurosta solidaginis*. *Journal of Comparative Physiology B* **164**, 247-255.

Joanisse, D. R. and Storey, K. B. (1996). Oxidative stress and antioxidants in overwintering larvae of cold-hardy goldenrod gall insects. *Journal of Experimental Biology* **199**, 1483-1491.

Joanisse, D. R. and Storey, K. B. (1998). Oxidative stress and antioxidants in stress and recovery of cold-hardy insects. *Insect Biochemistry and Molecular Biology* **28**, 23-30.

Kanehisa, M., Goto, S., Sato, Y., Furumichi, M. and Tanabe, M. (2011). KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Research* **40**, D109-D114.

Kikawada, T., Saito, A., Kanamori, Y., Fujita, M., Śnigórska, K., Watanabe, M. and Okuda, T. (2008). Dehydration-inducible changes in expression of two aquaporins in the sleeping chironomid, *Polypedilum vanderplanki*. *Biochimica et Biophysica Acta* **1778**, 514-520.

Kikawada, T., Saito, A., Kanamori, Y., Nakahara, Y., Iwata, K., Tanaka, D., Watanabe, M. and Okuda, T. (2007). Trehalose transporter 1, a facilitated and high-capacity trehalose transporter, allows exogenous trehalose uptake into cells. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 11585-11590.

Kim, M., Robich, R. M., Rinehart, J. P. and Denlinger, D. L. (2006). Upregulation of two actin genes and redistribution of actin during diapause and cold stress in the northern house mosquito, *Culex pipiens*. *Journal of Insect Physiology* **52**, 1226-1233.

King, A. M. and MacRae, T. H. (2015). Insect heat shock proteins during stress and diapause. *Annual Review of Entomology* **60**, 59-75.

Koštál, V., Berková, P. and Šimek, P. (2003). Remodelling of membrane phospholipids during transition to diapause and cold-acclimation in the larvae of *Chymomyza costata* (Drosophilidae). *Comparative Biochemistry and Physiology B* **135**, 407-419.

Koštál, V., Korbelová, J., Rozsypal, J., Zahradníčková, H., Cimlová, J., Tomčala, A. and Šimek, P. (2011). Long-term cold acclimation extends survival time at 0°C and modifies the metabolomic profiles of the larvae of the fruit fly *Drosophila melanogaster*. *PLoS One* **6**, e25025.

Koštál, V., Šimůnková, P., Kobelková, A. and Shimada, K. (2009). Cell cycle arrest as a hallmark of insect diapause: changes in gene transcription during diapause induction in the drosophilid fly, *Chymomyza costata*. *Insect Biochemistry and Molecular Biology* **39**, 875-883.

Koštál, V., Štětina, T., Poupardin, R., Korbelová, J. and Bruce, A. W. (2017). Conceptual framework of the eco-physiological phases of insect diapause development justified by transcriptomic profiling. *Proceedings of the National Academy of Sciences of the United States of America* **114**, 8532-8537.

Koštál, V., Urban, T., Řimnáčová, L., Berková, P. and Šimek, P. (2013). Seasonal changes in minor membrane phospholipid classes, sterols and tocopherols in overwintering insect, *Pyrrhocoris apterus*. *Journal of Insect Physiology* **59**, 934-941.

Lalouette, L., Williams, C., Hervant, F., Sinclair, B. J. and Renault, D. (2011). Metabolic rate and oxidative stress in insects exposed to low temperature thermal fluctuations. *Comparative Biochemistry and Physiology A* **158**, 229-234.

Langmead, B. and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods* **9**, 357-359.

Lee, R. E. (2010). A primer on insect cold-tolerance. In *Low Temperature Biology of Insects*, (eds. D. L. Denlinger and R. E. Lee), pp. 3-34. New York: Cambridge University Press.

Lee, R. E., Damodaran, K., Yi, S.-X. and Lorigan, G. A. (2006). Rapid cold-hardening increases membrane fluidity and cold tolerance of insect cells. *Cryobiology* **52**, 459-463.

Li, A., Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. A., Lee, R. E. and Denlinger, D. L. (2009a). Distinct contractile and cytoskeletal protein patterns in the Antarctic midge are elicited by desiccation and rehydration. *Proteomics* **9**, 2788-2798.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G. and Durbin, R. (2009b). The sequence alignment/map format and SAMtools. *Bioinformatics* **25**, 2078-2079.

Loewus, F. A. and Loewus, M. W. (1983). *Myo*-inositol: its biosynthesis and metabolism. *Annual Review of Plant Physiology* **34**, 137-161.

Lopez-Martinez, G., Benoit, J. B., Rinehart, J. P., Elnitsky, M. A., Lee, R. E. and Denlinger, D. L. (2009). Dehydration, rehydration, and overhydration alter patterns of gene expression in the Antarctic midge, *Belgica antarctica*. *Journal of Comparative Physiology B* **179**, 481-491.

Lu, M.-X., Liu, Z.-X., Cui, Y.-D. and Du, Y.-Z. (2014). Expression patterns of three heat shock proteins in *Chilo suppressalis* (Lepidoptera: Pyralidae). *Annals of the Entomological Society of America* **107**, 667-673.

Luo, W. and Brouwer, C. (2013). Pathview: an R/Bioconductor package for pathway-based data integration and visualization. *Bioinformatics* **29**, 1830-1831.

Luo, W., Friedman, M. S., Shedden, K., Hankenson, K. D. and Woolf, P. J. (2009). GAGE: generally applicable gene set enrichment for pathway analysis. *BMC Bioinformatics* **10**, 161.

MacMillan, H. A., Guglielmo, C. G. and Sinclair, B. J. (2009). Membrane remodeling and glucose in *Drosophila melanogaster*: a test of rapid cold-hardening and chilling tolerance hypotheses. *Journal of Insect Physiology* **55**, 243-249.

MacMillan, H. A., Knee, J. M., Dennis, A. B., Udaka, H., Marshall, K. E., Merritt, T. J. and Sinclair, B. J. (2016). Cold acclimation wholly reorganizes the *Drosophila melanogaster* transcriptome and metabolome. *Scientific Reports* **6**, 28999.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet Journal* **17**, 10-12.

Mitsumasu, K., Kanamori, Y., Fujita, M., Iwata, K. i., Tanaka, D., Kikuta, S., Watanabe, M., Cornette, R., Okuda, T. and Kikawada, T. (2010). Enzymatic control of anhydrobiosis-related accumulation of trehalose in the sleeping chironomid, *Polypedilum vanderplanki*. *FEBS Journal* **277**, 4215-4228.

Moussian, B. (2010). Recent advances in understanding mechanisms of insect cuticle differentiation. *Insect Biochemistry and Molecular Biology* **40**, 363-375.

Mugnano, J. A., Lee, R. E. and Taylor, R. T. (1996). Fat body cells and calcium phosphate spherules induce ice nucleation in the freeze-tolerant larve of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). *Journal of Experimental Biology* **199**, 465-471.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B, Karandikar, M., Berman, K. and Cobb, M. H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocrine Reviews* **22**, 153-183.

Pegg, D. E. (2010). The relevance of ice crystal formation for the cryopreservation of tissues and organs. *Cryobiology* **60**, S36-44.

Peng, L., Wang, L., Yang, Y.-F., Zou, M.-M., He, W.-Y., Wang, Y., Wang, Q., Vasseur, L. and You, M.-S. (2017). Transcriptome profiling of the *Plutella xylostella* (Lepidoptera: Plutellidae) ovary reveals genes involved in oogenesis. *Gene* **637**, 90-99.

Philip, B. N. and Lee, R. E. (2010). Changes in abundance of aquaporin-like proteins occurs concomitantly with seasonal acquisition of freeze tolerance in the goldenrod gall fly, *Eurosta solidaginis*. *Journal of Insect Physiology* **56**, 679–685.

Philip, B. N., Yi, S.-X., Elnitsky, M. A. and Lee, R. E. (2008). Aquaporins play a role in desiccation and freeze tolerance in larvae of the goldenrod gall fly, *Eurosta solidaginis*. *Journal of Experimental Biology* **211**, 1114-1119.

Poupardin, R., Schöttner, K., Korbelová, J., Provazník, J., Doležel, D., Pavlinic, D., Beneš, V. and Koštál, V. (2015). Early transcriptional events linked to induction of diapause revealed by RNAseq in larvae of drosophilid fly, *Chymomyza costata*. *BMC Genomics* **16**, 720.

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., Pang, N., Forslund, K., Ceric, G., Clements, J. et al. (2011). The Pfam protein families database. *Nucleic Acids Research* **40**, D290-D301.

R Core Team. (2017). R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing.

Rinehart, J. P., Hayward, S. A. L., Elnitsky, M. A., Sandro, L. H., Lee, R. E. and Denlinger, D. L. (2006). Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 14223-14227.

Rinehart, J. P., Robich, R. M. and Denlinger, D. L. (2010). Isolation of diapause-regulated genes from the flesh fly, *Sarcophaga crassipalpis* by suppressive subtractive hybridization. *Journal of Insect Physiology* **56**, 603-609.

Rinehart, J. P., Yocum, G. D. and Denlinger, D. L. (2000). Developmental upregulation of inducible hsp70 transcripts, but not the cognate form, during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Insect Biochemistry and Molecular Biology* **30**, 515-521.

Risso, D., Ngai, J., Speed, T. P. and Dudoit, S. (2014). Normalization of RNA-seq data using factor analysis of control genes or samples. *Nature Biotechnology* **32**, 896-902.

Robinson, M. D., McCarthy, D. J. and Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139-140.

Sakurai, M., Furuki, T., Akao, K.-i., Tanaka, D., Nakahara, Y., Kikawada, T., Watanabe, M. and Okuda, T. (2008). Vitrification is essential for anhydrobiosis in an African chironomid, *Polypedilum vanderplanki*. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 5093-5098.

Sim, C. and Denlinger, D. L. (2008). Insulin signaling and FOXO regulate the overwintering diapause of the mosquito *Culex pipiens*. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 6777-6781.

Sim, C. and Denlinger, D. L. (2013). Juvenile hormone III suppresses forkhead of transcription factor in the fat body and reduces fat accumulation in the diapausing mosquito, *Culex pipiens*. *Insect Molecular Biology* **22**, 1-11.

Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V. and Zdobnov, E. M. (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*, btv351.

Sinclair, B. J., Bretman, A., Tregenza, T., Tomkins, J. L. and Hosken, D. J. (2011). Metabolic rate does not decrease with starvation in *Gryllus bimaculatus* when changing fuel use is taken into account. *Physiological Entomology* **36**, 84-89.

Sinclair, B. J. and Marshall, K. E. (2018). The many roles of fats in overwintering insects. *Journal of Experimental Biology* **221**, doi: 10.1242/jeb.161836.

Sørensen, J. G. and Holmstrup, M. (2013). Candidate gene expression associated with geographical variation in cryoprotective dehydration of *Megaphorura arctica*. *Journal of Insect Physiology* **59**, 804-811.

Stanley-Samuelson, D. W., Jurenka, R. A., Cripps, C., Blomquist, G. J. and de Renobales, M. (1988). Fatty acids in insects: composition, metabolism, and biological significance. *Archives of Insect Biochemistry and Physiology* **9**, 1-33.

Štětina, T., Hůla, P., Moos, M., Šimek, P., Šmilauer, P. and Koštál, V. (2018). Recovery from supercooling, freezing, and cryopreservation stress in larvae of the drosophilid fly, *Chymomyza costata*. *Scientific Reports* **8**, 4414.

Storey, J. M. and Storey, K. B. (1985). Freezing and cellular metabolism in the gall fly larva, *Eurosta solidaginis*. *Journal of Comparative Physiology B* **155**, 333-337.

Storey, K. B. and Storey, J. M. (1981). Biochemical strategies of overwintering in the gall fly larva, *Eurosta solidaginis*: effect of low temperature acclimation on the activities of enzymes of intermediary metabolism. *Journal of Comparative Physiology B* **144**, 191-199.

Storey, K. B. and Storey, J. M. (2010). Oxygen: stress and adaptation in cold hardy insects. In *Low Temperature Biology of Insects* (eds. D. L. Denlinger and R. E. Lee), pp. 141-165. New York: Cambridge University Press.

Suarez, R. K. and Moyes, C. D. (2012). Metabolism in the age of ‘omes’. *Journal of Experimental Biology* **215**, 2351-2357.

Supek, F., Bošnjak, M., Škunca, N. and Šmuc, T. (2011). REViGO summarizes and visualizes long lists of gene ontology terms. *PLoS One* **6**, e21800.

Tassone, E. E., Geib, S. M., Hall, B., Fabrick, J. A., Brent, C. S. and Hull, J. J. (2016). *De novo* construction of an expanded transcriptome assembly for the western tarnished plant bug, *Lygus hesperus*. *GigaScience* **5**, 6.

Teets, N. M. and Denlinger, D. L. (2013). Autophagy in Antarctica: combating dehydration stress in the world's southernmost insect. *Autophagy* **9**, 629-631.

Teets, N. M., Kawarasaki, Y., Lee, R. E. and Denlinger, D. L. (2013). Expression of genes involved in energy mobilization and osmoprotectant synthesis during thermal and dehydration stress in the Antarctic midge, *Belgica antarctica*. *Journal of Comparative Physiology B* **183**, 189-201.

Teets, N. M., Peyton, J. T., Colinet, H., Renault, D., Kelley, J. L., Kawarasaki, Y., Lee, R. E. and Denlinger, D. L. (2012a). Gene expression changes governing extreme dehydration tolerance in an Antarctic insect. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 20744-20749.

Teets, N. M., Peyton, J. T., Ragland, G. J., Colinet, H., Renault, D., Hahn, D. A. and Denlinger, D. L. (2012b). Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. *Physiological Genomics* **44**, 764-777.

Theil, E. C. (1987). Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annual Review of Biochemistry* **56**, 289-315.

Theissinger, K., Falckenhayn, C., Blande, D., Toljamo, A., Gutekunst, J., Makkonen, J., Jussila, J., Lyko, F., Schrimpf, A. and Schulz, R. (2016). *De novo* assembly and annotation of the freshwater crayfish *Astacus astacus* transcriptome. *Marine Genomics* **28**, 7-10.

Tomioka, K. and Matsumoto, A. (2010). A comparative view of insect circadian clock systems. *Cellular and Molecular Life Sciences* **67**, 1397-1406.

Torabinejad, J. and Gillaspy, G. E. (2006). Functional genomics of inositol metabolism. In *Biology of Inositols and Phosphoinositides* (eds. A. Lahiri Majumder and B. B. Biswas), pp. 47-70. Amsterdam: Springer.

Torson, A. S., Yocum, G. D., Rinehart, J. P., Kemp, W. P. and Bowsher, J. H. (2015). Transcriptional responses to fluctuating thermal regimes underpinning differences in survival in the solitary bee *Megachile rotundata*. *Journal of Experimental Biology* **218**, 1060-1068.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., Pimentel, H., Salzberg, S. L., Rinn, J. L. and Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protocols* **7**, 562-578.

Wang, L., Ahsan, M. A. and Chen, M. (2017). A generalized approach for measuring relationships among genes. *Journal of Integrative Bioinformatics* **14**.

Wang, Q. and Chen, G. (2017). Fuzzy soft subspace clustering method for gene co-expression network analysis. *International Journal of Machine Learning and Cybernetics* **8**, 1157-1165.

Weeda, E., Koopmanschap, A., De Kort, C. and Beenakkers, A. T. (1980). Proline synthesis in fat body of *Leptinotarsa decemlineata*. *Insect Biochemistry* **10**, 631-636.

Wolkers, W. F., Walker, N. J., Tablin, F. and Crowe, J. H. (2001). Human platelets loaded with trehalose survive freeze-drying. *Cryobiology* **42**, 79-87.

Xing, L., Guo, M., Liu, X., Wang, C., Wang, L. and Zhang, Y. (2017). An improved Bayesian network method for reconstructing gene regulatory network based on candidate auto selection. *BMC Genomics* **18**, 844.

Yi, S.-X., Benoit, J. B., Elnitsky, M. A., Kaufmann, N., Brodsky, J. L., Zeidel, M. L., Denlinger, D. L. and Lee, R. E. (2011). Function and immuno-localization of aquaporins in the Antarctic midge *Belgica antarctica*. *Journal of Insect Physiology* **57**, 1096-1105.

Young, M. D., Wakefield, M. J., Smyth, G. K. and Oshlack, A. (2010). Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biology* **11**, R14.

Zachariassen, K. E., Baust, J. G. and Lee, R. E. (1982). A method for quantitative determination of ice nucleating agents in insect hemolymph. *Cryobiology* **19**, 180-184.

Zachariassen, K. E., Kristiansen, E., Pedersen, S. A. and Hammel, H. T. (2004). Ice nucleation in solutions and freeze-avoiding insects-homogeneous or heterogeneous? *Cryobiology* **48**, 309-321.

Zhang, G., Storey, J. M. and Storey, K. B. (2011). Chaperone proteins and winter survival by a freeze tolerant insect. *Journal of Insect Physiology* **57**, 1115-1122.

Chapter 4

4 Development of a method to knock down gene expression in *Gryllus veletis* using RNA interference

I have prepared this chapter as a manuscript for submission.

4.1 Introduction

Freeze tolerance, the ability to survive internal ice formation, is a widespread but poorly understood phenomenon (Chapter 1). Specifically, we know very little about how insects protect their cells from the challenges associated with low temperatures and ice (Chapter 1). By manipulating the abundance of low molecular weight cryoprotectants in *Gryllus veletis* crickets (*via* injection), I demonstrated that cryoprotectants function in freeze tolerance, but are not sufficient for surviving internal ice formation (Chapter 2). In Chapter 3, I hypothesized that several additional molecules contribute to preserving cell integrity at low temperatures and when frozen, including molecular chaperones (e.g. heat shock proteins; HSPs), antioxidants, and cytoskeletal regulators. Functional genetics experiments have revealed that cytoprotective proteins are necessary for survival at low temperatures [*HSP70* knockdown reduces cold tolerance of the temperate flesh fly (Rinehart et al., 2007)], and under dry conditions [knockdown of late embyrogenesis abundant proteins eliminates brine shrimp desiccation tolerance (Toxopeus et al., 2014)]. However, no one has manipulated gene expression to determine which molecules preserve cell integrity and promote survival of freeze-tolerant insects, limiting our understanding of how insects survive internal ice formation.

4.1.1 RNA interference

RNA interference (RNAi) is a commonly-used functional genetics tool in non-model organisms (Fire et al., 1998; van der Krol et al., 1990). This tool suppresses gene expression in a sequence-specific manner, using endogenous cellular machinery to prevent translation of proteins from target mRNA (Scott et al., 2013). We can therefore use this tool to investigate the impact of temporary knockdown in a variety of eukaryotic systems, including insects (Scott et al., 2013). In this section, I will review the general

cellular mechanisms underlying RNAi, and the factors that facilitate successful implementation of this technique in insects.

When double-stranded RNA (dsRNA) enters a eukaryotic cell, the cell degrades mRNA complementary to that dsRNA *via* a conserved cellular mechanism of RNAi (Bally et al., 2018). The enzyme Dicer cleaves intracellular long dsRNA (> 100 bp) into short interfering RNAs (siRNAs; 20-25 bp). These siRNAs then cause degradation of complementary mRNA *via* the following steps: 1) the siRNA interacts with proteins to form the RNA-induced silencing complex (RISC); 2) a nuclease in RISC degrades one of the siRNA strands, and the remaining strand ('guide strand') binds to complementary sections of mRNA; 3) an enzyme in RISC, Slicer or Argonaute, cleaves the mRNA; 4) cellular nucleases further degrade the message (Bally et al., 2018). Researchers can therefore use RNAi to prevent production of new protein from mRNA complementary to dsRNA of their design. Nematodes and plants further amplify the RNAi response *via* RNA-dependent RNA polymerases (RdRp) that replicate siRNA molecules (Bally et al., 2018). No canonical RdRp has been detected in insects (Bally et al., 2018), and it is therefore unclear how insects initiate prolonged and systemic RNAi (e.g. Cao et al., 2018; Li et al., 2018),

The efficacy of RNAi as a research tool in insects may vary due to dsRNA stability *in vivo*, and the extent to which the insect cells internalize and process this dsRNA. To initiate RNAi, researchers design and introduce long dsRNA or siRNAs into the organism, typically *via* injection or feeding (Scott et al., 2013). In many species, using injection rather than feeding as a delivery method results in more effective knockdown (greater reduction of target transcript abundance), especially when high gut nuclease activity causes dsRNA degradation (Singh et al., 2017; Wang et al., 2016). Hemolymph nucleases may also degrade dsRNA (e.g. in *Spodoptera frugiperda*), reducing dsRNA stability and consequently decreasing cellular uptake of dsRNA (Shukla et al., 2016). If nuclease activity limits RNAi, one can improve knockdown efficiency by inhibiting nuclease activity or increasing the dose of dsRNA (Guo et al., 2015; Spit et al., 2017). Hemolymph dsRNA or siRNA may enter cells *via* endocytosis or transmembrane transport, although the process is not well-characterized in insects (Jose, 2015). Some

tissues may be insensitive to RNAi because they are impermeable to dsRNA, such as ovary cells of *Locusta migratoria* (Ren et al., 2014). In *Spodoptera frugiperda* cell lines, dsRNA enters cells *via* endocytosis, but then remains trapped in endosomes and is not further processed to siRNA (Yoon et al., 2017). These endogenous differences among species and tissue types can account for some of the variability in RNAi efficacy across insects (Shukla et al., 2016; Singh et al., 2017; Wang et al., 2016).

If the organism and tissue of interest are amenable to RNAi, it is important to select appropriate target genes and design effective dsRNA constructs. Because RNAi only prevents production of new protein and does not accelerate degradation of existing proteins, it is best to use this technique to knockdown genes that encode proteins with a short half-life. In this case, RNAi should decrease both mRNA and protein abundance, validated by RT-qPCR and semi-quantitative Western blotting, respectively (Scott et al., 2013). Most insect RNAi studies use dsRNA constructs complementary to between 300 and 520 nucleotides of the target mRNA (Scott et al., 2013). However, incomplete complementarity between siRNAs and the target mRNA (e.g. due to allelic differences; Jackson et al., 2003; Joseph and Osman, 2012) and processing of dsRNA to siRNA with secondary structure (Horn and Boutros, 2010) can reduce RNAi efficacy. It is therefore common practice to design and test multiple dsRNA constructs for the same target in RNAi experiments to maximize the probability of successful knockdown (Horch et al., 2017).

4.1.2 Model system and genes of interest

Gryllus veletis is an emerging laboratory model for studying the mechanisms underlying insect freeze tolerance (Chapter 2), and is potentially amenable to genetic manipulation (Chapter 1). Acclimation (six weeks of decreasing temperature and photoperiod) induces freeze tolerance in late instar juveniles, which naturally overwinter in a freeze-tolerant state (Chapter 2). During this acclimation, *G. veletis* accumulates low molecular weight cryoprotectants (Chapter 2), and differentially regulates over 3,000 putative genes in fat body tissue (Chapter 3). I hypothesized that many of the genes (e.g. cryoprotectant transporters, antioxidants, molecular chaperones and cytoskeletal regulators) upregulated

during acclimation facilitate *G. veletis* chill tolerance and freeze tolerance (Chapter 3). To test these hypotheses, I aimed to develop a protocol for RNAi in this model system.

RNA interference has been used with moderate success in gryllid crickets (Horch et al., 2017), including *Gryllus texensis* (Singh et al., 2017) and *Gryllus bimaculatus* (Danbara et al., 2010; Meyering-Vos et al., 2006; Miyawaki et al., 2004). For example, injection of between 0.5 and 3 μg dsRNA into *G. bimaculatus* adults reduced *Allatostatin-A* transcript abundance by c. 50 % within three days in fat body, ovaries, and nervous system tissue (Meyering-Vos et al., 2006). In *G. bimaculatus*, RNAi has revealed key mechanisms of segment patterning during embryo development (Mito et al., 2006; Miyawaki et al., 2004; Shinmyo et al., 2005), as well as neuroendocrine control of physiology (Meyering-Vos et al., 2006; Meyering-Vos and Müller, 2007) and circadian regulation of behaviour (Danbara et al., 2010; Moriyama et al., 2008) in adults.

To develop a protocol for RNAi in *G. veletis* juveniles, I selected five target genes whose knockdown could facilitate testing of hypotheses about the mechanisms underlying insect freeze tolerance. Specifically, I chose genes whose products are hypothesized to promote cryoprotectant accumulation (*Pyrroline-5-carboxylate reductase*; *P5CR*), facilitate cryoprotectant transport (*Trehalose transporter-1*; *Tret-1*), stabilize the cytoskeleton (*Supervillin*), prevent protein denaturation and aggregation (*Heat shock protein 70*; *HSP70*), or reduce oxidative stress (*Ferritin*; Table 4.1; Chapter 3). *Ferritin*, *Supervillin*, and *Tret-1* were all highly expressed during acclimation (Table 4.1; Chapter 3), and I therefore predict that their knockdown is most likely to reduce freeze tolerance.

Table 4.1. Target genes for RNA interference (RNAi) in this study. Targets were selected because their knockdown could be used to test specific hypotheses about freeze tolerance. Most targets were upregulated in freeze-tolerant (acclimated) *Gryllus veletis* (as indicated by fold change > 2), and abundantly expressed (as indicated by large transcript count; Chapter 3). P5C, pyrroline-5-carboxylate.

Target Target ID[a]	Fold change[b]	Transcript count (percentile)[c]	Hypothesized protein function	Reference
Ferritin Gvel_51670_c2_g1_i1	3.16	1,674,853 (100th)	Iron ion chelator that inhibits reactive oxygen species (ROS) production *via* Fenton reaction	Theil (1987)
Heat shock protein 70 Gvel_4691_c0_g1_i1	0.61	19,849 (98th)	Molecular chaperone that reduces protein denaturation, aggregation	Rinehart et al. (2007)
P5C reductase Gvel_69512_c0_g1_i1	n/a	46 (15th)	Proline synthesis enzyme that promotes cryoprotectant accumulation	Weeda et al. (1980)
Supervillin Gvel_64991_c0_g1_i1	2.36	54,109 (99th)	Actin-binding protein that stabilizes the cytoskeleton	Wulfkuhle et al. (1999)
Trehalose transporter-1 Gvel_70154_c0_g4_i3	3.43	86,403 (99th)	Trehalose transmembrane transporter that facilitates cryoprotectant distribution	Kikawada et al. (2007)

[a]Unique identifier of target in *G. veletis* transcriptome (Chapter 3).
[b]Relative increase in transcript abundance in *G. veletis* fat body after six weeks of fall-like acclimation (Chapter 3), expressed as $\log_2$(fold change).
[c]Transcript count is a measure of transcript abundance (from Chapter 3); the first number is the sum of transcript (contig) read counts across three biological replicates of acclimated crickets; each biological replicate includes fat body RNA from five freeze-tolerant *G. veletis*; percentile of transcript abundance is relative to all differentially-regulated genes (indicated in parentheses).

4.1.3 Objectives

In this chapter, my goal was to further develop the utility of *G. veletis* as a freeze tolerance model by developing an RNAi protocol. Specifically, my objectives were to:

1) design dsRNA constructs complementary to the target genes;
2) determine whether dsRNA injected into the hemolymph of juvenile *G. veletis* entered fat body tissue; and
3) test the conditions (construct sequence, dose, timing) under which RNAi reduced transcript abundance of target genes in fat body tissue.

I knocked down *Ferritin*, *Supervillin* and *Tret-1* transcript abundance in fat body tissue three days after injection of dsRNA into the hemolymph. I suggest further studies to test whether knockdown affects freeze tolerance, and additional methods development to knock down *HSP70* and *P5CR*.

4.2 Materials and methods

4.2.1 Animals and rearing

I reared the laboratory colony of *G. veletis* at 25 ºC, 14:10 L:D photoperiod, 70 % RH, as described previously (Coello Alvarado et al., 2015). To induce freeze tolerance, I transferred fifth-instar juveniles (approximately eight weeks post-hatch) into a Sanyo MIR 154 incubator (Sanyo Scientific, Bensenville, IL, USA) for a six week acclimation (decreasing temperature and photoperiod), described in Chapter 2. I haphazardly selected male crickets three days after the end of acclimation for RNAi experiments. These crickets are freeze tolerant, and therefore this protocol can be used to test if RNAi eliminates freeze tolerance.

4.2.2 dsRNA synthesis and injection

I designed and synthesized one dsRNA construct each to target *P5CR* and *HSP70* mRNA, and multiple dsRNA constructs to target *Ferritin*, *Supervillin*, and *Tret-1* mRNA (Table 4.2). I also synthesized dsRNA complementary to *Green fluorescent protein* (GFP) as a negative control (Toxopeus et al., 2014), which should not target any endogenous insect mRNA (Scott et al., 2013). I synthesized these dsRNA constructs

using the steps outlined in Fig. 4.1A, which included synthesizing *G. veletis* cDNA, amplifying templates for dsRNA synthesis, and reverse transcribing dsRNA from those templates.

To synthesize cDNA from *G. veletis* fat body mRNA, I extracted RNA from *G. veletis* fat body tissue as described previously (Chapter 3). Briefly, I dissected c. 10 mg fat body from an individual late instar cricket, flash froze the sample in a 1.7 ml centrifuge tube in liquid nitrogen, homogenized the sample with a plastic micropestle in 100 µl TRIzol (ThermoFisher Scientific, Mississauga, ON, Canada), and extracted RNA according to the manufacturer's instructions. I measured absorbance at 260 nm to determine the RNA concentration. I then treated samples with DNase (to remove any contaminating genomic DNA) and synthesized cDNA from 500 ng RNA using the iScript gDNA Clear cDNA Synthesis Kit (BioRad, Mississauga, ON, Canada) according to manufacturer's instructions. This process should reverse transcribe all mRNA in the sample into cDNA.

I generated the templates for dsRNA synthesis *via* PCR of *G. veletis* cDNA (for all dsRNA synthesis except *GFP*) or the pEGFP-N1 plasmid (for *GFP* dsRNA synthesis; Clontech, Mountain View, CA, USA). I amplified these products using the primers in Table 4.2 and GeneDirex Taq DNA polymerase (FroggaBio, Toronto, ON, Canada) according to manufacturer's instructions. I used the following reaction conditions: an initial denaturation of 94 °C for 5 min; 35 to 45 cycles of denaturation at 94 °C for 30 s, incubation at the annealing temperature (T_A) for 30 s, and extension at 72 °C for 1 min; with a final extension at 72 °C for 5 min. The forward and reverse primers for each target included a T7 RNA polymerase promoter at the 5' end, required for dsRNA synthesis. For details on the T_A and number of cycles used for each primer pair, see Table 4.2.

Table 4.2. Primers used to amplify templates for double stranded RNA (dsRNA) construct synthesis, including the annealing temperature (T_A) and number of cycles (Cyc) in the PCR reaction, and the predicted length (in base pairs; bp) of the PCR product. The 5' end of each primer (first line) contains the binding sequence for T7 RNA polymerase, allowing synthesis of a dsRNA construct from each PCR product; the 3' end of the primer (second line) is complementary to the target. See Appendix C for sequence details of each construct. ds, double-stranded; P5C, pyrroline-5-carboxylate.

Target Construct	Forward primer (5' to 3')	Reverse primer (5' to 3')	T_A (°C)	Cyc[b]	Length (bp)
Ferritin					
ds*Ferritin*.1	TAATACGACTCACTATAGGGAG CAAATGCTCCCATCTCAG	TAATACGACTCACTATAGGGAG TCGAGTCACTTTGGAGGA	65	35	356
ds*Ferritin*.2	TAATACGACTCACTATAGGGAG AATGAATGCATTGCCCTA	TAATACGACTCACTATAGGGAG CCCACATCAGGATTTTCA	60/65	5/35	400
Green fluorescent protein[a]					
ds*GFP*	TAATACGACTCACTATAGGGAG ACACATGAAGCAGCACGACTT	TAATACGACTCACTATAGGGAG AAGTTCACCTTGATGCCGTTTC	60/65	5/35	313
Heat shock protein 70					
ds*HSP70*	TAATACGACTCACTATAGGGAG ACTTCGTTTCCACCCTA	TAATACGACTCACTATAGGGAG ATTTGAGATGCGAATTAGA	60/65	5/35	379
P5C reductase					
ds*P5CR*	TAATACGACTCACTATAGGGAG ATCTGCAATACCGAATA	TAATACGACTCACTATAGGGAG AGATCTCTGGGTAAAC	55/60	5/40	488
Supervillin					
ds*Supervillin*.1	TAATACGACTCACTATAGGGAG TACCCAGCAGTTCTTGGA	TAATACGACTCACTATAGGGAG AGCAGTCAGATGGCAAG	60/65	5/35	409
ds*Supervillin*.2	TAATACGACTCACTATAGGGAG TACAAGCACGGCTTCTTC	TAATACGACTCACTATAGGGAG TTCTGTAGGGCAGTTCCA	65	35	438
ds*Supervillin*.3	TAATACGACTCACTATAGGGAG TTTGCCAATTGTTCCACT	TAATACGACTCACTATAGGGAG CTGAAGGCGATGAGAATG	60/65	5/35	402
ds*Supervillin*.4	TAATACGACTCACTATAGGGAG TCCTCCCATTTTGTGCTA	TAATACGACTCACTATAGGGAG TGCCAGTGTTGCCTTATT	60/65	5/35	353
Trehalose transporter Tret-1					
ds*Tret-1*.1	TAATACGACTCACTATAGGGAG ACTGCTGGAACCACT	TAATACGACTCACTATAGGGAG CTCCTCGACTCTAACA	60	40	378
ds*Tret-1*.2	TAATACGACTCACTATAGGGAG GCCATAGGCATAGGTTT	TAATACGACTCACTATAGGGAG CTTCGTCGTCTTCGTTA	60	40	392
ds*Tret-1*.3	TAATACGACTCACTATAGGGAG TCCGATGTTGCCTAGTGT	TAATACGACTCACTATAGGGAG CACGCTCTCAGTCACTCC	60	40	367

[a]Primers from Toxopeus et al. (2014);
[b]Number of cycles in the PCR program. When two numbers are indicated, they correspond to the two numbers in the T_A column; e.g. 5/35 Cyc of T_A = 60/65 °C indicates five cycles with T_A = 60 °C, followed by 35 cycles with T_A = 65 °C.

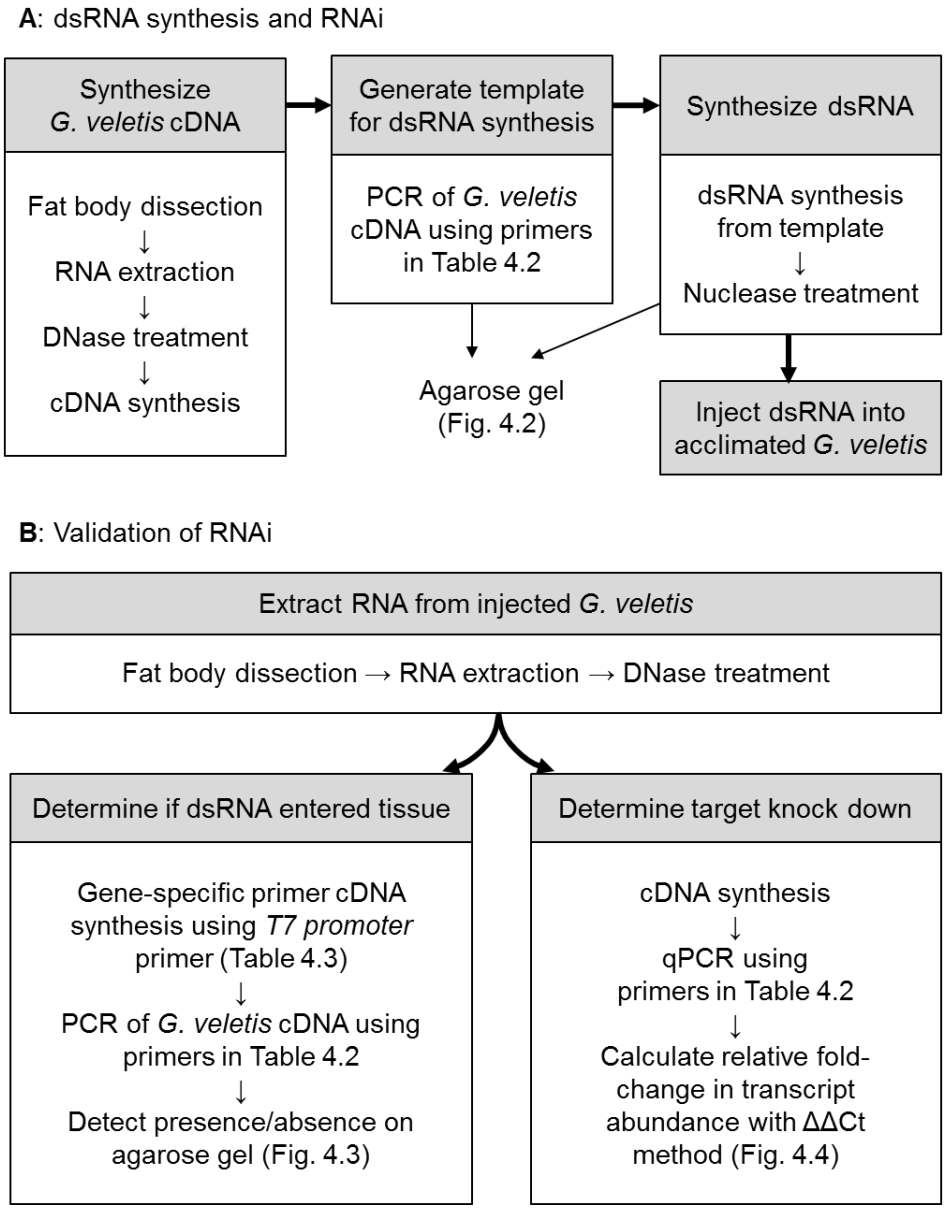

Figure 4.1. Methods work flow for RNA interference (RNAi) in *Gryllus veletis*. (A) To conduct RNAi, I synthesized dsRNA constructs, and injected these constructs into acclimated *G. veletis*. **(B)** I then validated whether dsRNA entered tissue (bottom left) and knocked down transcript abundance (bottom right).

I synthesized dsRNA from 1 µl template (PCR product) using the Ambion MEGAscript RNAi Kit (ThermoFisher Scientific) according to manufacturer's instructions. The dsRNA was synthesized in a single reaction at 37 °C for 4 h. I included the kit's nuclease treatment (to degrade template and any unannealed single-stranded RNA), but did not complete the kit's purification step, because it reduced the yield to less than 10 ng/µl. To confirm that PCR and dsRNA products were the expected size and purity, I resolved the products on a 1.3 % agarose gel in TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) stained with ethidium bromide, and imaged the gel using a BioRad gel doc and Quantity One software. The dsRNA templates (PCR products) were also sequenced at the London Regional Genomics Centre (Robarts Research, London, ON, Canada).

To initiate RNAi, I injected dsRNA into crickets three days after the end of acclimation. I used a 10 µl gastight Hamilton syringe (Hamilton Company, Reno, NV, USA) with a 30-gauge disposable needle (BD Canada, Mississauga ON, Canada) to inject crickets under the pronotum. I injected 5 µl of either 0.1, 0.3 or 0.6 µg/µl dsRNA diluted in nuclease-free water, resulting in the injection of 0.5, 1.5, or 3 µg dsRNA, respectively. I washed the Hamilton syringe with acetone and nuclease-free water between injections of different dsRNA constructs. I transferred injected crickets into individual mesh-covered 180 ml transparent cups (Polar Plastics, Summit Food Distributors, London, ON, Canada) containing rabbit food, water, and shelters made from egg cartons for up to three days at room temperature (c. 22 °C). All crickets survived this injection treatment.

4.2.3 Validation of dsRNA penetration into fat body tissue

To validate that dsRNA entered fat body tissue, I used gene specific primer (GSP) RT-PCR (Ren et al., 2014; Swevers et al., 2014) to detect and amplify dsRNA from fat body of dsRNA-injected crickets (Fig. 4.1B). I extracted and DNase-treated RNA extracted from the fat body of injected crickets, as described in Section 4.2.2. I then reverse transcribed the dsRNA using the QuantaBio qScript Flex cDNA Synthesis Kit (VWR International, Mississauga, ON, Canada) with 1 µl (500 ng) of DNase-treated RNA and 1 µM *T7 promoter* primer (Table 4.3). This GSP is complementary to the T7 promoter at the 5' end of all dsRNA constructs used in this study, so will generate cDNA from dsRNA constructs only. Following the recommended incubation of RNA, GSP and GSP

enhancer at 65 °C for 5 min, I modified the manufacturer's protocol by adding an incubation step of 1 min at 47 °C (the T_A of *T7 promoter* primer) to facilitate primer annealing. I then resumed the recommended protocol by transferring the sample to 42 °C, adding reverse transcriptase and its reaction mix, incubating the sample at 42 °C for 60 min, followed by 85 °C for 5 min. I used 0.5 µl of this GSP cDNA as a template for PCR, using the primers in Table 4.2 and protocol described in Section 4.2.2, and resolved the products on a 1.3 % agarose gel.

I used this GSP RT-PCR method to detect *GFP* and *HSP70* dsRNA in *G. veletis* fat body tissue 0.5, 2, 6, 24, and 72 h post-injection. In addition, to validate the method itself I generated three control samples. For negative controls, I used RNA extracted from crickets that were never injected (should contain no dsRNA), and the DNA template for *GFP* or *HSP70* dsRNA synthesis (cannot make cDNA from this product after a DNase treatment). For positive controls, I spiked RNA extracts from uninjected crickets with 0.01 µg/µl *GFP* or *HSP70* dsRNA. I treated each control sample alongside the RNA extracted from injected *G. veletis*.

4.2.4 Validation of knockdown

To determine whether dsRNA injection decreased transcript abundance for each target, I used RT-qPCR of fat body tissue RNA extracts one, two, or three days post-injection (Fig. 4.1B). I extracted RNA and reverse transcribed cDNA as described in Section 4.2.2, and performed qPCR using the primers in Table 4.3. Prior to processing my experimental samples, I determined efficiencies of each qPCR primer pair (Table 4.3) by conducting qPCR on ten-fold (10^{-1} to 10^{-6}) and two-fold (2^{-1} to 2^{-6}) dilution series of cDNA from uninjected *G. veletis*. I conducted qPCR in 20 µl reactions with Sso Advanced SYBR Green Supermix (BioRad) using 4 µl of cDNA template, and 0.4 mM primers in a Rotor-Gene 6000 system (Corbett Research, Cambridge, UK). I set up technical duplicates of each reaction and used the following reaction conditions: initial denaturation at 94 °C for 5 min; 40 cycles of 94 °C for 15 s, T_A (see Table 4.3) for 15 s, 72 °C for 20 s; followed by melt curve determination *via* ramping from 65 °C to 95 °C in increments of 0.5 °C. I used the Rotor-Gene software v.1.7 to determine Ct (cycle threshold) values for each sample in the dilution series, and to determine the primer efficiency using the slope of the Ct value as a function of template concentration (Toxopeus et al., 2014).

To quantify transcript abundance of target genes from dsRNA-injected crickets, I conducted RT-qPCR on three to six crickets. I processed three technical replicates of each biological replicate, using the reaction conditions above, with 1/50 dilutions of cDNA as template, and the T_A associated with an efficiency between 90 and 110 % (Table 4.3). I used the Rotor-Gene software to determine Ct values for each reaction, and excluded biological replicates whose standard deviation among Ct of technical replicates exceeded 0.2 (Newman et al., 2017). I normalized the expression of each target gene against two housekeeping genes, *Elongation factor 1β* (*Ef1β*) and *Ribosomal protein L18* (*Rpl18*), and calculated the relative normalized expression (ΔΔCt) compared to control-injected (*GFP* dsRNA) crickets in each experiment (Toxopeus et al., 2014). I compared relative transcript abundance of each target gene following injection with dsRNA constructs using one-tailed Welch's t-tests (*P5CR* and *HSP70*) or one-way ANOVAs with planned contrasts (*Ferritin*, *Supervillin* and *Tret-1*) in R v3.2.2 (R Core Team, 2017).

Table 4.3. Primers used for quantitative PCR (qPCR) and gene-specific primed (GSP) cDNA synthesis, including the annealing temperature (T_A,) used in qPCR cycle or cDNA synthesis, and the efficiencies of qPCR primers.

Target [a]	Forward primer (5' to 3')	Reverse primer (5' to 3')	T_A (°C)	Efficiency (%)
Elongation factor 1β[a]	CGCTCAATATGGTTGTTGGA	CCATCGAAAGATTTGATGTGG	55	110
Ferritin	AGTGAACCGACCAGGTTTTG	GCCAAAGCATTCTTCAGAGC	55	99
Heat shock protein 70	GACTCGCATTCCATTCCTGT	AGTCGAGCGTGAGGTGTTCT	55	99
P5C reductase	CGCGACCTTTGATTTCAGGT	GCTCCGAGGAAACCGATTTT	55	101
Ribosomal protein L18[a]	GAGAAGTTGACGGCTGTGGT	CGGCCTTGCATAAGAACAGT	55	99
Supervillin	CCTTTGCATCACAGGGTTTT	GGTCTCTCTTGGCTCGTGTC	55	107
Trehalose transporter-1	AGCGAGTGATGGCTGGTTAT	GATGAGCTCGATCACGAACA	58	91
T7 promoter[b]	TAATACGACTCACTATAGGG	-	47	-

[a]Primers for housekeeping genes;
[b]Primer for gene-specific primer (GSP) cDNA synthesis, targeting the 3' region (on both strands) of any dsRNA constructs;

4.3 Results

4.3.1 dsRNA persists in fat body tissue for up to three days

The dsRNA constructs generated from *G. veletis* cDNA were the expected length (Fig. 4.2, Table 4.2), and sequencing of the dsRNA templates confirmed that they were complementary to the target transcript (Appendix C). Using the GSP RT-PCR method I developed to reverse transcribe and amplify dsRNA, I detected dsRNA in the positive control, and did not detect dsRNA in either of the negative controls (Fig. 4.3). Following injection of *G. veletis* with *GFP* or *HSP70* dsRNA, I detected dsRNA in RNA extractions from *G. veletis* fat body for up to three days (Fig. 4.3).

4.3.2 RNAi reduces transcript abundance of *Ferritin*, *Supervillin*, and *Tret-1*

I reduced target transcript abundance by at least 80 % in fat body of *G. veletis* three days post-injection with 0.5 μg of dsRNA targeting *Ferritin* (ds*Ferritin*.2), *Supervillin* (ds*Supervillin*.1 and 2) and *Tret-1* (ds*Tret-1*.3) (Fig. 4.4). The other dsRNA constructs for *Ferritin*, *Supervillin* and *Tret-1* either resulted in less effective reduction of transcript abundance (ds*Supervillin*.3 and 4) or did not alter transcript abundance (ds*Ferritin*.1, ds*Tret-1*.1 and 2) relative to control-injected crickets. Neither of the dsRNA constructs I designed to target *P5CR* or *HSP70* for knockdown altered transcript abundance (Fig. 4.4), even at higher dsRNA concentrations (i.e. 3 μg ds*P5CR*; Fig. 4.5) or within a shorter timeframe (i.e. 1 or 2 d post-injection with ds*P5CR*; Fig. 4.5).

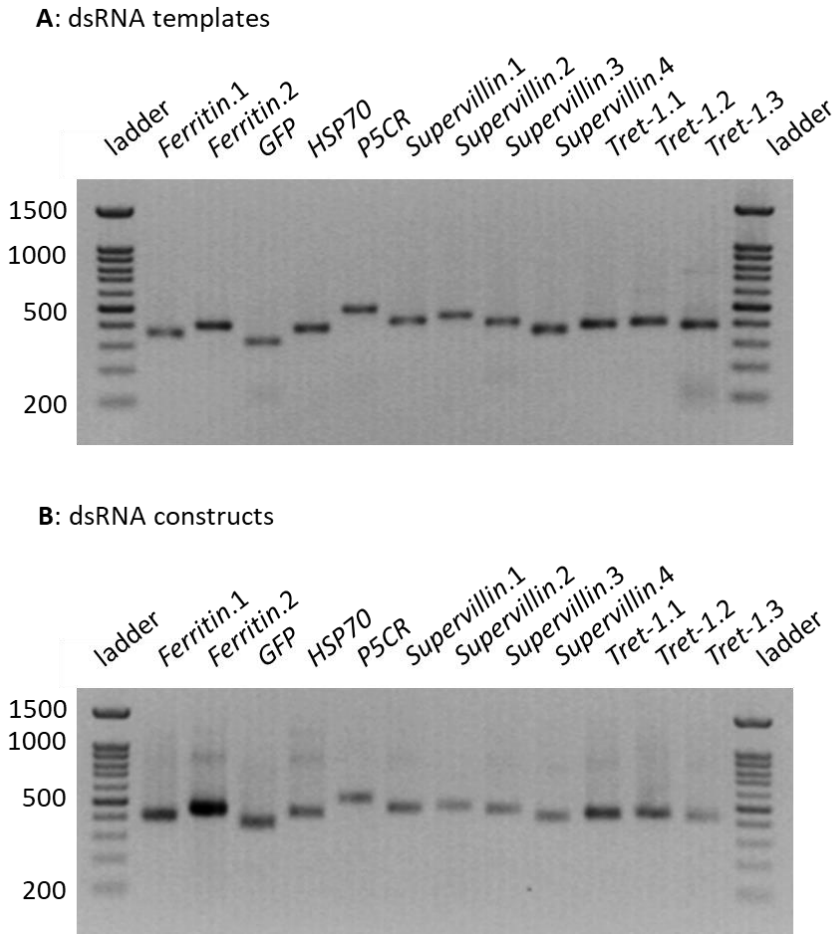

Figure 4.2. Agarose gel of dsRNA templates and dsRNA constructs. Each band represents the **(A)** dsRNA template or **(B)** dsRNA construct, with 100 bp DNA ladder (FroggaBio) included in the first and last lanes to estimate product size in base pairs (bp).

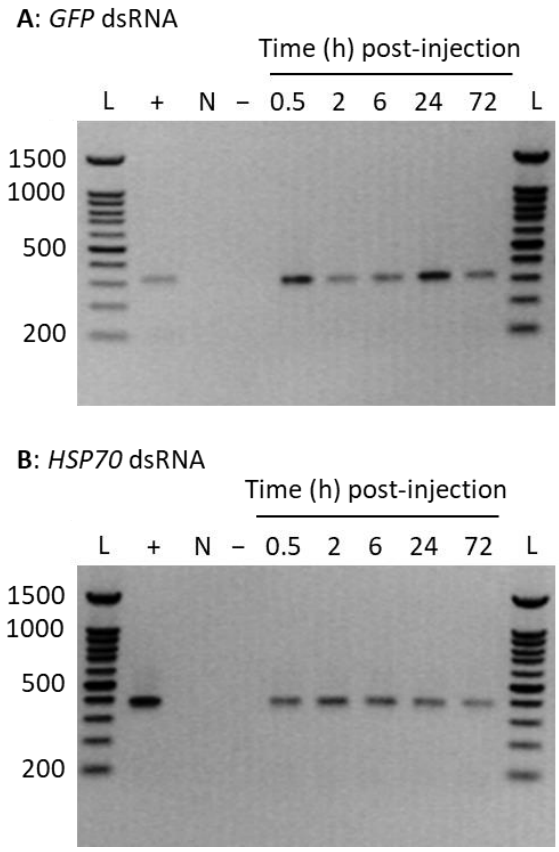

Figure 4.3. Agarose gel of *GFP* and *HSP70* dsRNA in *Gryllus veletis* fat body post-injection, amplified by GSP RT-PCR. Each band represents **(A)** *GFP* or **(B)** *HSP70* dsRNA reverse transcribed and amplified from control samples, or from fat body RNA extracts three days following dsRNA injection, with 100 bp DNA ladder (L; FroggaBio) included in the first and last lanes to estimate product size in base pairs (bp). Control samples include a positive control (+) of RNA from uninjected cricket spiked with *GFP* or *HSP70* dsRNA, and two negative controls: DNA template for *GFP* or *HSP70* dsRNA synthesis (N), and RNA from uninjected cricket (−). GFP, green fluorescent protein; HSP70, heat shock protein 70.

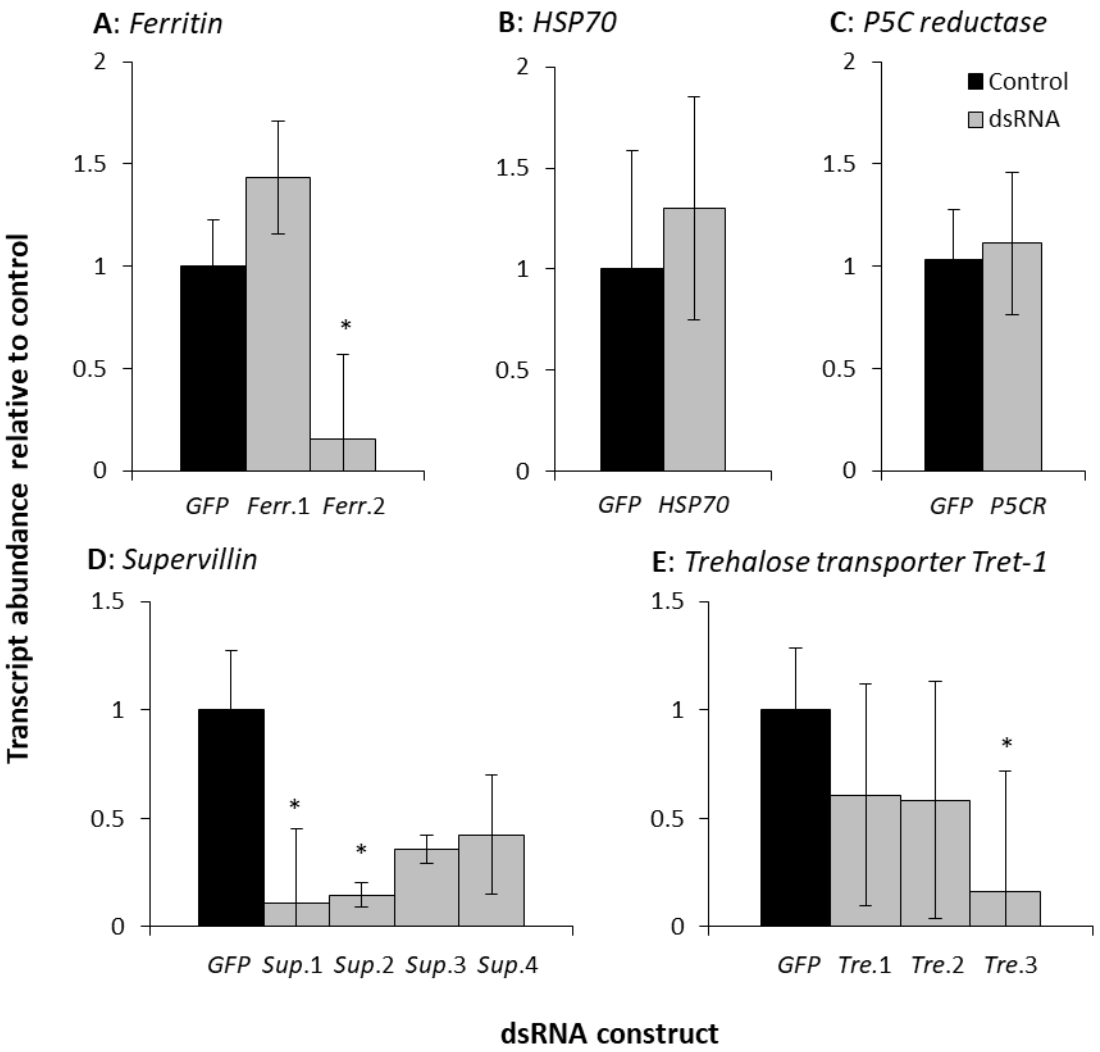

Figure 4.4. Transcript abundance of RNAi targets (A) *Ferritin*, (B) *HSP70*, (C) *P5C reductase*, (D) *Supervillin*, and (E) *Trehalose transporter Tret-1* three days after injection with dsRNA. dsRNA constructs included *GFP* dsRNA (control), or dsRNA constructs targeting the gene of interest (abbreviated from Table 4.2). Transcript abundance of the target gene is normalized to housekeeping genes *Elongation factor 1β* (*Ef1β*) and *Ribosomal protein L18* (*Rpl18*), and displayed relative to expression in control-injected crickets. Each bar represents the mean transcript abundance from three (*HSP70*, *P5CR*) or five (*Ferritin*, *Supervillin*, *Tret-1*) crickets ± SE. Asterisks indicate that relative transcript abundance is lower ($P < 0.05$) than in control crickets injected with *GFP* dsRNA (*Ferritin*: $F_{2,13} = 29.78$, $P < 0.001$; *HSP70*: $t_5 = 0.44$, $P = 0.358$; *P5CR*: $t_5 = 1.26$, $P = 0.139$, *Supervillin*: $F_{4,21} = 4.88$, $P = 0.007$; *Tret-1*: $F_{3,17} = 4.94$, $P = 0.013$). Ferr, ferritin; GFP, green fluorescent protein; HSP70, heat shock protein 70, P5CR, pyrroline-5-carboxylate reductase, Sup; supervillin; Tre, trehalose transporter Tret-1.

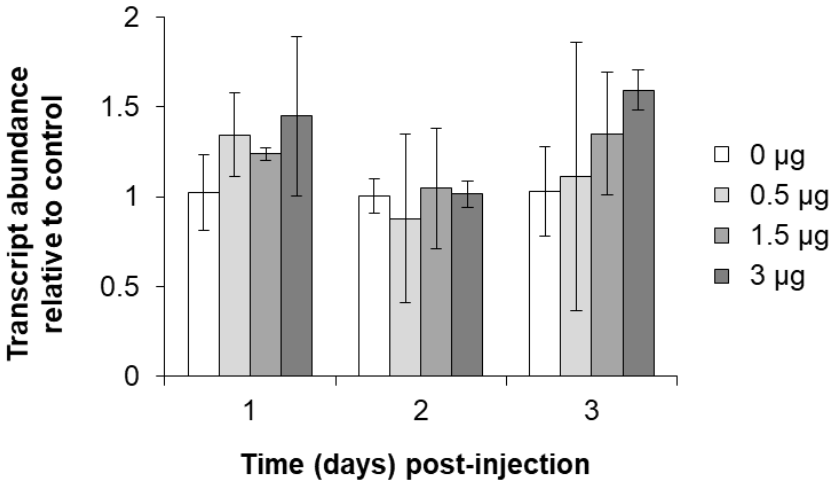

Figure 4.5. Relative abundance of *P5C reductase* transcripts in *Gryllus veletis* fat body, following injection with 0 µg, 0.5 µg, 1.5 µg, or 3 µg *P5CR* dsRNA. Transcript abundance is normalized to housekeeping genes *Elongation factor 1β* (*Ef1β*) and *Ribosomal protein L18* (*Rpl18*), and displayed relative to expression of *P5CR* in crickets injected with no dsRNA. Each bar represents the mean transcript abundance from three crickets ± SE. P5C, pyrroline-5-carboxylate

4.4 Discussion

Functional genetics experiments that manipulate molecular correlates of freeze tolerance have considerable potential to advance our understanding of how insects survive freezing. The spring field cricket *G. veletis* is a good candidate for these studies: we can rear them and induce freeze tolerance in the laboratory (Chapter 2), I have identified many changes in gene expression associated with freeze tolerance (Chapter 3), and gryllids appear to be generally amenable to RNAi (Horch et al., 2017; Singh et al., 2017). In this chapter, I synthesized 14 dsRNA constructs to target five genes of interest *via* RNAi. I confirmed that dsRNA injected into the hemolymph entered the tissue of interest (fat body), and that select dsRNA constructs reduced transcript abundance of *Ferritin*, *Supervillin* and *Tret-1* (but not *P5CR* or *HSP70*) for up to three days post-injection. In this section, I discuss factors that may drive variability of the RNAi response in *G. veletis*, and suggest future experiments for the knockdowns that were successful.

4.4.1 RNAi efficacy in *G. veletis* is construct-specific

For RNAi to be successful, dsRNA must be sufficiently stable *in vivo* and must be taken up by cells, which I evaluated in *G. veletis*. Gene specific priming to reverse transcribe dsRNA from biological samples can be used to track dsRNA stability in hemolymph (Ren et al., 2014) and cells (Swevers et al., 2014), when followed by PCR amplification of the reverse-transcribed dsRNA. Using this concept, I developed a GSP RT-PCR protocol to detect dsRNA in *G. veletis* fat body tissue in the three days post-injection, verifying that injected dsRNA was sufficiently stable and taken up by *G. veletis* fat body cells. The GSP RT-PCR protocol uses readily available molecular biology tools (cDNA synthesis and PCR) to track dsRNA persistence, and is likely more accessible than tools that use radiolabelling (Shukla et al., 2016) or fluorescent labelling (Yoon et al., 2017). The protocol I developed was appropriately discerning – i.e. I could detect dsRNA when it was present in RNA samples, and didn't encounter any false positives when dsRNA was absent.

I applied the GSP RT-PCR method to examine the stability and uptake of a construct that did not knock down target transcript abundance (as determined by RT-qPCR): *HSP70*

dsRNA. I confirmed that *HSP70* dsRNA entered fat body tissue within 6 h of injection, and could be detected up to three days post-injection, similar to *GFP* dsRNA. This suggests that the limited RNAi success with the *HSP70* dsRNA construct was not due to rapid degradation of dsRNA by hemolymph nucleases (e.g. within 60 min in *S. frugiperda* hemolymph; Shukla et al., 2016), nor failure of the tissue to take up the construct (Ren et al., 2014). I therefore hypothesize that ds*HSP70* either was not sufficiently processed to siRNA (Yoon et al., 2017), or was processed to siRNA that failed to initiate transcript degradation (Jackson et al., 2003; Joseph and Osman, 2012). Alternatively, ds*HSP70* may have knocked down the target transcript, but failed to knock down other isoforms of the transcript (which were subsequently detected by RT-qPCR).

I determined the conditions under which RNAi was successful by quantifying the relative abundance of target mRNAs using RT-qPCR. In *G. bimaculatus*, injection of between 0.5 and 3 μg dsRNA knocks down transcript abundance of *Allatostatin-A* by c. 50 % in ovaries, fat body, and brain tissue after three days (Meyering-Vos et al., 2006). Similarly, injection of 0.5 μg of ds*Ferritin*.2, ds*Supervillin*.1 and 2, or ds*Tret-1*.3 reduced target transcript abundance in *G. veletis* fat body tissue by at least 80 % three days post-injection. Therefore, *G. veletis* appears to be similarly amenable to RNAi as this other gryllid. Two targets (*HSP70* and *P5CR*) were not knocked down, similar to a study in *G. bimaculatus* embryos, in which RNAi of *Armadillo*, but not *Wingless* or *Hedgehog*, was successful (Miyawaki et al., 2004). The failure of ds*P5CR* to reduce transcript abundance was not dose or time-dependent, but future studies should consider these parameters when designing and testing new dsRNA constructs.

Neither the ds*HSP70* nor ds*P5CR* appeared to reduce target transcript abundance, which may stem from ineffective construct design (failed RNAi) or ineffective detection methods. Transcripts of *P5CR* were much less abundant than other targets in this study (46 read counts across fat body from 15 crickets; Table 4.1). Given this low transcript abundance, RT-qPCR may not be sufficiently sensitive to detect further reduction in transcript abundance. Further protocol development is required to confirm whether transcript knockdown reduced protein abundance (using semi-quantitative Western blotting; King et al., 2013; Toxopeus et al., 2014) or activity (using enzyme activity

assays; Yang et al., 2017) or resulted in other predicted biochemical effects (e.g. reduced cryoprotectant abundance; Yang et al., 2017).

RNAi efficacy for *Ferritin*, *Supervillin* and *Tret-1* varied among constructs, supporting the principle that RNAi success can be limited by dsRNA construct design. *Supervillin* was the most amenable to RNAi (all four dsRNA constructs caused at least partial knockdown), which may in part stem from its lower transcript abundance relative to *Ferritin* and *Tret-1* (Table 4.1). The *Ferritin* and *Tret-1* constructs that were ineffective (ds*Ferritin.1*, ds*Tret-1.1*, ds*Tret-1.2*) were of similar length and composition to the constructs that effectively reduced transcript abundance (ds*Ferritin.2*, ds*Tret-1.3*) (Table 4.2, Appendix C). I speculate that low construct efficacy was caused by sequence-specific properties, reinforcing the principle that one should design and test multiple constructs to ensure successful RNAi. I suggest further developing RNAi of *HSP70* and *P5CR* by testing dsRNA constructs different to the ones I designed in this chapter.

4.4.2 Leveraging RNAi to understand freeze tolerance

To continue using RNAi in future studies of *G. veletis* freeze tolerance, I suggest additional validation and development of the methods outlined in this chapter. For example, it is important to determine whether dsRNA injection results in reduced protein abundance (King et al., 2013; Toxopeus et al., 2014) and how this abundance changes with time, because reduced transcript abundance alone is unlikely to impact freeze tolerance. If RNAi reduces target protein abundance (due to reduced protein synthesis), I suggest experiments in this section that will determine the impact of this knockdown on *G. veletis* freeze tolerance at the whole animal, cellular, and subcellular levels.

In Chapter 1, I framed freeze tolerance as surviving the processes of cooling, freezing and thawing, each of which is hypothesized to challenge survival. I suggest using RNAi to test the extent to which freeze-tolerant *G. veletis* modify these processes and mitigate these challenges *via* the physiological changes correlated with acclimation (Chapters 2, 3). For example, both low temperatures (Lalouette et al., 2011; Rojas and Leopold, 1996) and thawing (Doelling et al., 2014; Storey and Storey, 2010) are hypothesized to cause oxidative stress. I predict that knockdown of the iron chelator *Ferritin* would increase

reactive oxygen species (ROS) production and therefore oxidative damage (Lopez-Martinez et al., 2008) during acclimation (i.e. at low temperatures), and after a freeze-thaw cycle, likely reducing freeze tolerance. Similarly, low temperatures can depolymerize the actin cytoskeleton (Des Marteaux et al., 2018), and I hypothesize that freezing further exacerbates this stress. I predict that knockdown of the actin-binding *Supervillin* would reduce cytoskeletal stability (increase cytoskeletal depolymerization) during acclimation and freezing. These experiments would address both the questions of whether the hypothesized challenges drive mortality of frozen and thawed insects, and whether these cytoprotective genes mitigate those challenges, furthering our understanding of the challenges and mechanisms associated with freeze tolerance.

I hypothesized that multiple mechanisms contribute to freeze tolerance (Chapter 1), and in *G. veletis* those mechanisms may require substantial time to develop/mature (Chapters 2, 3). Becoming freeze-tolerant requires a full six weeks of acclimation (Chapter 2), and RNAi could be used to understand how the processes associated with acclimation interact with each other, and contribute to freeze tolerance. For example, I hypothesized that differential expression of cytoskeleton proteins and regulators may cause cytoskeletal remodelling during acclimation that preserves cell integrity in the cold and frozen state (Chapter 3). *Supervillin* encodes an actin-binding protein (Table 4.1) and is expressed early in acclimation (Chapter 3). Accumulation of this protein may be prerequisite for cytoskeletal remodelling late in acclimation. I speculate that knockdown of *Supervillin* early in acclimation may interfere with this putative remodelling, while knockdown of *Supervillin* late in acclimation will not. Similarly, I hypothesized that Tret-1 facilitates trehalose distribution throughout the body during acclimation and freezing/thawing (Table 4.1; Chapter 3). I predict that *Tret-1* knockdown during acclimation will prevent trehalose export from fat body tissue, limiting freeze tolerance. If Tret-1 is required for cryoprotectant transport during freezing, I predict that knockdown of *Tret-1* at the end of acclimation will reduce cell and whole animal survival when frozen. Thus, RNAi can be used to determine the extent to which changes during acclimation are necessary for freeze tolerance, and whether further changes during the freeze-thaw cycle (e.g. cryoprotectant transport during freezing) facilitate survival of internal ice formation.

4.5 Conclusions

In this chapter, I developed a protocol for RNAi (and the validation thereof) in *G. veletis*, generating a functional genetics tool that will facilitate future work investigating the mechanisms underlying insect freeze tolerance. By injecting custom dsRNA constructs, I knocked down transcript abundance of *Ferritin*, *Supervillin*, and *Tret-1* for at least three days in freeze-tolerant juveniles of this species. Investigating the impact of these knockdowns on oxidative stress, cytoskeletal stability, and cryoprotectant transport (respectively) will provide insight into three rarely-studied aspects of insect freeze tolerance. This chapter therefore provides a tool for better understanding how insects protect their cells from the challenges associated with low temperatures and ice.

4.6 References

Bally, J., Fishilevich, E., Bowling, A. J., Pence, H. E., Narva, K. E. and Waterhouse, P. M. (2018). Improved insect-proofing: expressing double-stranded RNA in chloroplasts. *Pest Management Science* **74**, 1751-1758.

Cao, M., Gatehouse, J. A. and Fitches, E. C. (2018). A systematic study of RNAi effects and dsRNA stability in *Tribolium castaneum* and *Acyrthosiphon pisum*, following injection and ingestion of analogous dsRNAs. *International Journal of Molecular Sciences* **19**, 1079.

Coello Alvarado, L. E., MacMillan, H. A. and Sinclair, B. J. (2015). Chill-tolerant *Gryllus* crickets maintain ion balance at low temperatures. *Journal of Insect Physiology* **77**, 15-25.

Danbara, Y., Sakamoto, T., Uryu, O. and Tomioka, K. (2010). RNA interference of timeless gene does not disrupt circadian locomotor rhythms in the cricket *Gryllus bimaculatus*. *Journal of Insect Physiology* **56**, 1738-1745.

Des Marteaux, L. E., Stinziano, J. R. and Sinclair, B. J. (2018). Effects of cold acclimation on rectal macromorphology, ultrastructure, and cytoskeletal stability in *Gryllus pennsylvanicus* crickets. *Journal of Insect Physiology* **104**, 15-24.

Doelling, A. R., Griffis, N. and Williams, J. B. (2014). Repeated freezing induces oxidative stress and reduces survival in the freeze-tolerant goldenrod gall fly, *Eurosta solidaginis*. *Journal of Insect Physiology* **67**, 20-27.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806-811.

Guo, W.-C., Fu, K.-Y., Yang, S., Li, X.-X. and Li, G.-Q. (2015). Instar-dependent systemic RNA interference response in *Leptinotarsa decemlineata* larvae. *Pesticide Biochemistry and Physiology* **123**, 64-73.

Horch, H., Liu, J., Mito, T., Popadić, A. and Watanabe, T. (2017). Protocols in the cricket. In *The Cricket as a Model Organism* (eds. H. Horch, T. Mito, A. Popadić, H. Ohuchi and S. Noji), pp. 327-370. Tokyo: Springer.

Horn, T. and Boutros, M. (2010). E-RNAi: a web application for the multi-species design of RNAi reagents–2010 update. *Nucleic Acids Research* **38**, W332-W339.

Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., Li, B., Cavet, G. and Linsley, P. S. (2003). Expression profiling reveals off-target gene regulation by RNAi. *Nature Biotechnology* **21**, 635-637.

Jose, A. M. (2015). Movement of regulatory RNA between animal cells. *Genesis* **53**, 395-416.

Joseph, T. T. and Osman, R. (2012). Thermodynamic basis of selectivity in guide-target-mismatched RNA interference. *Proteins: Structure, Function, and Bioinformatics* **80**, 1283-1298.

Kikawada, T., Saito, A., Kanamori, Y., Nakahara, Y., Iwata, K., Tanaka, D., Watanabe, M. and Okuda, T. (2007). Trehalose transporter 1, a facilitated and high-capacity trehalose transporter, allows exogenous trehalose uptake into cells. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 11585-11590.

King, A. M., Toxopeus, J. and MacRae, T. H. (2013). Functional differentiation of small heat shock proteins in diapause-destined *Artemia* embryos. *FEBS Journal* **280**, 4761-4772.

Lalouette, L., Williams, C., Hervant, F., Sinclair, B. J. and Renault, D. (2011). Metabolic rate and oxidative stress in insects exposed to low temperature thermal fluctuations. *Comparative Biochemistry and Physiology A* **158**, 229-234.

Li, H., Bowling, A. J., Gandra, P., Rangasamy, M., Pence, H. E., McEwan, R. E., Khajuria, C., Siegfried, B. D. and Narva, K. E. (2018). Systemic RNAi in western corn rootworm, *Diabrotica virgifera virgifera*, does not involve transitive pathways. *Insect Science* **25**, 45-56.

Lopez-Martinez, G., Elnitsky, M. A., Benoit, J. B., Lee, R. E. and Denlinger, D. L. (2008). High resistance to oxidative damage in the Antarctic midge *Belgica antarctica*, and developmentally linked expression of genes encoding superoxide dismutase, catalase and heat shock proteins. *Insect Biochemistry and Molecular Biology* **38**, 796-804.

Meyering-Vos, M., Merz, S., Sertkol, M. and Hoffmann, K. H. (2006). Functional analysis of the allatostatin-A type gene in the cricket *Gryllus bimaculatus* and the armyworm *Spodoptera frugiperda*. *Insect Biochemistry and Molecular Biology* **36**, 492-504.

Meyering-Vos, M. and Müller, A. (2007). RNA interference suggests sulfakinins as satiety effectors in the cricket *Gryllus bimaculatus*. *Journal of Insect Physiology* **53**, 840-848.

Mito, T., Okamoto, H., Shinahara, W., Shinmyo, Y., Miyawaki, K., Ohuchi, H. and Noji, S. (2006). *Krüppel* acts as a gap gene regulating expression of *hunchback* and *even-skipped* in the intermediate germ cricket *Gryllus bimaculatus*. *Developmental Biology* **294**, 471-481.

Miyawaki, K., Mito, T., Sarashina, I., Zhang, H., Shinmyo, Y., Ohuchi, H. and Noji, S. (2004). Involvement of Wingless/Armadillo signaling in the posterior sequential segmentation in the cricket, *Gryllus bimaculatu*s (Orthoptera), as revealed by RNAi analysis. *Mechanisms of Development* **121**, 119-130.

Moriyama, Y., Sakamoto, T., Karpova, S. G., Matsumoto, A., Noji, S. and Tomioka, K. (2008). RNA interference of the clock gene period disrupts circadian rhythms in the cricket *Gryllus bimaculatus*. *Journal of Biological Rhythms* **23**, 308-318.

R Core Team. (2017). R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing.

Ren, D., Cai, Z., Song, J., Wu, Z. and Zhou, S. (2014). dsRNA uptake and persistence account for tissue-dependent susceptibility to RNA interference in the migratory locust, *Locusta migratoria*. *Insect Molecular Biology* **23**, 175-184.

Rinehart, J. P., Li, A., Yocum, G. D., Robich, R. M., Hayward, S. A. L. and Denlinger, D. L. (2007). Up-regulation of heat shock proteins is essential for cold survival during insect diapause. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 11130-11137.

Rojas, R. R. and Leopold, R. A. (1996). Chilling injury in the housefly: evidence for the role of oxidative stress between pupariation and emergence. *Cryobiology* **33**, 447-458.

Scott, J. G., Michel, K., Bartholomay, L. C., Siegfried, B. D., Hunter, W. B., Smagghe, G., Zhu, K. Y. and Douglas, A. E. (2013). Towards the elements of successful insect RNAi. *Journal of Insect Physiology* **59**, 1212-1221.

Shinmyo, Y., Mito, T., Matsushita, T., Sarashina, I., Miyawaki, K., Ohuchi, H. and Noji, S. (2005). Caudal is required for gnathal and thoracic patterning and for posterior elongation in the intermediate-germband cricket *Gryllus bimaculatus*. *Mechanisms of Development* **122**, 231-239.

Shukla, J. N., Kalsi, M., Sethi, A., Narva, K. E., Fishilevich, E., Singh, S., Mogilicherla, K. and Palli, S. R. (2016). Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects. *RNA Biology* **13**, 656-669.

Singh, I. K., Singh, S., Mogilicherla, K., Shukla, J. N. and Palli, S. R. (2017). Comparative analysis of double-stranded RNA degradation and processing in insects. *Scientific Reports* **7**, 17059.

Spit, J., Philips, A., Wynant, N., Santos, D., Plaetinck, G. and Broeck, J. V. (2017). Knockdown of nuclease activity in the gut enhances RNAi efficiency in the Colorado potato beetle, *Leptinotarsa decemlineata*, but not in the desert locust, *Schistocerca gregaria*. *Insect Biochemistry and Molecular Biology* **81**, 103-116.

Storey, K. B. and Storey, J. M. (2010). Oxygen: stress and adaptation in cold hardy insects. In *Low Temperature Biology of Insects* (eds. D. L. Denlinger and R. E. Lee), pp. 141-165. New York: Cambridge University Press.

Swevers, L., Kolliopoulou, A., Li, Z., Daskalaki, M., Verret, F., Kalantidis, K., Smagghe, G. and Sun, J. (2014). Transfection of BmCPV genomic dsRNA in silkmoth-derived Bm5 cells: Stability and interactions with the core RNAi machinery. *Journal of Insect Physiology* **64**, 21-29.

Theil, E. C. (1987). Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annual Review of Biochemistry* **56**, 289-315.

Toxopeus, J., Warner, A. H. and MacRae, T. H. (2014). Group 1 LEA proteins contribute to the desiccation and freeze tolerance of *Artemia franciscana* embryos during diapause. *Cell Stress and Chaperones* **19**, 939-948.

van der Krol, A. R., Mur, L. A., Beld, M., Mol, J. N. M. and Stuitje, A. R. (1990). Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *The Plant Cell* **2**, 291-299.

Wang, K., Peng, Y., Pu, J., Fu, W., Wang, J. and Han, Z. (2016). Variation in RNAi efficacy among insect species is attributable to dsRNA degradation *in vivo*. *Insect Biochemistry and Molecular Biology* **77**, 1-9.

Weeda, E., Koopmanschap, A., De Kort, C. and Beenakkers, A. T. (1980). Proline synthesis in fat body of *Leptinotarsa decemlineata*. *Insect Biochemistry* **10**, 631-636.

Wulfkuhle, J. D., Donina, I. E., Stark, N. H., Pope, R. K., Pestonjamasp, K. N., Niswonger, M. L. and Luna, E. J. (1999). Domain analysis of supervillin, an F-actin bundling plasma membrane protein with functional nuclear localization signals. *Journal of Cell Science* **112**, 2125-2136.

Yang, M., Zhao, L., Shen, Q., Xie, G., Wang, S. and Tang, B. (2017). Knockdown of two trehalose-6-phosphate synthases severely affects chitin metabolism gene expression in the brown planthopper *Nilaparvata lugens*. *Pest Management Science* **73**, 206-216.

Yoon, J.-S., Gurusamy, D. and Palli, S. R. (2017). Accumulation of dsRNA in endosomes contributes to inefficient RNA interference in the fall armyworm, *Spodoptera frugiperda*. *Insect Biochemistry and Molecular Biology* **90**, 53-60.

Chapter 5

5 General Discussion

Freeze tolerance has evolved multiple times, facilitating survival of low temperatures and unpredictable environments (Chapter 1). Despite the discovery of freeze tolerance over 200 years ago, our current understanding of how insects survive internal ice formation is limited due to a focus on targeted descriptive studies rather than mechanism-driven research. In Chapter 1, I proposed that we could better approach this research by framing freeze tolerance as the ability to survive a process of cooling, freezing, thawing, and recovery; determining the challenges associated with these processes; and identifying mechanisms to mitigate the challenges. I suggested that the best tool for much of this work would be a model organism that is amenable to manipulations in the laboratory (Chapter 1). In my thesis, I investigated how physiological changes during acclimation contribute to surviving the process of cooling, freezing, thawing, and recovery.

I characterized and manipulated freeze tolerance in the spring field cricket *Gryllus veletis*, which becomes freeze-tolerant as a late instar juvenile in the autumn, or in response to a six week acclimation in the lab (Chapter 2). I identified physiological and molecular correlates of acclimation, including metabolic rate suppression, changes in ice nucleation, cryoprotectant accumulation, and altered expression of more than 3,000 genes (Chapters 2, 3). By manipulating cryoprotectant concentrations *in vivo* and *ex vivo*, I demonstrated that *myo*-inositol, proline, and trehalose enhanced whole animal and/or tissue (fat body) freeze tolerance, likely *via* non-colligative mechanisms (Chapter 2). Importantly, each cryoprotectant appeared to function *via* unique mechanisms, and no cryoprotectant was sufficient for freeze tolerance (Chapter 2). In the final experimental chapter, I developed a protocol for RNA interference (RNAi) in juvenile *G. veletis* to facilitate future studies that test whether and how the differential gene expression identified in Chapter 3 contributes to freeze tolerance (Chapter 4). In this chapter I synthesize the three experimental chapters in this thesis within the context of the ‘processes’ framework (Chapter 1), and identify key directions for future research investigating the mechanisms underlying insect freeze tolerance.

5.1 The value of descriptive and functional approaches in understanding freeze tolerance

Observational studies that identify molecular correlates of freeze tolerance, (e.g. by investigating seasonal changes; Chapter 1) are useful for generating hypotheses about the mechanisms underlying insect freeze tolerance. However, these studies are often targeted, quantifying common low molecular weight cryoprotectants (e.g. Toxopeus et al., 2016), or genes known to facilitate stress tolerance [e.g. heat shock proteins (HSPs; Lu et al., 2014; Zhang et al., 2011)], thereby limiting this exploratory observation. In addition, there are relatively few studies that test the hypotheses that these molecular correlates of freeze tolerance contribute to surviving internal ice formation (see Koštál et al., 2016; 2012; 2011). In this section, I highlight the value of combining broad descriptive studies and functional approaches that test hypotheses to better understand the mechanisms underlying freeze tolerance.

5.1.1 Descriptive studies with '-omics' technologies

The advent of various '-omics' approaches (e.g. metabolomics, transcriptomics, proteomics) has facilitated identification of cryoprotective molecules and differentially-regulated genes that would not be identified in targeted descriptive studies. I identified over 3,000 differentially-expressed genes in acclimated *G. veletis* (Chapter 3), expanding our understanding of the molecular correlates of freeze tolerance beyond those identified in targeted studies (Izumi et al., 2006; Lu et al., 2014; Philip et al., 2008; Zhang et al., 2011). By using a broad metabolomics screen (for > 50 metabolites), I determined that freeze-tolerant *G. veletis* accumulates *myo*-inositol, proline and trehalose (Chapter 2). Proline and trehalose are relatively common low molecular weight cryoprotectants in freeze-tolerant Orthoptera (Neufeld and Leader, 1998; Ramløv et al., 1992; Toxopeus et al., 2016), which I likely would have detected in a targeted study comparing the hemolymph composition of freeze-tolerant and freeze-intolerant *G. veletis*. However, *myo*-inositol is a rarely-studied cryoprotectant (Purać et al., 2016; Tanaka and Tanaka, 1997) that I likely would not have identified *via* a more traditional approach (e.g. targeted spectrophotometric assays). Indeed, there may be additional cryoprotectants in freeze-tolerant *G. veletis* hemolymph (Chapter 2), given that I did not determine the source of c.

120 mOsm of hemolymph osmolality (Fig. 2.6). I suggest metabolomics screening to identify less-common cryoprotectants in other freeze-tolerant insects with (unexplained) elevated hemolymph osmolality.

‘-Omics’ technologies can help generate hypotheses based on multiple molecular correlates (e.g. altered expression of many genes in a single biological process/pathway) rather than single genes/proteins/metabolites. For example, *Belgica antarctica* differentially-regulates multiple cytoskeletal proteins during cryoprotective dehydration (identified by proteomics; Li et al., 2009), providing a strong basis for the hypothesis that modifying cytoskeletal composition preserves cell integrity under low water activity. In addition, combining multiple ‘-omics’ tools can provide a stronger basis for these hypotheses: cold-tolerant *Sarcophaga bullata* appears to alter the activity of several metabolic pathways during recovery from cold shock, coordinated at the levels of gene expression (based on transcriptomics) and metabolism (based on metabolomics; Teets et al., 2012). In my thesis, some of the transcriptional changes during acclimation (e.g. downregulation of metabolic enzymes; Chapter 3) support physiological correlates of freeze tolerance in *G. veletis* (e.g. metabolic rate suppression; Chapter 2). I also generated hypotheses about additional processes during acclimation that may facilitate freeze tolerance (Fig. 3.4). These processes should be validated at the cellular/physiological level, for example by testing whether acclimation increases membrane fluidity, cytoskeletal stability, cell permeability to cryoprotectants, and antioxidant capacity (Chapter 3).

To better understand the processes that regulate freeze tolerance, I suggest using ‘-omics’ techniques to characterize other comparisons within *G. veletis*. For example, to determine which changes during acclimation are induced by light or temperature cues, one could characterize the transcriptome and/or metabolome of *G. veletis* during six week acclimations that only modify temperature or photoperiod (Chapter 2). In addition, future studies could examine potential neuroendocrine control of these changes, as well as whether any of these changes are regulated at post-transcriptional level (e.g. by comparing the phosphoproteome; Teets and Denlinger, 2016) rather than transcriptional

level. Characterizing the cues and processes that regulate acclimation will improve our understanding of how insects modify their physiology to become freeze-tolerant.

5.1.2 Functional studies to test the mechanisms underlying freeze tolerance

To test the hypotheses generated in descriptive studies, researchers can experimentally manipulate molecule abundance, and determine whether the manipulations enhance or reduce freeze tolerance. Low molecular weight metabolites are particularly amenable to laboratory manipulation experiments: researchers can increase their abundance *in vivo* using diet manipulation (Koštál et al., 2016) or injection (Benoit et al., 2009; Rosendale et al., 2016). For example, Koštál et al. (2016; 2012; 2011) demonstrated that the amino acids proline and arginine facilitate freeze tolerance in drosophilid larvae. By manipulating cryoprotectant concentrations in *G. veletis*, I tested three hypotheses: 1) cryoprotectants are sufficient for freeze tolerance, 2) cryoprotectants enhance freeze tolerance, and 3) cryoprotectants are functionally redundant (Chapter 2). No cryoprotectant (alone or in combination) was sufficient to confer freeze tolerance on unacclimated *G. veletis* (Chapter 2). However, these manipulative experiments demonstrated that *myo*-inositol, proline, and trehalose enhance freeze tolerance of acclimated *G. veletis*, likely *via* different mechanisms (Chapter 2). This challenges the hypothesis that low molecular weight cryoprotectants contribute to freeze tolerance *via* largely colligative mechanisms (Chapter 1).

Given that no cryoprotectant was sufficient for freeze tolerance (Chapter 2), a combination of mechanisms is likely required to confer freeze tolerance on a freeze-intolerant insect. For example, Koštál et al. (2016; 2012) demonstrated that a combination of elevated dietary proline, a dormancy-inducing cold-acclimation regime, and a high supercooling point (SCP; c. -0.5 °C) confers freeze tolerance on larvae of the normally chill-susceptible *Drosophila melanogaster*. A similar approach in *G. veletis* or a related (but chill-susceptible) species (e.g. *Gryllus pennsylvanicus*; Des Marteaux and Sinclair, 2016) could identify the combination of mechanisms that are sufficient for freeze tolerance. If, for example, a combination of cold acclimation, cryoprotectant injection, and inoculation of freezing at high subzero temperatures also induces freeze

tolerance in *G. pennsylvanicus*, this would provide strong support that this combination of mechanisms typically confers freeze tolerance.

Experiments that reduce the abundance/activity of molecules can identify factors that are necessary for freeze tolerance. For example, functional genetics experiments that prevent gene expression using RNAi (Scott et al., 2013) or CRISPR-Cas9 (Gratz et al., 2013) have facilitated our understanding of the genes and mechanisms underlying stress tolerance in the mosquito *Anopheles gambiae* (Liu et al., 2013) and *D. melanogaster* (Newman et al., 2017), respectively. Similarly, decreasing enzyme activity with pharmacological inhibitors can reveal the role of metabolic pathways in physiological processes (e.g. Beuster et al., 2011; Hossain et al., 2014; Tisdale and Threadgill, 1984). In Chapter 4, I identified appropriate targets for RNAi from the *G. veletis* transcriptome (Chapter 3), and developed a protocol to knock down transcript abundance of these targets. This is the first documented manipulation of gene expression in a freeze-tolerant model, and I encourage further use of RNAi and other functional experiments in *G. veletis* to test hypotheses concerning the mechanisms underlying freeze tolerance, as outlined in Section 5.2 and Table 5.1.

5.2 Revisiting hypotheses about the mechanisms underlying insect freeze tolerance

Of the freeze tolerance mechanisms identified in Chapter 1, my thesis provides support for controlling ice formation, stabilizing cells and macromolecules, and preventing accumulation of harmful metabolites (Chapters 2, 3). In this section, I review this evidence, and suggest future experiments to functionally test the mechanisms underlying *G. veletis* freeze tolerance and the extent to which those mechanisms are interdependent.

5.2.1 Controlling ice, stabilizing molecules, and regulating metabolism

Like many freeze-tolerant insects (Zachariassen and Kristiansen, 2000), acclimated *G. veletis* elevate their SCP, appearing to accumulate ice-nucleating agents (INAs) in their hemolymph and gut tissue (Chapter 2), which could support increased control of ice formation (Chapter 1). Supercooling point elevation is not sufficient for freeze tolerance:

deacclimated *Eleodes blanchardi* (Zachariassen and Hammel, 1976) and unacclimated *G. veletis* inoculated with silver iodide (AgI; Chapter 2) have high SCPs, but do not survive freezing. However, a more nuanced set of manipulations could test whether controlling the ice formation process is necessary for freeze tolerance. For example, rather than using AgI to inoculate ice formation at c. -4 °C, one could attempt to induce freezing at c. -0.5 °C *via* inoculation with external ice, in combination with a very slow cooling rate of 0.1 °C min^{-1} (Koštál et al., 2016). If controlling ice formation is important for freeze tolerance, I predict that this freezing protocol will improve survival of frozen *G. veletis*, potentially by reducing damage to cells/tissues (Table 5.1). Conversely, if controlling ice formation is necessary for freeze tolerance, knocking down production of ice-nucleating agents (INAs; e.g. *via* RNAi of transcripts encoding INAs) will reduce or eliminate freeze in *G. veletis* (Table 5.1). I suggested a number of approaches to identify *G. veletis* INAs in Chapter 3, which is a prerequisite for manipulations that reduces the abundance of these INAs.

I suggest that *G. veletis* is an appropriate model to examine whether controlling ice content facilitates freeze tolerance. Acclimation increased *G. veletis* hemolymph osmolality by c. 250 mOsm (Fig. 2.6), which could colligatively reduce ice content by more than 10 % (e.g. from 78 % to 65 %; Fig. 1.5), at least based on interspecific comparisons (Chapter 1). I therefore predict that freeze-tolerant *G. veletis* has lower ice content than freeze-intolerant *G. veletis* (Table 5.1), which may facilitate freeze tolerance (Chapter 1). Conversely, accumulation of high ice contents may cause mortality, for example at the lower lethal temperature (LLT; Chapter 1). Mortality at low temperatures appears to correlate with a critical ice content in *Eurosta solidaginis* (Lee and Lewis, 1985), while LLT is independent of ice content in *Hemideina maori* (Ramløv and Westh, 1993), and the relationship between ice content and survival of drosophilid larvae varies with acclimation (Rozsypal et al., 2018). To systematically investigate the impact of ice content on mortality in *G. veletis*, one could inject highly soluble but non-cryoprotective osmolytes, measure the resulting ice content (e.g. *via* calorimetry; Lee and Lewis, 1985), and determine whether ice content appears to drive mortality (Table 5.1). In Chapter 2, glucose was not cryoprotective, and future work could use one or more injections of saturated glucose solution to elevate hemolymph osmolality (and reduce ice content).

Table 5.1. Predictions for experiments that test the hypothesized mechanisms underlying freeze tolerance in *Gryllus veletis*. '-' indicates that a manipulative experiment is not appropriate in *G. veletis*, or is not yet possible based on technical limitations. Ch., chapter; DGE, differential gene expression; FT, freeze-tolerant; FI, freeze-intolerant; INA, ice-nucleating agent; LMW, low molecular weight; SCP, supercooling point; HSP70, heat shock protein 70; ROS, reactive oxygen species.

Mechanism	Predictions		
Effect of acclimation[a]	Comparative experiment	Manipulation to enhance freeze tolerance[b]	Manipulation to reduce freeze tolerance
1. Controlling ice formation			
Potential INA synthesis, suggested by SCP elevation (Ch. 2)	FT crickets synthesize INA molecules (Ch. 3), while FI crickets do not	Inoculation of ice at high temperatures + slow cooling will confer/improve freeze tolerance	Knock down of INA production in acclimated crickets will reduce survival of frozen FT crickets
2. Controlling ice content			
Elevated hemolymph osmolality, due to cryo-protectant synthesis (Ch. 2)	FT crickets have lower ice content than FI crickets	Reducing ice content will confer/improve freeze tolerance	-
3. Stabilizing cells and macromolecules			
Accumulation of LMW cryoprotectants that non-colligatively enhance freeze tolerance (Ch. 2)	Relative to FI crickets, FT crickets upregulate activity of cryo-protectant synthesis enzymes (Ch. 3)	Increasing cryoprotectant abundance will reduce cell death and macromolecule damage in frozen FT crickets	Inhibition of cryoprotectant synthesis enzyme activity during acclimation will increase cell and macromolecule damage in frozen FT crickets
Upregulation of trehalose transporter *Tret-1* (Ch. 3)	FT crickets export trehalose from fat body to hemolymph, import from hemolymph into other tissues (Ch. 3)	-	Knockdown of *Tret-1* (Ch. 4) will prevent hemolymph trehalose accumulation, increase cell and macromolecule damage in frozen FT crickets
Cytoskeletal remodelling, suggested by DGE of cytoskeletal regulators (Ch. 3)	FT cricket cytoskeleton is more resistant to depolymerization at low temperatures (Ch. 3)	-	Knockdown of actin stabilizer *Supervillin* (Ch. 4) will reduce cytoskeleton stability, increase cell damage in frozen FT crickets

Table 5.1 continued

Mechanism	Predictions		
Effect of acclimation[a]	Comparison experiment	Manipulation to enhance freeze tolerance[b]	Manipulation to reduce freeze tolerance
3. Stabilizing cells and macromolecules			
Cell membrane remodelling, suggested by DGE of membrane lipid synthesis genes (Ch. 3)	FT crickets retain membrane fluidity at low temperatures better than FI crickets (Ch. 3)	-	Knockdown of membrane lipid synthesis genes (e.g. *stearoyl CoA desaturase*) during acclimation will reduce membrane fluidity, increase cell damage in frozen FT crickets
Upregulation of molecular chaperone *HSP70* (Ch. 3)	FT crickets accumulate less protein damage (aggregation) when frozen than FI crickets	-	Knockdown of *HSP70* (Ch. 4) will increase protein aggregation, reduce survival of frozen FT crickets
4. Managing biochemical processes			
Suppressed metabolic rate (Ch. 2)	FT crickets accumulate fewer metabolic end products (e.g. lactate, ROS) in the frozen state than FI crickets	Greater metabolic suppression (variation among FT crickets) will improve freeze tolerance	-
Putative antioxidants/ detoxifying enzymes, suggested by (Ch. 3)	FT crickets accumulate less oxidative damage when frozen than FI crickets	-	Knockdown of *Ferritin* (Ch. 4) or *Catalase* will increase oxidative damage, reduce survival of frozen FT crickets
Metabolic restructuring, suggested by metabolomics (Ch. 2) and DGE of metabolic enzymes (Ch. 3)	FT crickets catabolize more lipids than FI crickets, upregulate cryoprotectant synthesis pathways (Ch. 3)	-	-

[a]Changes during acclimation (Chapters 2 and 3) that may support mechanisms underlying freeze tolerance proposed in Chapter 1;
[b]"Confer" = make a FI cricket survive freezing; "improve" = increase whole animal survival or reduce damage in FT crickets frozen to the lethal limits

Freeze-tolerant *G. veletis* better protected their fat body tissue (*in vivo*) from damage/death during freezing and thawing compared to freeze-intolerant *G. veletis* (Chapter 2), suggesting that this is a good system to examine the mechanisms that stabilize cells and their macromolecules in the frozen state. Low molecular weight cryoprotectants protect macromolecules *in vitro* (Arakawa and Timasheff, 1982; Arakawa and Timasheff, 1983; Gekko and Timasheff, 1981; Rudolph and Crowe, 1985), and cytoprotective proteins such as HSP70 are necessary for surviving low temperatures (Rinehart et al., 2007). However, no one has examined the role of these molecules (and others) in protecting cells of freeze-tolerant insects *in vivo*. I hypothesize that both the accumulation of low molecular weight cryoprotectants (trehalose, proline and *myo*-inositol; Chapter 2) and expression of cytoprotective genes (e.g. HSP70; Chapter 3) during acclimation help stabilize cells and macromolecules at low temperatures and when frozen, therefore facilitating whole animal freeze tolerance. I suggest several functional experiments to explore this hypothesis in *G. veletis* in Table 5.1, building on the work from my thesis. For example, to test the prediction that high hemolymph [trehalose] improves survival at the LLT and lethal time (Lt) by stabilizing cells and macromolecules (Chapter 2), one could inject *G. veletis* with trehalose and measure *in vivo* cell survival (*via* live-dead staining; Chapter 2) and macromolecule protection (using the methods outlined in Table 1.5, e.g. protein aggregation) following a freeze treatment (Table 5.1). Similarly, to test the function of cytoprotective genes, future work could knock down their expression using RNAi (Chapter 4), and determine the impact on freeze-induced damage in *G. veletis*. For example, if the actin-binding protein Supervillin is necessary for stabilizing the cytoskeleton, I predict that RNAi-mediated knockdown of *Supervillin* will increase cytoskeleton depolymerization (Des Marteaux et al., 2018) and cell death in frozen *G. veletis* (Table 5.1).

Several physiological correlates of acclimation (Chapters 2, 3) could prevent or mitigate metabolic dysregulation in frozen *G. veletis*, providing support for the freeze tolerance mechanism of managing biochemical processes. While freeze-tolerant insects often suppress their metabolic rate (e.g. Irwin et al., 2001), and/or have high antioxidant capacity (e.g. Joanisse and Storey, 1996; Lopez-Martinez et al., 2008), the impact of

these physiological correlates on surviving freezing has not been well-explored. I hypothesize that upregulation of antioxidants and cytochrome P450s facilitates freeze tolerance by decreasing build-up of reactive oxygen species (ROS) and other harmful metabolites (Chapter 3). For example, I predict that RNAi of *Ferritin* (Chapter 4) will increase oxidative damage associated with freezing and thawing (as measured by assays in Table 1.5), reducing freeze tolerance (Table 5.1). I hypothesize that general downregulation of metabolism (Chapter 2) could reduce energy use in the frozen state, as well as the accumulation of harmful metabolic end products. It would be challenging to modify whole animal metabolic rate *via* functional studies. However, metabolic rate varies among freeze-tolerant *G. veletis* (Fig. 2.1), and future work could therefore determine whether low metabolic rates are correlated with low build-up of harmful metabolites and minimal energy drain (Table 5.1).

5.2.2 How do the timing and interactions of mechanisms influence freeze tolerance?

Each of the mechanisms above is unlikely to function independently, and it will be important to consider the potential interdependence among mechanisms when interpreting the results of functional studies. Some physiological changes during acclimation may affect multiple mechanisms. For example, trehalose accumulation likely helps stabilize macromolecules, but will also colligatively reduce ice content, and may impact biochemical processes (if catabolized as an energy source). Therefore, experiments that knock down trehalose production may reduce freeze tolerance in multiple ways. Similarly, many of the mechanisms are likely supported by multiple changes during acclimation. For example, in Chapter 2, I hypothesized that low molecular weight cryoprotectants can best stabilize macromolecules and cells if they accumulate both intracellularly and extracellularly. The accumulation of cryoprotectant transporters (e.g. *Tret-1*; Chapter 3) likely improves protection of cells and macromolecules by facilitating appropriate distribution of cryoprotectants. Due to this hypothesized interdependence among factors, I predict that inhibiting one process or preventing accumulation of one molecule will readily cause loss of freeze tolerance,

whereas many manipulations may be required to confer freeze tolerance on unacclimated *G. veletis*.

Describing and manipulating acclimation on a finer-resolution time scale may better identify factors that are necessary for freeze tolerance. The majority of changes in gene expression occur in the first three weeks of acclimation (Chapter 3), before *G. veletis* is freeze-tolerant (Chapter 2), suggesting that some long-term changes must be initiated early in acclimation to confer freeze tolerance. I hypothesize that *G. veletis* can only acquire freeze tolerance after these long-term changes are enacted for a threshold time, e.g. crickets require a threshold acclimation time to accumulate adequate quantities of molecular chaperones for cytoprotection when frozen. By characterizing acclimation over smaller time intervals (e.g. every week), future work could identify these potential thresholds. This would be further strengthened by manipulative experiments that systematically interfere with/prevent the potential thresholds. For example, if a threshold accumulation of HSP70 is required for freeze tolerance, I predict that knocking down its expression prior to reaching that threshold will prevent acquisition of freeze tolerance. If knocking down this gene has no effect on freeze tolerance, this suggests that it is not necessary for freeze tolerance, and that other molecules with functional overlap may compensate for the knockdown. This approach can identify which of the changes during acclimation (e.g. in Chapter 3) are necessary for freeze tolerance in a time/threshold-dependent manner.

5.3 Approaches to characterize the processes and challenges of freeze tolerance

While I focused my thesis on how changes during acclimation may facilitate freeze tolerance (Chapters 2, 3), the hypotheses I (and others) have generated are all based on assumptions about the processes and challenges associated with freezing, which are rarely empirically validated (Chapter 1). In this section, I outline future research to characterize the processes and challenges of freezing in *G. veletis* and other models. Once the processes and challenges are better characterized, we can extend our functional studies to determine the mechanisms by which low molecular weight cryoprotectants and changes in gene expression alter processes and mitigate the associated challenges.

5.3.1 Characterizing processes associated with freeze tolerance

To test the hypothesis that freeze-tolerant insects 'control' the process of ice formation (Chapter 1), I suggest further characterizing the processes of freezing and thawing, and determining whether freeze-tolerant *G. veletis* modify those processes. Ice and water distribution during freezing and thawing may be 'passive', i.e. driven by simple physical and chemical principles, and guided by molecules accumulated during acclimation (e.g. INAs; Chapter 2). Alternatively, freeze-tolerant insects may have an 'active' response to freezing and/or thawing, similar to freeze-tolerant ectotherms that mobilize glucose stores during freezing (Pedersen and Holmstrup, 2003; Storey and Storey, 1984), or insects that synthesize cryoprotectants in the frozen state (Walters et al., 2009). While it would be challenging (given current technologies; Table 1.5) to directly study the processes of ice nucleation, propagation, and melting, I suggest investigating whether freezing and thawing are 'active' or 'passive' indirectly *via* measurements of biological processes that use or generate ATP (Table 5.2). For example, researchers can measure metabolic rate in real time during the freeze-thaw cycle (see Sinclair et al., 2004; Sinclair et al., 2013), and can use a 'snapshot' approach (i.e. flash-freeze *G. veletis* samples during cooling, freezing, and thawing) to measure changes in metabolite concentrations [e.g. to detect cryoprotectant synthesis (Walters et al., 2009), energy reserve depletion (Storey and Storey, 1985)] and gene expression (Chapter 3). If cooling, freezing and thawing are passive, I predict that both freeze-tolerant and freeze-intolerant *G. veletis* will reduce activity of these processes as temperature decreases and ice content increases, and reactivate them during thawing (Table 5.2). However, if *G. veletis* actively control freezing or thawing, I predict that freeze-tolerant insects will elevate activity of processes that use or generate ATP during the freeze-thaw process (Table 5.2). Investigating whether *G. veletis* expend energy or actively alter physiological processes during the freeze-thaw cycle will reveal whether the changes during acclimation are sufficient for freeze tolerance, or whether post-acclimation mechanisms are necessary to mitigate the challenges of cooling, freezing and thawing.

Table 5.2. Predicted responses of freeze-intolerant (FI) and freeze-tolerant (FT) *Gryllus veletis* during the processes of cooling, freezing, thawing, and recovery, which are hypothesized to be passive or active. 'Biological processes' can include ATP-generating metabolism (estimated by measuring whole animal metabolic rate), and indicators of processes that use ATP such as cryoprotectant synthesis, active transport (e.g. ion pumps), transcription and translation, accumulation of harmful metabolic end products (e.g. lactate, reactive oxygen species), and depletion of ATP and energy reserves such as carbohydrates and lipids. '-' indicates that no prediction is relevant to the hypothesis.

Hypothesis	Cooling and freezing	Thawing	Post-thaw
The freeze-thaw and post-thaw processes are passive in FI crickets	Activity of biological processes in FI crickets will decrease as temperature decreases and ice forms	Activity of biological processes in FI crickets will increase as ice melts and temperature increases, but likely at a low rate (due to freeze injury)	Activity of biological processes in FI crickets post-thaw will be the same as unfrozen FI crickets, or lower (due to freeze injury)
The freeze-thaw and post-thaw processes are passive in FT crickets	Activity of biological processes in FT crickets will decrease as temperature decreases and ice forms, similar to FI crickets	Activity of biological processes in FT crickets will increase as ice melts and temperature increases, reversing the changes that happened during cooling and freezing	Activity of biological processes in FT crickets post-thaw will be the same as unfrozen FT crickets at the same temperature
The freeze-thaw process (or parts thereof) is active in FT crickets	Activity of biological processes will be higher in FT than FI crickets during cooling, ice formation, or in the frozen state	Activity of biological processes will increase in FT crickets during thawing, above what would be predicted due to increasing temperature and decreasing ice content	-
The post-thaw process is active in FT crickets	-	-	Activity of biological processes in FT crickets post-thaw will be higher than unfrozen FT crickets at the same temperature

Freeze tolerance research has historically focused on mechanisms that prevent freeze injury rather than those that repair it (Chapter 1), and I suggest that *G. veletis* is an appropriate model in which to explore the extent to which post-thaw repair and recovery processes are necessary for freeze tolerance. Post-thaw processes vary among freeze-tolerant species: thawed insects may recover motility rapidly (within 30 min; Štětina et al., 2018) or slowly (several days; Salt, 1961); and metabolic rate during recovery may increase (Zachariassen et al., 1979), decrease (Block et al., 1998), or vary over time (Štětina et al., 2018). *Gryllus veletis* has a lengthy post-thaw recovery period, requiring at least two days to regain full motility. To determine whether recovery is an 'active' process (Table 5.2), future studies should characterize repair (e.g. autophagy; Table 1.5) and restoration of physiological processes (e.g. metabolic rate, ion balance, neuromuscular function, metabolite composition, gene expression, etc.) during this post-thaw period. I predict that *G. veletis* exposed to conditions that cause more damage (e.g. being frozen to lower temperatures or for longer times) will require more repair and recovery (cf. Štětina et al., 2018).

5.3.2 Characterizing challenges associated with freeze tolerance

To investigate the challenges caused by low temperatures and ice in freeze-tolerant crickets (Chapter 1), I suggest further characterizing perturbations or damage at the (sub)cellular level associated with the freeze-thaw process. Fat body cell death after freezing and thawing was high in freeze-intolerant *G. veletis*, as well as freeze-tolerant *G. veletis* frozen to their lethal limits (Chapter 2). However, further investigation is required to determine the cause (e.g. low temperatures, ice, metabolic processes), timing (e.g. during cooling, freezing, thawing), and type of damage (e.g. which tissues/organelles/ macromolecules are damaged). I therefore advocate using the techniques listed in Table 1.5 to measure subcellular damage (e.g. ultrastructural changes, protein denaturation, DNA damage, and oxidative damage) at multiple stages of the freeze-thaw cycle, and determine whether that damage is repaired during post-thaw recovery. I predict that freeze-tolerant *G. veletis* accumulate less irreparable damage than freeze-intolerant *G. veletis*, due to protective changes during acclimation and/or the freeze-thaw process.

Characterizing how freeze injury changes with temperature, ice content and time spent frozen, can provide insight into the causes of damage and mortality at the lethal limits.

Both ice and low temperatures are hypothesized to challenge cellular and macromolecule stability (Chapter 1). I predict that macromolecule damage (e.g. protein denaturation, membrane damage) increases as temperature decreases and ice content increases (Fig. 5.1A, B). Given that high ice content correlates with low temperature, I suggest manipulating *G. veletis* hemolymph osmolality to compare cellular and subcellular damage in freeze-tolerant crickets with different ice contents at the same temperature. If low temperature is the primary driver of damage, all *G. veletis* frozen to a common temperature should exhibit similar damage, regardless of ice content. However, if ice content is the primary driver of damage, crickets with higher osmolyte concentrations (lower ice content) should have less cell damage at a given temperature (e.g. Fig. 5.1A).

Continued metabolism in the frozen state may challenge survival (Chapter 1), for example by build-up of harmful metabolites that damage macromolecules, or depletion of fuel reserves required for repair and recovery. I therefore predict that *G. veletis* accumulates harmful metabolites (e.g. ROS, lactate) leading to macromolecule damage (e.g. oxidative damage, as measured by protein carbonylation, lipid peroxidation; Table 1.5) that increases as a function of time spent frozen (Fig. 5.1C). In addition, I predict that *G. veletis* depletes fuel reserves (e.g. lipids or carbohydrates) in the frozen state over time (Fig. 5.1D). These descriptive experiments could inform our understanding of the mechanisms of mortality at the lethal limits, by determining which types of damage are correlated with the LLT and Lt (Fig. 5.1).

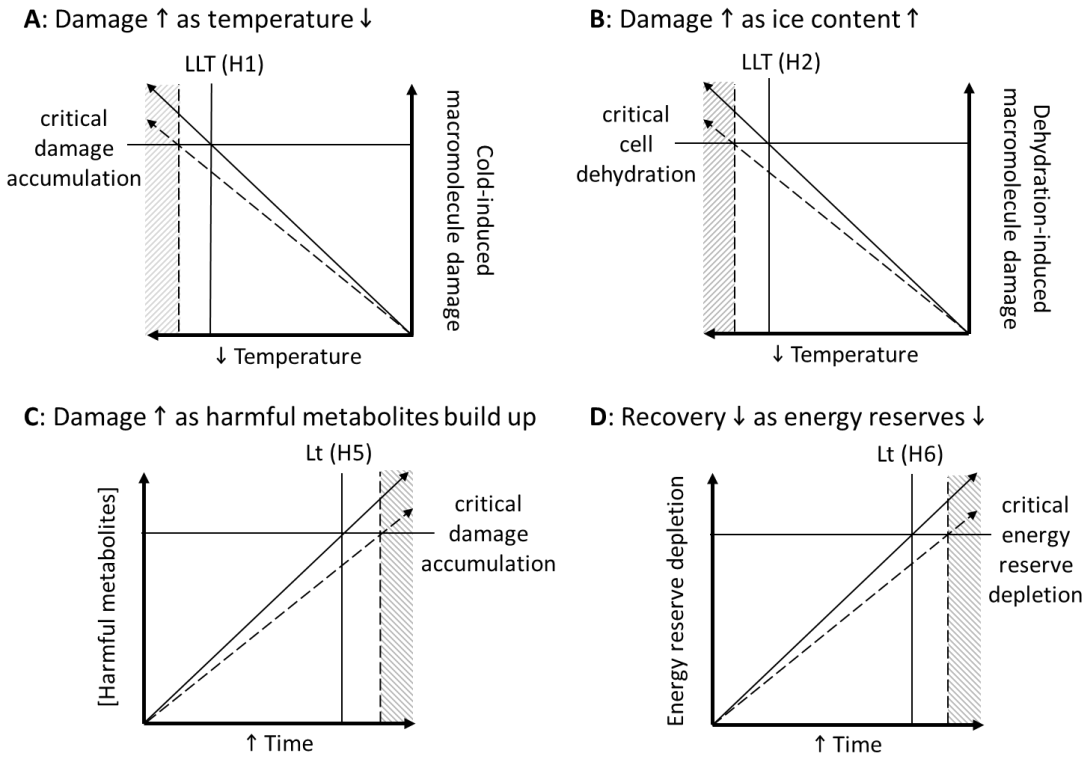

Figure 5.1. Predictions of how challenges change with (A, B) temperature and (C, D) time when *Gryllus veletis* is frozen, and the hypothesized relationship between challenges and mortality at the lethal limits. Solid line arrows indicate that challenge increases with temperature or time, reaching a critical threshold (horizontal solid lines) that causes mortality at the lower lethal temperature (LLT) or lethal time (Lt) (vertical solid lines). Hypothesis (H) number in parentheses refers to hypothesis identified in Table 1.4. Dashed line arrows indicate reduced challenge (e.g. due to experimental manipulation), resulting in a decreased LLT or increased Lt (vertical dashed lines).

Better characterization of the challenges of freezing and thawing will facilitate further functional studies that examine whether molecular correlates of freeze tolerance protect against specific challenges during the freeze-thaw cycle, and whether protective mechanisms fail at the lethal limits. For example, if death at the Lt is caused by accumulation of oxidative damage over time, I predict that knocking down expression of antioxidant enzymes (Chapter 4) will increase macromolecule damage and decrease the Lt of *G. veletis* (Table 5.1). Similarly, if death at the LLT is correlated with a critical level of macromolecule damage, I predict that knocking down synthesis of macromolecule stabilizers (e.g. trehalose, HSP70) will increase macromolecule damage (e.g. protein aggregation) and increase the LLT (Table 5.1).

5.4 Concluding remarks

In this thesis, I used a combination of descriptive and functional studies to advance our understanding of freeze tolerance in a novel model, *G. veletis*. However, substantial future research is required to clearly determine the mechanisms underlying freeze tolerance. In particular, I encourage continued use of *G. veletis* to characterize the processes and challenges associated with freezing and thawing, as well as the process of post-thaw recovery. This will facilitate experiments that test how the mechanisms outlined in this thesis alter these processes and mitigate the associated challenges. I hope that the work in this thesis inspires continued use of a combination of exploratory and hypothesis-testing experiments in *G. veletis* and other freeze-tolerant models, advancing our understanding of how insects survive internal ice formation.

5.5 References

Arakawa, T. and Timasheff, S. N. (1982). Stabilization of protein structure by sugars. *Biochemistry* **21**, 6536-6544.

Arakawa, T. and Timasheff, S. N. (1983). Preferential interactions of proteins with solvent components in aqueous amino acid solutions. *Archives of Biochemistry and Biophysics* **224**, 169-177.

Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. A., Lee, R. E. and Denlinger, D. L. (2009). Dehydration-induced cross tolerance of *Belgica antarctica* larvae to cold and heat is facilitated by trehalose accumulation. *Comparative Biochemistry and Physiology A* **152**, 518-523.

Beuster, G., Zarse, K., Kaleta, C., Thierbach, R., Kiehntopf, M., Steinberg, P., Schuster, S. and Ristow, M. (2011). Inhibition of alanine aminotransferase *in silico* and *in vivo* promotes mitochondrial metabolism to impair malignant growth. *Journal of Biological Chemistry* **286**, 22323-22330.

Block, W., Worland, M. R. and Bale, J. (1998). Respiratory responses to chilling and freezing in two sub-antarctic insects. *Cryobiology* **37**, 163-6.

Des Marteaux, L. E. and Sinclair, B. J. (2016). Ion and water balance in *Gryllus* crickets during the first twelve hours of cold exposure. *Journal of Insect Physiology* **89**, 19-27.

Des Marteaux, L. E., Stinziano, J. R. and Sinclair, B. J. (2018). Effects of cold acclimation on rectal macromorphology, ultrastructure, and cytoskeletal stability in *Gryllus pennsylvanicus* crickets. *Journal of Insect Physiology* **104**, 15-24.

Gekko, K. and Timasheff, S. N. (1981). Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. *Biochemistry* **20**, 4667-4676.

Gratz, S. J., Cummings, A. M., Nguyen, J. N., Hamm, D. C., Donohue, L. K., Harrison, M. M., Wildonger, J. and O'Connor-Giles, K. M. (2013). Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. *Genetics* **194**, 1029-1035.

Hossain, M. I., Roulston, C. L. and Stapleton, D. I. (2014). Molecular basis of impaired glycogen metabolism during ischemic stroke and hypoxia. *PLoS One* **9**, e97570.

Irwin, J. T., Bennett, V. A. and Lee, R. E. (2001). Diapause development in frozen larvae of the goldenrod gall fly, *Eurosta solidaginis* Fitch (Diptera: Tephritidae). *Journal of Comparative Physiology B* **171**, 181-188.

Izumi, Y., Sonoda, S., Yoshida, H., Danks, H. V. and Tsumuki, H. (2006). Role of membrane transport of water and glycerol in the freeze tolerance of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). *Journal of Insect Physiology* **52**, 215-220.

Joanisse, D. R. and Storey, K. B. (1996). Oxidative stress and antioxidants in overwintering larvae of cold-hardy goldenrod gall insects. *Journal of Experimental Biology* **199**, 1483-1491.

Koštál, V., Korbelová, J., Poupardin, R., Moos, M. and Šimek, P. (2016). Arginine and proline applied as food additives stimulate high freeze tolerance in larvae of *Drosophila melanogaster*. *Journal of Experimental Biology* **219**, 2358-2367.

Koštál, V., Šimek, P., Zahradníčková, H., Cimlová, J. and Štětina, T. (2012). Conversion of the chill susceptible fruit fly larva (*Drosophila melanogaster*) to a freeze tolerant organism. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 3270-3274.

Koštál, V., Zahradníčková, H. and Šimek, P. (2011). Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata*, survive cryopreservation in liquid nitrogen. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 13041-13046.

Lee, R. E. and Lewis, E. A. (1985). Effect of temperature and duration of exposure on tissue ice formation in the gall fly, *Eurosta solidaginis* (Diptera, Tephritidae). *Cryo-Letters* **6**, 25-34.

Li, A., Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. A., Lee, R. E. and Denlinger, D. L. (2009). Distinct contractile and cytoskeletal protein patterns in the Antarctic midge are elicited by desiccation and rehydration. *Proteomics* **9**, 2788-2798.

Liu, K., Dong, Y., Huang, Y., Rasgon, J. L. and Agre, P. (2013). Impact of trehalose transporter knockdown on *Anopheles gambiae* stress adaptation and susceptibility to *Plasmodium falciparum* infection. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 17504-17509.

Lopez-Martinez, G., Elnitsky, M. A., Benoit, J. B., Lee, R. E. and Denlinger, D. L. (2008). High resistance to oxidative damage in the Antarctic midge *Belgica antarctica*, and developmentally linked expression of genes encoding superoxide dismutase, catalase and heat shock proteins. *Insect Biochemistry and Molecular Biology* **38**, 796-804.

Lu, M.-X., Liu, Z.-X., Cui, Y.-D. and Du, Y.-Z. (2014). Expression patterns of three heat shock proteins in *Chilo suppressalis* (Lepidoptera: Pyralidae). *Annals of the Entomological Society of America* **107**, 667-673.

Neufeld, D. S. and Leader, J. P. (1998). Freezing survival by isolated Malpighian tubules of the New Zealand alpine weta *Hemideina maori*. *Journal of Experimental Biology* **201**, 227-236.

Newman, C. E., Toxopeus, J., Udaka, H., Ahn, S., Martynowicz, D. M., Graether, S. P., Sinclair, B. J. and Percival-Smith, A. (2017). CRISPR-induced null alleles show that *Frost* protects *Drosophila melanogaster* reproduction after cold exposure. *Journal of Experimental Biology* **220**, 3344-3354.

Pedersen, P. and Holmstrup, M. (2003). Freeze or dehydrate: only two options for the survival of subzero temperatures in the Arctic enchytraeid *Fridericia ratzeli*. *Journal of Comparative Physiology B* **173**, 601-609.

Philip, B. N., Yi, S.-X., Elnitsky, M. A. and Lee, R. E. (2008). Aquaporins play a role in desiccation and freeze tolerance in larvae of the goldenrod gall fly, *Eurosta solidaginis*. *Journal of Experimental Biology* **211**, 1114-1119.

Purać, J., Kojić, D., Petri, E., Popović, Ž. D., Grubor-Lajšić, G. and Blagojević, D. P. (2016). Cold adaptation responses in insects and other arthropods: an "Omics" approach. In *Short Views on Insect Genomics and Proteomics* (eds. C. Raman M. R. Goldsmith and T. A. Agunbiade), pp. 89-112. New York: Springer.

Ramløv, H., Bedford, J. J. and Leader, J. P. (1992). Freezing tolerance of the New Zealand alpine weta, *Hemideina maori* Hutton [Orthoptera; Stenopelmatidae]. *Journal of Thermal Biology* **17**, 51-54.

Ramløv, H. and Westh, P. (1993). Ice formation in the freeze tolerant alpine weta *Hemideina maori* Hutton (Orthoptera, Stenopelmatidae). *Cryo-Letters* **14**, 169-176.

Rinehart, J. P., Li, A., Yocum, G. D., Robich, R. M., Hayward, S. A. L. and Denlinger, D. L. (2007). Up-regulation of heat shock proteins is essential for cold survival during insect diapause. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 11130-11137.

Rosendale, A. J., Romick-Rosendale, L. E., Watanabe, M., Dunlevy, M. E. and Benoit, J. B. (2016). Mechanistic underpinnings of dehydration stress in the American dog tick revealed through RNA-Seq and metabolomics. *Journal of Experimental Biology* **219**, 1808-1819.

Rozsypal, J., Moos, M., Šimek, P. and Koštál, V. (2018). Thermal analysis of ice and glass transitions in insects that do and do not survive freezing. *Journal of Experimental Biology*, doi: 10.1242/jeb.170464.

Rudolph, A. S. and Crowe, J. H. (1985). Membrane stabilization during freezing: the role of two natural cryoprotectants, trehalose and proline. *Cryobiology* **22**, 367-377.

Salt, R. W. (1961). A comparison of injury and survival of larvae of *Cephus cinctus* Nort. after intracellular and extracellular freezing. *Canadian Journal of Zoology* **39**, 349-357.

Scott, J. G., Michel, K., Bartholomay, L. C., Siegfried, B. D., Hunter, W. B., Smagghe, G., Zhu, K. Y. and Douglas, A. E. (2013). Towards the elements of successful insect RNAi. *Journal of Insect Physiology* **59**, 1212-1221.

Sinclair, B. J., Klok, C. J. and Chown, S. L. (2004). Metabolism of the sub-Antarctic caterpillar *Pringleophaga marioni* during cooling, freezing and thawing. *Journal of Experimental Biology* **207**, 1287-1294.

Sinclair, B. J., Williams, C. M., MacMillan, H. A., Marshall, K. E. and Storey, K. B. (2013). Real-time measurements of metabolic rate during freezing and thawing of the wood frog, *Rana sylvatica*: implications for overwinter energy use. *Journal of Experimental Biology* **216**, 292-302.

Štětina, T., Hůla, P., Moos, M., Šimek, P., Šmilauer, P. and Koštál, V. (2018). Recovery from supercooling, freezing, and cryopreservation stress in larvae of the drosophilid fly, *Chymomyza costata*. *Scientific Reports* **8**, 4414.

Storey, J. M. and Storey, K. B. (1985). Freezing and cellular metabolism in the gall fly larva, *Eurosta solidaginis*. *Journal of Comparative Physiology B* **155**, 333-337.

Storey, K. B. and Storey, J. M. (1984). Biochemical adaptation for freezing in the wood frog, *Rana sylvatica*. *Journal of Comparative Physiology B* **155**, 29-36.

Tanaka, K. and Tanaka, S. (1997). Winter survival and freeze tolerance in a northern cockroach, *Periplaneta japonica* (Blattidae: Dictyoptera). *Zoological Science* **14**, 849-853.

Teets, N. M. and Denlinger, D. L. (2016). Quantitative phosphoproteomics reveals signaling mechanisms associated with rapid cold hardening in a chill-tolerant fly. *Journal of Proteome Research* **15**, 2855-2862.

Teets, N. M., Peyton, J. T., Ragland, G. J., Colinet, H., Renault, D., Hahn, D. A. and Denlinger, D. L. (2012). Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. *Physiological Genomics* **44**, 764-777.

Tisdale, M. and Threadgill, M. (1984). (+/-) 2, 3-Dihydroxypropyl dichloroacetate, an inhibitor of glycerol kinase. *Cancer Biochemistry Biophysics* **7**, 253-259.

Toxopeus, J., Lebenzon, J. E., McKinnon, A. H and Sinclair, B. J. (2016). Freeze tolerance of *Cyphoderris monstrosa* (Orthoptera: Prophalangopsidae). *The Canadian Entomologist* **148**, 668-672.

Walters, K. R., Sformo, T., Barnes, B. M. and Duman, J. G. (2009). Freeze tolerance in an arctic Alaska stonefly. *Journal of Experimental Biology* **212**, 305-312.

Zachariassen, K. E. and Hammel, H. T. (1976). Nucleating agents in the haemolymph of insects tolerant to freezing. *Nature* **262**, 285-287.

Zachariassen, K. E., Hammel, H. T. and Schmidek, W. (1979). Studies on freezing injuries in *Eleodes blanchardi* beetles. *Comparative Biochemistry and Physiology A* **63**, 199-202.

Zachariassen, K. E. and Kristiansen, E. (2000). Ice nucleation and antinucleation in nature. *Cryobiology* **41**, 257-279.

Zhang, G., Storey, J. M. and Storey, K. B. (2011). Chaperone proteins and winter survival by a freeze tolerant insect. *Journal of Insect Physiology* **57**, 1115-1122.

Appendices

Appendix A: Chapter 2 supplementary material

A.1 Supplementary Methods

To measure cryoprotectant concentrations in hemolymph samples from *Gryllus veletis*, I used the following spectrophotometric assays:

Glucose – I determined hemolymph glucose concentrations using the Glucose Assay Reagent (Sigma Aldrich, Missisauga, ON, Canada), according to manufacturer's instructions. Briefly, I diluted hemolymph samples by 1:50 to 1:1000 in Tris buffer (TB; 5 mM Tris, 137 mM NaCl, 2.7 mM KCl), vortexed to mix, incubated at 70 °C for 10 min, and centrifuged at 20,000 × g for 3 min. I added 10 µl of supernatant to 90 µl of Glucose Assay Reagent (Sigma Aldrich), incubated the reaction in flat-bottomed 96-well-plates for 20 min at room temperature (c. 22 °C), and measured absorbance in a microplate spectrophotometer (SpectraMax 340PC, Molecular Devices, Sunnydale, CA, USA) at 340 nm. I determined glucose concentration in each sample by comparison to a standard curve range of 0.16 to 0.01 mg/ml glucose diluted in TB.

Glycerol – I determined hemolymph glycerol concentrations using Free Glycerol Reagent (Sigma Aldrich), according to manufacturer's instructions (Crosthwaite et al., 2011). Briefly, I diluted hemolymph samples by 1:50 to 1:1000 in 0.05 % Tween-20, and vortexed to mix. I added 30 µl of sample to 100 µl of Free Glycerol Reagent (Sigma Aldrich), incubated the reaction in flat-bottomed 96-well-plates for 15 min at room temperature, and measured absorbance at 540 nm. I determined glycerol concentration in each sample by comparison to a standard curve range of 0.5 mM to 0.031 mM glycerol diluted in 0.05 % Tween-20.

myo-Inositol – I determined hemolymph *myo*-inositol concentrations using the *myo*-Inositol Assay Kit (Megazyme, Bray, Ireland), according to manufacturer's instructions. Briefly, I diluted hemolymph samples by 1:50 to 1:1000 in distilled deionized H_2O (ddH_2O), vortexed to mix, incubated at 70 °C for 10 min, and centrifuged at 20,000 × g

for 3 min. I added 15 µl of solution A (hexokinase solution) to 20 µl of sample, and incubated the reaction at room temperature for 15 min. I then added 100 µl of solution B (inositol dehydrogenase/diaphorase solution), and incubated the reaction at room temperature for 10 min. I transferred 100 µl of this mixture into flat-bottomed 96-well-plates, and measured absorbance at 492 nm. I determined *myo*-inositol concentration in each sample by comparison to a standard curve range of 0.25 to 0.016 mg/ml *myo*-inositol diluted in ddH_2O.

Polyethylene glycol – I measured hemolymph polyethylene glycol (PEG) concentrations using the assay described by Nag et al. (1996). Briefly, I diluted hemolymph samples 1:25 in ddH_2O, and vortexed to mix. I added 500 µl of chloroform and 500 µl of ammonium ferrothiocyanate reagent (0.1 M $FeCl_3$, 0.4 M NH_4SCN) to 50 µl of sample in 1.7 ml microcentrifuge tubes. I mixed the sample vigorously on a benchtop shaker (700 rpm) for 30 min at room temperature, and centrifuged at 600 × g for 2 min. I transferred 100 µl of the lower chloroform layer into flat-bottomed 96-well-plates, and measured absorbance at 510 nm. I determined PEG concentration in each sample by comparison to a standard curve range of 1.25 to 0.078 mM PEG-8000 (Sigma Aldrich) diluted in ddH_2O.

Proline – I measured hemolymph proline concentration using the assay described by Carillo and Gibon (2011). Briefly, I diluted hemolymph samples by 1:50 to 1:1000 in 40 % ethanol, and vortexed to mix. Samples were incubated at 4 °C overnight (18 to 24 h), and then centrifuged at 14,000 × g for 5 min. I added 40 µl of sample to 100 µl of ninhydrin solution [1 % (w/v) ninhydrin in 60 % acetic acid (v/v) and 20 % ethanol (v/v)], and incubated the reaction at 95 °C for 20 min. I centrifuged samples at 600 × g for 1 min, transferred 100 µl of supernatant into flat-bottomed 96-well-plates, and measured absorbance at 520 nm. I determined proline concentration in each sample by comparison to a standard curve range of 0.5 mM to 0.016 mM proline diluted in 40 % ethanol.

Trehalose – I measured hemolymph trehalose concentrations using the assay described by Tennessen et al. (2014). Briefly, I diluted hemolymph samples by 1:50 to 1:1000 in Tris

buffer (TB), vortexed to mix, incubated at 70 °C for 10 min, and centrifuged at 20,000 × g for 3 min. For each sample, I added 30 µl of trehalase stock [TS; 0.3 % (v/v) porcine trehalase enzyme (Sigma Alrich) in TB] to a 30 µl aliquot of supernatant to lyse trehalose into glucose monomers. I added 30 µl of TB to a second aliquot of supernatant. Samples were incubated at 37 °C overnight (18 to 24 h), centrifuged at 20,000 × g for 3 min, and processed using the Glucose Assay Reagent, as described above. I determined baseline glucose hemolymph concentration by comparing samples incubated in TB to a standard curve range of 0.16 to 0.1 mg/ml glucose diluted in TB. I calculated trehalose hemolymph concentration by comparing samples incubated in 1:1 TB:TS to a standard curve range of 0.16 to 0.1 mg/ml trehalose diluted in 1:1 TB:TS, and subtracting the signal from baseline glucose hemolymph.

A.2 Supplementary References

Carillo, P. and Gibon, Y. (2011). Protocol: extraction and determination of proline. [online] https://www.researchgate.net/publication/211353600_PROTOCOL_Extraction_and_determination_of_proline [accessed 28 June 2018].

Crosthwaite, J. C., Sobek, S., Lyons, D. B., Bernards, M. A. and Sinclair, B. J. (2011). The overwintering physiology of the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). *Journal of Insect Physiology* **57**, 166-173.

Nag, A., Mitra, G. and Ghosh, P. C. (1996). A colorimetric assay for estimation of polyethylene glycol and polyethylene glycolated protein using ammonium ferrothiocyanate. *Analytical Biochemistry* **237**, 224-231.

Tennessen, J. M., Barry, W. E., Cox, J. and Thummel, C. S. (2014). Methods for studying metabolism in *Drosophila*. *Methods* **68**, 105-115.

A.3 Supplementary Tables

Table A.1. Freeze tolerance of fifth-instar *Gryllus veletis* following acclimation, acclimatization, or laboratory manipulations. Treatments are described in methods section of Chapter 2. Crickets were defined as freeze tolerant if > 75 % survived being frozen at -8 °C for 1.5 h. *N*, sample size; AgI, silver iodide. Data from McKinnon (2015).

Treatment	*N*	*N* frozen	*N* survived	Freeze tolerant?
Laboratory acclimations				
Zero weeks control[a]	24	24	0	No
Three weeks control[a]	24	22	0	No
Six weeks control[a]	48	43	0	No
Three weeks acclimation[b]	24	24	3	No
Six weeks acclimation[b]	24	24	22	**Yes**
Six weeks acclimation temperature only[c]	8	8	0	No
Six weeks acclimation photoperiod only[d]	8	8	0	No
One week cold shock	8	8	0	No
Rapid cold hardening	8	8	0	No
Field acclimatizations				
Outdoor 2013-a[e]	6	6	6	**Yes**
Outdoor 2013-b[e]	8	8	8	**Yes**
Outdoor 2014	5	5	5	**Yes**
Laboratory manipulations				
External AgI (inoculation)	8	8	0	No
Gut AgI (diet)	24	24	0	No
Hemolymph AgI (injection)	8	8	0	No

[a]Fig. 2.1A, red temperature and photoperiod; [b]Fig. 2.1A, blue temperature and photoperiod; [c]Fig.2. 1A, blue temperature, red photoperiod; [d]Fig. 2.1A, red temperature, blue photoperiod; [e]Fig. 2.1B.

Table A.2. Gas exchange and water loss parameters in freeze-tolerant (FI) and freeze-intolerant (FT) fifth instar juvenile *Gryllus veletis*. Mean ± SE at each temperature is presented. CWL, cuticular water loss; *N*, sample size; Q_{10}, measure of thermal sensitivity; RWL, respiratory water loss; $\dot{V}CO_2$, rate of CO_2 emission; WLR, water loss rate.

Parameter	Freeze-intolerant		Freeze-tolerant	
	5 °C	15 °C	5 °C	15 °C
N	8		8	
Fresh mass (mg)	173.9 ± 19.5		178.7 ± 11.7	
Q_{10}	2.27 ± 0.29		1.77 ± 0.38	
$\dot{V}CO_2$ (µl h^{-1}) [a,b]	12.8 ± 2.4	35.5 ± 3.6	7.9 ± 1.0	25.6 ± 4.2
WLR (mg h^{-1}) [a,b]	0.411 ± 0.100	0.739 ± 0.144	0.194 ±0.043	0.334 ±0.09
RWL (mg h^{-1}) [a,b]	0.030 ± 0.022	0.114 ±0.031	0.013 ±0.010	0.055 ±0.019
CWL (mg h^{-1}) [a,b]	0.382 ±0.079	0.625 ±0.128	0.192 ±0.038	0.278 ± 0.035
RWL as a percentage total water loss (%) [c]	4.0 ± 2.0	16.6 ± 2.8	3.9 ±2.2	17.4 ± 6.3

[a]Signficant difference ($P < 0.05$) in parameter between FI and FT crickets.
[b]Signficant difference ($P < 0.05$) in parameter between crickets at 5 °C and 15 °C.
[c]Significant interaction ($P < 0.05$) of acclimation and test temperature on parameter.

Table A.3. Melting point (T_m), thermal hysteresis (TH) and ice crystal morphology of hemolymph samples from freeze-tolerant and freeze-intolerant fifth-instar juvenile *Gryllus veletis*, determined by nanolitre osmometry. *N*, sample size. Data from McKinnon (2015).

Treatment	*N*	Mean ± SE T_m (°C)[a]	Mean TH (°C)[b]	*N* nymphs with hexagonal crystal growth
Laboratory conditions				
Six weeks control	8	-0.72 ± 0.05	0.00	0
Six weeks acclimation	8	-1.14 ± 0.05	0.00	0

[a]Temperature at which last ice crystal melts when warming frozen hemolymph, corrected for concentration of anticoagulant.
[b]Difference between T_m and freezing point (temperature at which ice crystal begins to grow when cooled from the T_m).

Table A.4. Hemolymph concentrations (mM) of metabolites, as determined by GC/LC-MS and GC-FID. The concentrations were determined from three biological replicates (numbered) for each treatment group of fifth instar male *Gryllus veletis* nymphs maintained under control or acclimation conditions for zero, three, or six weeks. '-' indicates not detected.

	0 weeks control			3 weeks control			6 weeks control			3 weeks acclimated			6 weeks acclimated		
Metabolite	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Arginine	0.92	1.11	1.26	1.84	0.71	0.84	1.47	1.53	1.24	0.96	0.74	0.77	0.96	1.42	0.70
Glutamine	2.63	1.63	2.83	1.93	1.62	2.57	7.25	3.39	2.19	2.16	2.78	3.06	1.03	2.74	1.49
Serine	0.86	1.48	1.28	0.99	0.75	0.73	1.02	0.72	1.51	1.23	1.64	0.73	0.62	0.61	0.32
Asparagine	0.59	0.46	0.51	0.41	0.23	0.39	0.54	0.49	0.36	0.28	0.29	0.29	0.25	0.48	0.28
Threonine	0.56	0.37	0.43	0.46	0.28	0.35	0.60	0.79	0.38	0.30	0.26	0.34	0.15	0.37	0.23
Alanine	1.17	0.65	0.49	1.70	0.55	0.68	1.36	0.99	0.94	0.91	0.61	0.50	1.54	2.87	1.48
Putrescine	0.03	0.02	0.03	0.04	0.02	0.02	0.05	0.04	0.06	0.02	0.01	0.01	0.03	0.03	0.01
Aspartic acid	0.10	0.05	0.06	0.06	0.04	0.04	0.04	0.06	0.07	0.03	0.03	0.03	0.05	0.05	0.04
Ornithine	0.04	0.02	0.02	0.03	0.02	0.02	0.02	0.03	0.03	0.02	0.02	0.02	0.02	0.03	0.02
Proline	4.34	3.79	4.37	4.62	3.36	2.82	8.18	5.99	6.14	15.92	17.07	17.26	6.20	21.88	19.71
Glutamic acid	0.18	0.13	0.17	0.20	0.12	0.11	0.13	0.15	0.34	0.15	0.15	0.17	0.21	0.19	0.21
Methionine	0.23	0.21	0.17	0.17	0.07	0.14	0.20	0.27	0.11	0.16	0.08	0.10	0.18	0.23	0.11
Histidine	0.10	0.01	0.02	0.15	0.09	0.07	0.28	0.29	0.25	0.16	0.07	0.09	0.18	0.31	0.19
Glutathione (GSH)	0.25	0.13	0.21	0.21	0.15	0.15	0.15	0.13	0.31	0.09	0.10	0.07	0.12	0.08	0.09
Cysteine	0.06	0.03	0.04	0.06	0.04	0.07	0.06	0.07	0.06	0.03	0.03	0.02	0.04	0.03	0.02
Cystathionine (CTH)	0.03	0.01	0.01	0.02	-	0.01	-	0.04	-	-	-	-	0.02	0.02	0.01
Tryptophan	0.11	0.14	0.16	0.15	0.14	0.17	0.17	0.29	0.20	0.10	0.10	0.14	0.19	0.19	0.11
Phenylalanine	0.38	0.56	0.48	0.36	0.32	0.31	0.86	0.55	0.47	0.33	0.33	0.27	0.35	0.40	0.50
Tyrosine	1.37	1.70	1.63	1.14	1.42	1.15	3.78	1.90	2.56	1.33	1.56	1.57	0.88	1.78	1.44
Lysine	1.26	1.21	1.16	1.30	0.75	1.35	0.92	1.40	1.28	2.28	1.21	1.91	2.39	2.50	1.72
Fumaric acid	0.10	0.06	0.04	0.13	0.04	0.06	0.17	0.09	0.04	0.01	0.01	0.01	0.03	0.02	0.01
Succinic acid	0.17	0.15	0.07	0.10	0.05	0.09	0.21	0.21	0.07	0.07	0.04	0.07	0.05	0.07	0.05
Lactic acid	2.54	0.46	0.99	1.82	0.53	0.53	0.32	0.71	0.43	0.37	0.33	0.24	0.50	0.44	0.44
Maleic acid	0.09	0.05	0.06	0.08	0.05	0.06	0.09	0.06	0.07	0.04	0.04	0.03	0.04	0.03	0.03
Glycine	2.38	1.83	1.95	3.46	1.71	2.63	4.37	3.67	2.46	3.24	2.01	2.88	1.44	3.24	1.00
Aminobutyric acid	-	-	-	-	-	-	0.01	0.02	-	0.02	-	0.02	0.03	0.04	0.02

Table A.4. continued

	0 weeks control			3 weeks control			6 weeks control			3 weeks acclimated			6 weeks acclimated		
Metabolite	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Valine	0.92	0.64	0.93	0.96	0.81	0.82	2.01	1.46	0.97	1.43	1.31	1.29	1.29	2.24	1.54
beta-Alanine	0.04	0.04	0.03	0.06	-	-	0.07	0.06	-	0.02	-	-	0.02	0.03	0.03
Ketoglutaric acid	0.61	0.66	0.52	0.64	0.45	0.49	0.83	0.98	0.62	0.50	0.40	0.47	0.38	0.45	0.41
Leucine	0.53	0.49	0.59	0.74	0.48	0.57	1.02	0.87	0.69	1.10	0.82	1.11	1.18	1.93	1.19
Isoleucine	0.46	0.42	0.43	0.64	0.38	0.43	0.82	0.79	0.53	0.93	0.58	0.77	0.94	1.58	0.90
Malic acid	0.10	0.07	0.09	0.09	0.05	0.05	0.06	0.12	0.08	0.04	0.04	0.03	0.08	0.06	0.04
Aconitic acid	0.15	0.15	0.14	0.21	0.14	0.14	0.39	0.21	0.25	0.27	0.29	0.36	0.34	0.39	0.30
Myristic acid (C14:0)	0.72	0.33	0.24	0.18	0.16	0.17	0.12	0.11	0.23	0.18	0.19	0.14	0.15	0.11	0.10
Citric acid	0.93	1.27	1.22	1.31	0.92	0.98	2.80	1.44	2.49	1.94	1.08	1.68	3.72	3.42	2.49
Palmitic acid (C16:0)	0.44	0.16	0.20	0.23	0.18	0.18	0.10	0.22	0.17	0.12	0.14	0.08	0.15	0.12	0.10
Stearic acid (C18:0)	0.25	0.14	0.20	0.23	0.21	0.23	0.13	0.31	0.24	0.14	0.18	0.09	0.18	0.15	0.13
Oleic acid (C18:1n9)	0.09	0.06	0.08	0.11	0.08	0.09	0.05	0.16	0.10	0.05	0.07	0.04	0.07	0.07	0.06
Linoleic acid (C18:2n6)	-	0.04	0.06	0.15	0.01	-	0.01	0.25	0.07	0.01	0.02	0.03	0.02	0.10	0.09
Pyruvic acid	0.55	0.22	0.37	0.72	0.23	0.24	0.24	0.32	0.46	0.20	0.22	0.15	0.25	0.22	0.20
Glycerol	0.49	0.17	0.40	0.19	0.31	0.10	0.16	0.30	0.23	-	-	0.06	-	-	-
Threitol	0.25	0.20	0.24	0.22	0.18	0.20	0.24	0.27	0.31	0.17	0.16	0.15	0.12	0.11	0.09
Ribose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ribitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fructose	0.58	0.25	0.15	0.35	-	0.13	-	0.87	-	-	-	-	-	-	-
Glucose	1.58	1.54	1.60	3.22	0.40	0.41	0.41	4.12	0.47	0.04	0.63	0.04	0.07	0.05	0.05
***chiro*-Inositol**	1.01	0.30	0.30	1.56	0.45	0.51	0.44	1.38	0.44	1.49	1.55	2.05	1.02	0.87	1.79
***scyllo*-Inositol**	0.86	0.28	0.17	7.75	0.70	0.84	0.87	2.56	0.24	6.51	9.11	12.46	5.43	4.68	13.16
***myo*-Inositol**	1.55	0.82	0.38	3.81	0.64	0.65	0.83	2.39	0.48	13.13	22.01	22.72	11.38	10.18	30.60
Sucrose	0.42	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	14.33	8.94	13.00	11.82	7.96	9.67	23.46	19.25	14.49	41.72	39.14	36.97	36.58	31.49	53.56
Maltose	-	-	-	-	-	-	-	0.35	1.65	-	-	-	-	-	-

Table A.5. Fat body concentrations (nmol/mg fresh mass) of metabolites, as determined by GC/LC-MS and GC-FID. The concentrations were determined from three biological replicates (numbered) for each treatment group of fifth instar male *Gryllus veletis* nymphs maintained under control or acclimation conditions for zero, three, or six weeks. '-' indicates not detected.

	0 weeks control			3 weeks control			6 weeks control			3 weeks acclimated			6 weeks acclimated		
Metabolite	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Arginine	2.53	2.59	4.07	3.48	5.34	1.96	1.75	1.10	2.17	2.45	3.89	4.16	6.70	5.07	5.47
Glutamine	0.62	0.41	0.77	0.86	0.95	0.55	1.21	0.47	0.72	1.11	1.74	1.97	2.37	2.10	1.79
Serine	0.48	0.55	1.06	0.55	0.48	0.24	0.50	0.25	0.33	0.86	0.77	0.24	0.81	0.55	0.61
Asparagine	0.15	0.14	0.23	0.15	0.15	0.11	0.14	0.08	0.12	0.14	0.24	0.25	0.37	0.33	0.34
Threonine	0.15	0.13	0.18	0.22	0.25	0.12	0.20	0.15	0.17	0.16	0.30	0.31	0.50	0.45	0.32
Alanine	0.41	0.26	0.40	0.33	0.42	0.36	0.42	0.26	0.36	0.38	0.47	0.36	1.27	0.98	1.17
Putrescine	0.01	0.02	0.02	0.03	0.04	0.03	0.03	0.01	0.02	0.04	0.05	0.04	0.04	0.05	0.05
Aspartic acid	0.34	0.21	0.21	0.29	0.17	0.10	0.09	0.09	0.12	0.13	0.14	0.13	0.33	0.21	0.31
Ornithine	0.02	0.03	0.03	0.02	0.04	0.02	0.02	0.01	0.02	0.03	0.03	0.04	0.04	0.03	0.05
Proline	1.62	2.14	2.92	4.96	4.52	2.48	3.29	1.72	3.20	7.25	9.14	13.51	9.90	7.13	16.26
Glutamic acid	0.75	1.23	1.80	2.73	2.01	1.18	1.69	0.84	1.42	1.94	2.94	3.96	3.19	2.54	2.89
Methionine	0.06	0.09	0.11	0.05	0.11	0.07	0.06	0.06	0.07	0.10	0.19	0.12	0.27	0.27	0.18
Histidine	0.16	0.13	0.09	0.17	-	0.12	0.11	0.06	0.20	0.08	0.21	0.51	0.49	0.49	0.50
Glutathione (GSH)	0.82	1.38	1.96	3.96	1.89	2.36	1.13	1.20	1.20	1.24	1.59	2.27	1.85	1.54	2.08
Cysteine	0.04	0.05	0.05	0.07	0.06	0.06	0.04	0.03	0.05	0.05	0.05	0.08	0.07	0.05	0.07
Cystathionine (CTH)	0.03	0.03	0.02	0.02	0.01	0.03	-	0.04	-	-	0.01	0.01	0.02	0.01	0.02
Tryptophan	0.21	0.52	0.73	0.56	1.05	0.50	0.82	0.53	0.78	0.44	0.74	0.85	0.82	0.66	0.68
Phenylalanine	0.20	0.55	0.62	0.25	0.87	0.22	0.55	0.22	0.53	0.51	1.57	0.45	1.10	1.45	1.39
Tyrosine	1.47	3.40	4.16	1.23	4.19	1.42	3.67	1.29	3.69	2.02	4.55	2.92	4.88	4.33	3.51
Lysine	0.31	0.55	0.75	1.02	1.37	0.78	0.43	0.27	0.73	0.90	1.39	2.49	2.84	2.30	2.38
Fumaric acid	0.07	0.03	0.05	0.06	0.05	0.05	0.03	0.06	0.02	0.02	0.03	0.02	0.06	0.03	0.04
Succinic acid	0.08	0.07	0.08	0.11	0.09	0.07	0.07	0.07	0.04	0.03	0.06	0.05	0.05	0.05	0.04
Lactic acid	0.85	1.07	0.55	1.06	0.96	0.49	0.24	0.38	0.34	1.16	0.61	0.44	1.00	0.65	0.63
Maleic acid	0.08	0.07	0.07	0.07	0.05	0.06	0.03	0.04	0.03	0.06	0.06	0.06	0.07	0.04	0.05
Glycine	0.60	0.70	0.88	1.49	1.05	0.90	1.14	0.76	0.81	1.44	1.09	1.43	1.31	1.06	0.96
Aminobutyric acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A.5. continued

	0 weeks control			3 weeks control			6 weeks control			3 weeks acclimated			6 weeks acclimated		
Metabolite	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Valine	0.21	0.22	0.34	0.53	0.60	0.27	0.63	0.27	0.36	0.73	1.09	0.91	4.08	1.61	1.72
beta-Alanine	-	-	-	0.03	0.02	0.03	0.02	0.03	0.01	-	-	-	-	-	0.03
Ketoglutaric acid	0.17	0.20	0.21	0.25	0.20	0.17	0.18	0.12	0.15	0.20	0.20	0.27	0.15	0.16	0.21
Leucine	0.19	0.24	0.38	0.32	0.68	0.23	0.52	0.18	0.47	0.71	1.54	1.19	2.46	2.40	2.05
Isoleucine	0.14	0.15	0.23	0.23	0.45	0.16	0.35	0.15	0.28	0.44	0.97	0.67	1.57	1.79	1.46
Malic acid	0.17	0.12	0.13	0.14	0.11	0.11	0.06	0.13	0.06	0.08	0.09	0.09	0.12	0.08	0.11
Aconitic acid	0.10	0.09	0.08	0.13	0.12	0.07	0.11	0.08	0.08	0.06	0.05	0.03	-	0.10	0.16
Myristic acid (C14:0)	0.53	0.33	0.27	0.20	0.23	0.16	0.06	0.11	0.11	0.28	0.19	0.18	0.16	0.19	0.18
Citric acid	0.23	0.26	0.39	0.25	0.50	0.23	0.51	0.29	0.42	0.40	0.37	0.33	0.76	0.42	1.02
Palmitic acid (C16:0)	0.35	0.24	0.22	0.24	0.18	0.21	0.09	0.21	0.10	0.31	0.14	0.12	0.25	0.11	0.19
Stearic acid (C18:0)	0.45	0.33	0.32	0.29	0.23	0.31	0.13	0.28	0.14	0.39	0.19	0.19	0.31	0.14	0.29
Oleic acid (C18:1n9)	0.18	0.14	0.11	0.12	0.09	0.11	0.05	0.16	0.06	0.16	0.08	0.08	0.14	0.06	0.12
Linoleic acid (C18:2n6)	0.16	0.16	0.05	0.07	0.03	0.03	0.03	0.34	0.04	0.09	0.07	0.05	0.03	0.03	0.04
Pyruvic acid	0.45	0.43	0.38	0.36	0.34	0.34	0.20	0.24	0.23	0.36	0.41	0.34	0.34	0.36	0.33
Glycerol	0.68	0.91	0.98	1.49	0.16	0.99	0.56	0.72	0.54	0.43	0.65	0.76	0.66	0.67	0.80
Threitol	0.29	0.24	0.21	0.19	0.12	0.20	0.12	0.10	0.10	0.22	0.24	0.26	0.32	0.20	0.23
Ribose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ribitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fructose	-	-	-	-	0.18	-	-	0.43	-	-	-	-	-	-	-
Glucose	0.58	0.30	0.54	0.42	1.72	0.23	0.35	2.28	0.25	-	-	-	0.42	-	0.12
***chiro*-Inositol**	0.15	0.13	0.13	0.83	0.30	0.40	0.20	0.30	0.15	0.48	0.54	0.80	0.61	0.41	1.20
***scyllo*-Inositol**	-	-	-	4.00	0.26	0.85	0.35	0.31	0.08	1.59	2.52	4.21	2.69	2.11	7.93
***myo*-Inositol**	-	-	-	3.88	0.43	1.31	0.41	0.46	0.17	3.72	6.20	8.25	6.90	6.35	19.10
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	1.11	-	-
Trehalose	1.80	3.00	3.76	5.53	6.63	4.73	5.47	4.03	5.63	12.35	16.02	15.50	18.84	15.85	40.73
Maltose	0.71	0.76	1.33	1.09	3.24	0.46	0.83	-	-	-	-	-	0.67	-	-

Table A.6. Generalized linear models testing the effect of elevated cryoprotectant concentrations on survival of freeze-tolerant (FT) and freeze-intolerant (FI) crickets and their fat body cells. Separate models were run for each group (FI, FT-LLT, and FT-Lt) within each sample (whole crickets, and fat body frozen *ex vivo*). LLT, lower lethal temperature treatment; Lt, lethal time treatment; PEG, polyethylene glycol. Significant effect of cryoprotectant on survival denoted by bold *P*-values.

Sample	FI		FT-LLT		FT-Lt	
Cryoprotectant	χ^2	*P*	χ^2	*P*	χ^2	*P*
Whole crickets						
myo-Inositol	0.000	1.000	0.847	0.397	0.007	0.994
Proline	0.000	1.000	0.899	0.369	2.558	**0.011**
Trehalose	0.000	1.000	3.162	**0.002**	1.922	**0.045**
Combination[a]	0.000	1.000	1.913	**0.045**	2.516	**0.013**
Glycerol	0.002	0.998	3.237	**0.001**	2.391	**0.017**
PEG	0.000	1.000	0.011	0.991	0.007	0.994
Glucose	0.000	1.000	0.305	0.761	0.008	0.994
Fat body frozen *ex vivo*[b]						
myo-Inositol	0.788	0.431	2.170	**0.030**	1.921	**0.045**
Proline	0.652	0.515	0.268	0.789	0.376	0.707
Trehalose	0.878	0.379	0.635	0.526	1.981	**0.048**
Combination[a]	1.290	0.197	2.611	**0.009**	2.329	**0.020**
Glycerol	3.517	**<0.001**	2.611	**0.009**	0.872	0.383
PEG	0.901	0.368	0.322	0.747	0.856	0.392
Glucose	0.299	0.765	0.215	0.829	0.588	0.556

[a]'Combination' is a combination of *myo*-inositol, proline, and trehalose.
[b]Cryoprotectants were present at 'high' concentrations (300 mM each, except 3 mM PEG)

A.4 Supplementary Figures

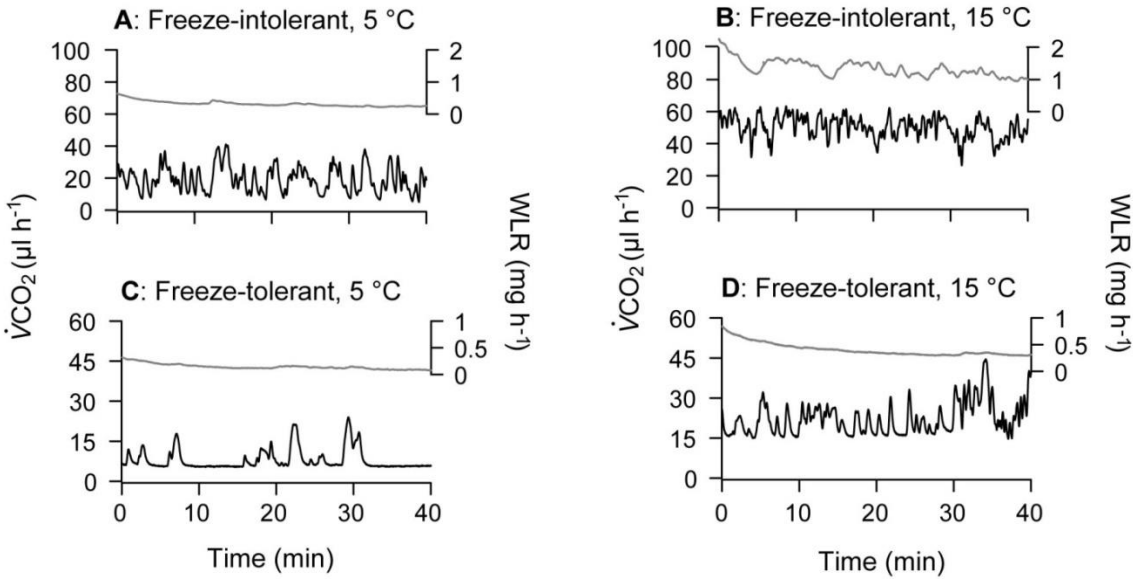

Figure A.1. Example respirometry traces from *Gryllus veletis*. Rates of CO_2 emission ($\dot{V}CO_2$; black) and water loss (WLR; grey) for representative freeze-intolerant **(A, B)** and freeze-tolerant **(C, D)** crickets exposed to 5 °C or 15 °C for 40 min. Activity (not shown) was recorded to determine which portions of a run should be excluded due to excessive animal movement.

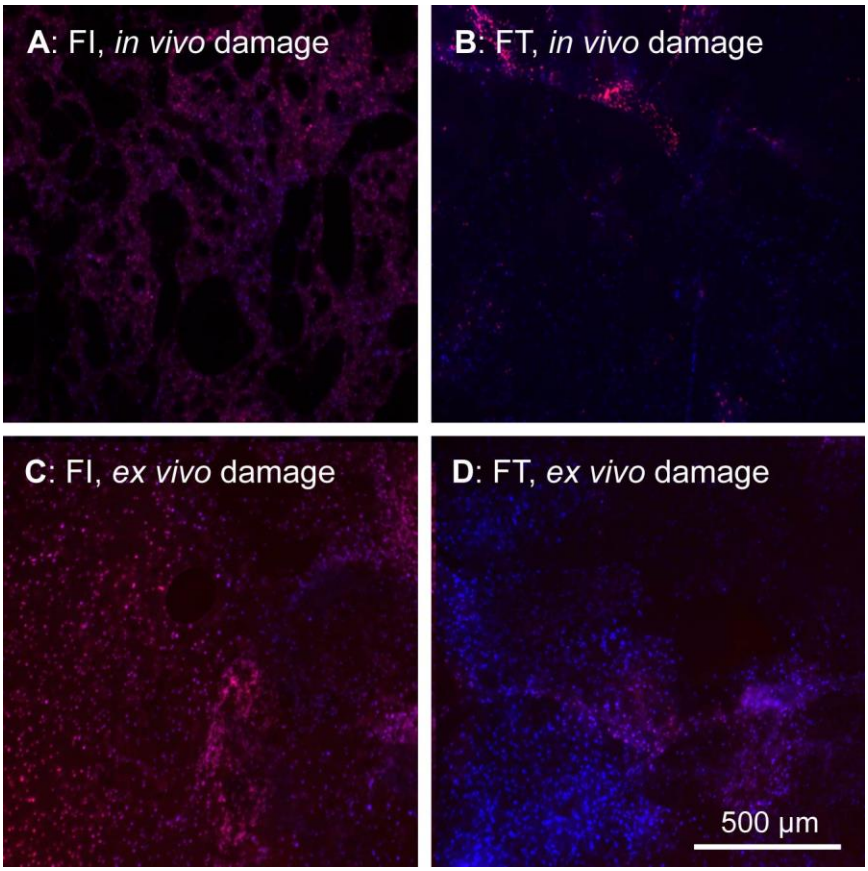

Figure A.2. Representative micrographs used to determine *Gryllus veletis* fat body cell (FBC) survival. (A) Freeze-intolerant (FI) and **(B)** freeze-tolerant (FT) *G. veletis* were frozen at -8 °C for 1.5 h, after which *in vivo* FBC damage was determined by live-dead staining. Fat body cells were dissected from **(C)** FI and **(D)** FT *G. veletis*, frozen *ex vivo* at -8 °C for 10 min, and then stained. Nuclei was stained with DAPI (blue, permeates all cells) and propidium iodide (red, permeates dead cells). Areas that appear purple/pink indicate cells with compromised plasma membranes. Scale is the same in all panels.

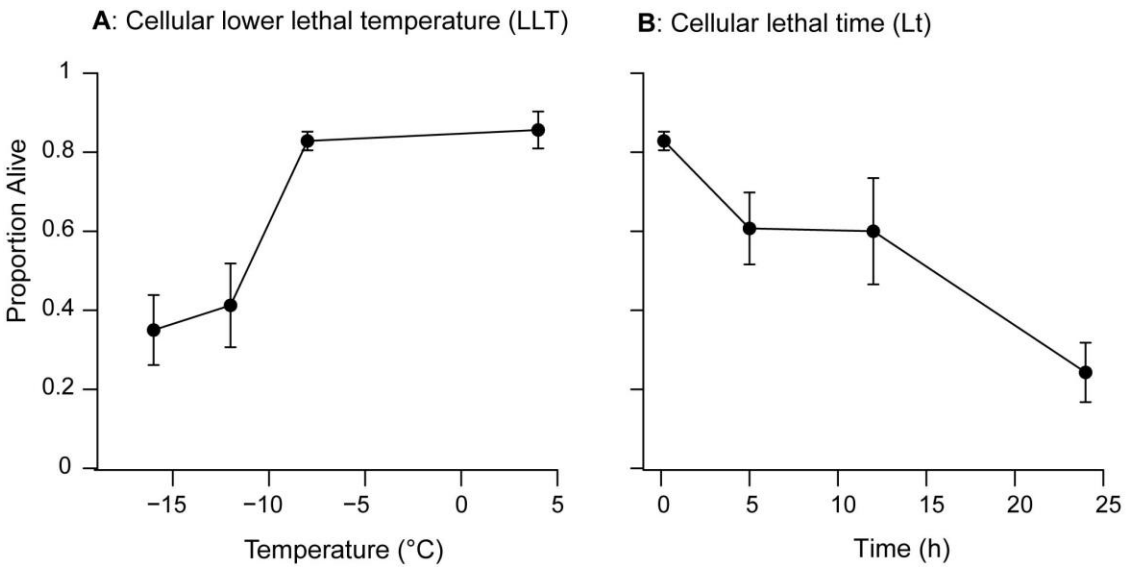

Figure A.3. Lethal limits of freeze-tolerant fat body cells (FBCs) frozen *ex vivo*. (A) Proportion of FBCs from freeze-tolerant (FT) crickets that survived 10 min *ex vivo* exposures to temperatures ranging from 4 °C to -16 °C. **(B)** Proportion of FBCs from FT crickets that survived following *ex vivo* exposure to -8 °C for times ranging from 10 min to 24 h. Each point represents the proportion of eight fat body samples ± SE. Low temperatures reduced cell survival (Wald χ^2 = 2.053, P = 0.04; LLT_{80} = -20.1 °C). Prolonged exposure to -8 °C reduced cell survival (Wald χ^2 = 1.630, P = 0.10; Lt_{80} = 32 h).

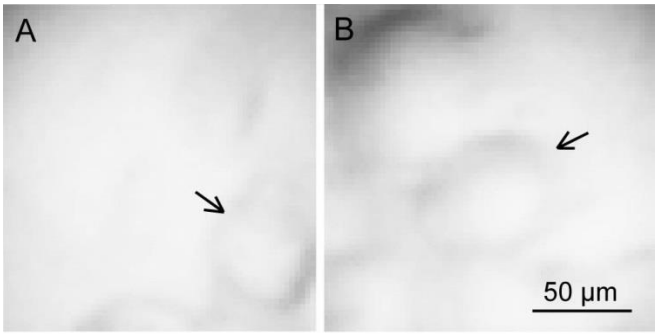

Figure A.4. Ice crystal morphology of *Gryllus veletis* hemolymph is unaffected by acclimation. Hemolymph samples (diluted 1:4 in anticoagulant, 3 % ascorbic acid) from **(A)** freeze-intolerant and **(B)** freeze-tolerant juvenile male *G. veletis* were frozen, and ice crystal shape was observed at the melting point in a nanolitre osmometer. Arrows indicate round ice crystals. No angular/specular ice crystals were observed.

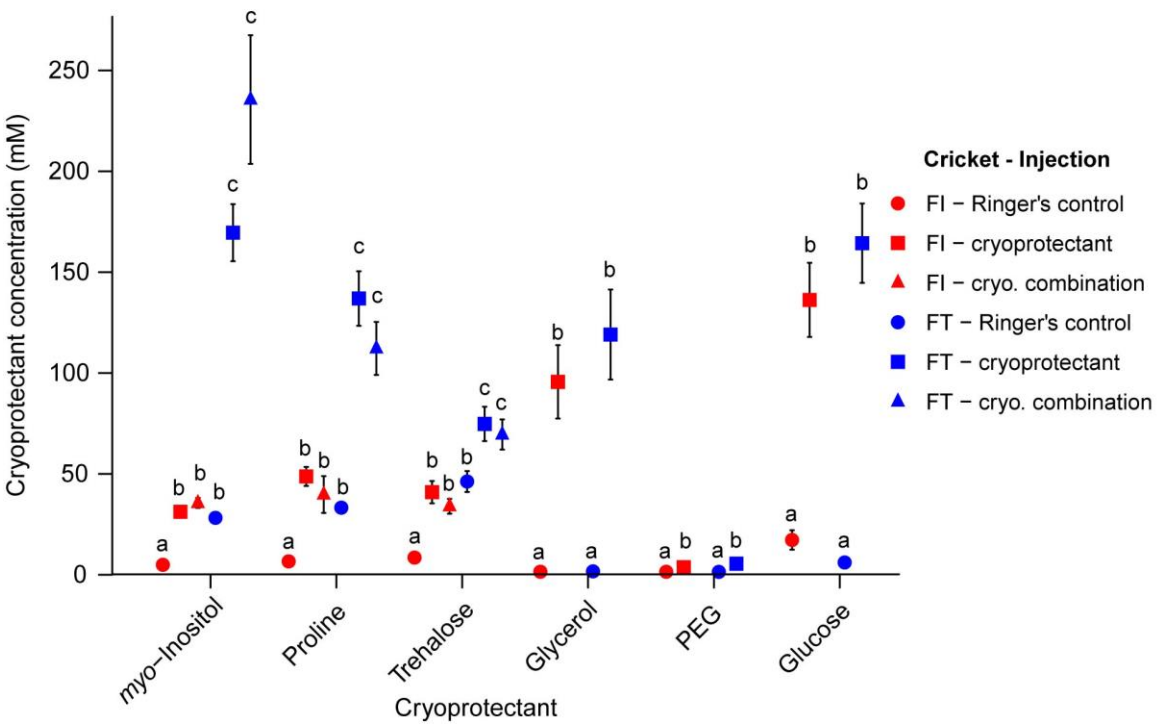

Figure A.5. Injection elevates hemolymph cryoprotectant concentrations. Hemolymph concentration of six cryoprotectants (determined by spectrophotometric assays) in freeze-intolerant (FI; red) or freeze-tolerant (FT; blue) crickets 15-30 min after they were injected with a Ringer's solution (control, ●), the cryoprotectant of interest (■), or a cryoprotectant combination (▲) of *myo*-inositol, proline, and trehalose. Each point represents the mean ± SE cryoprotectant concentration of 24 crickets. Small error bars are obscured by symbols. Different letters indicate significantly different concentrations of that cryoprotectant among treatments (ANOVA, Tukey's *post-hoc* test $P < 0.05$). Injection of cryoprotectants or combinations of cryoprotectants elevated hemolymph concentrations above that of control-injected crickets. Injection of *myo*-inositol, proline, trehalose or a combination of all three elevated these cryoprotectant levels in FI crickets to concentrations similar to control-injected FT crickets.

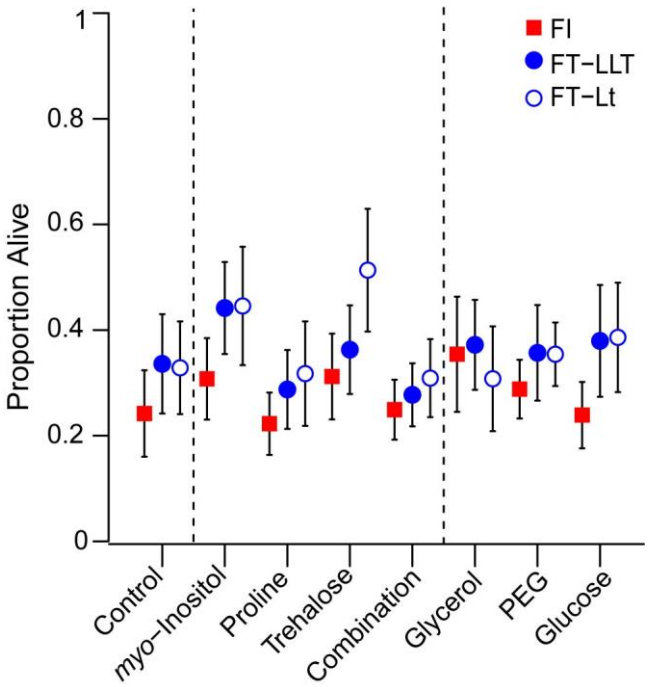

Figure A.6. Survival of fat body cells (FBCs) frozen *ex vivo* in physiologically-relevant concentrations of exogenous cryoprotectants. The proportion of live FBCs following dissection from freeze-intolerant (FI) and freeze-tolerant (FT) *Gryllus veletis* and freezing *ex vivo* in Grace's Insect Medium (control) or the indicated cryoprotectant in Grace's Insect Medium. Fat body from FI crickets was frozen to -8 °C for 10 min (FI), and fat body from FT crickets was frozen to the cellular LLT (FT-LLT) or cellular Lt (FT-Lt). Cryoprotectant concentrations were similar to those measured in FT cricket hemolymph (30 mM *myo*-inositol, 30 mM proline, 70 mM trehalose). For cryoprotectants not found in FT *G. veletis*, cryoprotectant concentrations were chosen to match analogous metabolites (30 mM glycerol and 0.3 mM PEG – similar to *myo*-inositol; 140 mM glucose – same number of glucose moieties as 70 mM trehalose). Each point represents the surviving proportion of FBCs from 24 crickets ± SE. No cryoprotectant increased cell survival (generalized linear models, all $P > 0.05$).

Appendix B: Chapter 3 supplementary material

B.1 Supplementary Tables

Table B.1. Summary of 16 RNA-Seq libraries, before and after trimming (removing sequences with fewer than 15 base pairs; bp).

Treatment group	Biological Replicate	Number of 125 bp paired-end reads	
		Pre-trimming	Post-trimming
Zero week control	1	39,570,233	39,452,800
	2	34,282,107	33,808,140
	3	59,964,440	59,624,943
Three weeks control	1	37,846,895	37,605,041
	2	40,078,110	39,897,840
	3	41,407,387	41,178,176
Six weeks control	1	44,473,989	43,614,069
	2	37,141,958	37,042,872
	3	47,953,663	47,353,430
Three weeks acclimation	1	40,061,666	39,760,627
	2	43,678,045	42,745,386
	3	35,947,266	35,392,548
Six weeks acclimation	1	44,812,452	44,689,304
	2	42,574,575	41,894,550
	3	37,442,754	37,086,872
Aggregate[a]	1	45,412,267	45,302,821
Total		**672,647,807**	**666,449,419**
Mean		**42,040,488**	**41,653,089**

[a]Includes RNA from *G. veletis* at various developmental stages: whole male and female adult crickets, first through fifth instar nymphs, fifth instar nymphs that had undergone chilling (0 °C for 1, 4, and 24 h), freezing (-8 °C for 1.5 h), thawing, dehydration (incubation at room temperature with silica gel for 1, 4, and 24 h), and an immune challenge (injection with heat-killed bacteria, recovery for 1, 6, and 24 h).

Table B.2. Selected genes of interest that were not differentially expressed in *Gryllus veletis* fat body during acclimation.

Function	Description	Contig ID
Potential cryoprotectant synthesis enzymes		
Inositol synthesis	Inositol-P synthase	Gvel_56451_c5_g2
Proline synthesis	P5C synthase	Gvel_66589_c0_g1
	P5C reductase	Gvel_69512_c0_g1
Trehalose synthesis	Bifunctional T6P synthase/phosphatase	Gvel_54376_c0_g1
Glycogen breakdown	Glycogen phosphorylase	Gvel_23709_c0_g1
	Glycogen phosphorylase	Gvel_38846_c0_g1
	Glycogen phosphorylase	Gvel_50699_c0_g1
Potential cryoprotectant transporters		
Proline transport	Sodium-dependent neutral amino acid transporter B(0)AT2	Gvel_26522_c1_g1
	Proton-coupled amino acid transporter 4	Gvel_42160_c0_g1
	Proton-coupled amino acid transporter 4	Gvel_73282_c0_g1
myo-Inositol transport	GPI inositol-deacylase	Gvel_10568_c0_g1
General amino acid transport		
	Putative sodium-coupled neutral amino acid transporter 7	Gvel_83253_c2_g1
	Sodium-coupled neutral amino acid transporter 9 homolog	Gvel_69575_c0_g1
	Sodium-dependent neutral amino acid transporter B(0)AT1	Gvel_74286_c0_g1
	Sodium-dependent neutral amino acid transporter B(0)AT3	Gvel_17082_c0_g1
	Putative sodium-coupled neutral amino acid transporter 11	Gvel_78773_c1_g1
	Proton-coupled amino acid transporter 4	Gvel_17696_c0_g1
	Proton-coupled amino acid transporter 4	Gvel_18085_c0_g1
	Proton-coupled amino acid transporter 4	Gvel_25506_c0_g1
	Proton-coupled amino acid transporter 4	Gvel_25679_c0_g1
	Proton-coupled amino acid transporter 4	Gvel_26291_c0_g1
	Proton-coupled amino acid transporter 4	Gvel_56726_c0_g1
	Amino acid transporter ANTL1	Gvel_19506_c0_g1
	b(0,+)-type amino acid transporter 1	Gvel_5247_c0_g1
	b(0,+)-type amino acid transporter 1	Gvel_42656_c0_g1
	b(0,+)-type amino acid transporter 1	Gvel_41737_c0_g1
	High affinity cationic amino acid transporter 1	Gvel_13471_c0_g1
	High affinity cationic amino acid transporter 1	Gvel_32998_c0_g1
	High affinity cationic amino acid transporter 1	Gvel_72649_c0_g1
	High affinity cationic amino acid transporter 1	Gvel_73575_c0_g1
	High affinity cationic amino acid transporter 1	Gvel_26563_c0_g1
	Cationic amino acid transporter 2	Gvel_5461_c0_g1
	Cationic amino acid transporter 2	Gvel_29535_c0_g1
	Cationic amino acid transporter 2	Gvel_73575_c0_g1
	Cationic amino acid transporter 3	Gvel_6717_c0_g2
	Cationic amino acid transporter 3	Gvel_58226_c0_g1
	Cationic amino acid transporter 4	Gvel_60049_c0_g1

Table B.2 continued

Function	Description	Contig ID
	Excitatory amino acid transporter 3	Gvel_28987_c0_g2
	Sodium-dependent nutrient amino acid transporter 1	Gvel_66850_c0_g1
	Sodium-dependent nutrient amino acid transporter 1	Gvel_60491_c0_g1
Aquaporins		
Water transport	Aquaporin AQPcic	Gvel_37176_c0_g1
	Aquaporin AQPcic	Gvel_46914_c0_g1
	Aquaporin AQPAe	Gvel_51308_c1_g1
	Aquaporin-12	Gvel_56044_c0_g1
Molecular Chaperones		
Heat shock proteins	10 kDa heat shock protein, mitochondrial	Gvel_78446_c0_g1_i1
	60 kDa heat shock protein, mitochondrial	Gvel_27658_c0_g1_i1
	60 kDa heat shock protein, mitochondrial	Gvel_56040_c0_g1_i1
	Heat shock protein 67B2	Gvel_79017_c0_g1_i1
	Heat shock protein HSP 90-alpha A2	Gvel_28035_c0_g1_i1
	Heat shock protein HSP 90-alpha	Gvel_68127_c0_g1_i1
	Heat shock protein Hsp-16.2	Gvel_37784_c0_g2_i1
	Heat shock protein Hsp-16.2	Gvel_37784_c0_g1_i1
	Heat shock protein 70	Gvel_78482_c1_g1_i1
	Heat shock 70 kDa protein	Gvel_78482_c2_g1_i1
	Heat shock cognate 70 kDa protein	Gvel_9203_c0_g1_i1
	Heat shock 70 kDa protein	Gvel_57949_c0_g3_i1
	Major heat shock 70 kDa protein Ab	Gvel_16103_c0_g1_i1
	Heat shock 70 kDa protein 1	Gvel_18794_c0_g1_i1
	Heat shock protein 70 B2	Gvel_61233_c0_g1_i1
	Heat shock protein 70 B2	Gvel_83898_c0_g1_i1
	Heat shock 70 kDa protein 4	Gvel_87722_c0_g1_i1
	Heat shock 70 kDa protein 4	Gvel_87722_c0_g1_i2
	Heat shock 70 kDa protein A	Gvel_57949_c0_g1_i1
	Heat shock 70 kDa protein A	Gvel_57949_c0_g2_i1
	Heat shock 70 kDa protein cognate 1	Gvel_60996_c0_g2_i1
	Heat shock 70 kDa protein cognate 2	Gvel_78482_c0_g1_i1
	Heat shock 70 kDa protein cognate 2	Gvel_19946_c0_g1_i1
	Heat shock cognate 71 kDa protein	Gvel_12474_c0_g1_i1
	Heat shock 70 kDa protein cognate 3	Gvel_60796_c0_g1_i1
	Heat shock 70 kDa protein cognate 3	Gvel_78480_c0_g1_i2
	Heat shock 70 kDa protein cognate 3	Gvel_78480_c0_g1_i1
	Heat shock 70 kDa protein cognate 3	Gvel_78770_c0_g1_i1
	Heat shock cognate 71 kDa protein	Gvel_22440_c0_g1_i1
	Heat shock 70 kDa protein cognate 4	Gvel_34771_c0_g1_i1
	Heat shock 70 kDa protein cognate 4	Gvel_31684_c0_g1_i1
	Heat shock 70 kDa protein 14	Gvel_57508_c0_g1_i1

Table B.2 continued

Function	Description	Contig ID
	Heat shock 70 kDa protein cognate 5	Gvel_51846_c0_g1_i1
	Heat shock 70 kDa protein cognate 5	Gvel_78850_c0_g1_i1
	Heat shock protein 83	Gvel_18505_c0_g1_i1
	Heat shock protein 83	Gvel_76432_c0_g1_i1
	Heat shock protein 83	Gvel_26469_c4_g9_i1
	Heat shock protein 90	Gvel_6014_c0_g1_i1
	Heat shock protein 75 kDa, mitochondrial	Gvel_87650_c0_g1_i1

Table B.3. Selected genes upregulated in *Gryllus veletis* fat body during acclimation, in addition to those discussed in the text.

Function	Description	Contig ID	Pattern
Putative ice binding proteins[a]			
C-type lectin domain protein	Hemolymph lipopolysaccharide-binding protein	Gvel_14378_c0_g1_i1	E
	Hemolymph lipopolysaccharide-binding protein	Gvel_73626_c0_g1_i2	E
	Hemolymph lipopolysaccharide-binding protein	Gvel_15098_c0_g1_i1	H
Putative transcription factors[b]			
General	ATP-dependent RNA helicase DHX36	Gvel_85658_c0_g2_i1	B
	CCHC-type zinc finger protein CG3800	Gvel_73663_c0_g1_i1	C
	CCHC-type zinc finger protein CG3800	Gvel_48092_c0_g1_i1	D
	Chorion transcription factor Cf2	Gvel_30148_c2_g1_i1	G
	Chromodomain-helicase-DNA-binding protein 1	Gvel_62761_c0_g1_i1	D
	CREB/ATF bZIP transcription factor	Gvel_22640_c0_g1_i1	C
	CXXC-type zinc finger protein 1	Gvel_82026_c0_g2_i1	G
	DNA-binding protein D-ETS-4	Gvel_16617_c0_g1_i1	H
	DNA-binding protein Ets97D	Gvel_35132_c0_g1_i1	D
	Gastrula zinc finger protein XlCGF26.1	Gvel_8013_c0_g2_i1	B
	Gastrula zinc finger protein XlCGF57.1	Gvel_44855_c2_g2_i1	G
	Gastrula zinc finger protein XlCGF57.1	Gvel_30226_c5_g4_i1	A
	General transcription factor IIE subunit 1	Gvel_62557_c0_g2_i1	D
	General transcription factor IIH subunit 1	Gvel_63875_c0_g1_i2	D
	General transcription factor IIH subunit 1	Gvel_63875_c0_g1_i3	E
	General transcription factor IIH subunit 4	Gvel_41185_c0_g1_i1	C
	Mediator of RNA polymerase II transcription subunit 13	Gvel_39856_c1_g1_i1	H
	Mediator of RNA polymerase II transcription s subunit 26	Gvel_43951_c0_g2_i1	C
	Mushroom body large-type Kenyon cell-specific protein 1	Gvel_55728_c0_g1_i1	E
	NFX1-type zinc finger-containing protein 1	Gvel_51372_c0_g1_i2	A
	Nucleolar transcription factor 1	Gvel_19437_c0_g1_i2	B
	RB-associated KRAB zinc finger protein	Gvel_53258_c0_g2_i1	B
	SAM pointed domain-containing Ets transcription factor	Gvel_22687_c0_g1_i2	H
	Transcription factor GATA-4	Gvel_33512_c0_g1_i1	H
	Transcription factor SOX-5	Gvel_70660_c0_g1_i1	H
	WD repeat-containing protein 43	Gvel_17203_c0_g1_i1	D
	Zinc finger MYND domain-containing protein 11	Gvel_78950_c0_g2_i1	C
	Zinc finger protein 26	Gvel_67773_c0_g1_i1	G
	Zinc finger protein 79	Gvel_78746_c1_g3_i1	A
	Zinc finger protein 84	Gvel_70614_c0_g1_i1	A
	Zinc finger protein 84	Gvel_50214_c1_g1_i1	B
	Zinc finger protein 84	Gvel_30148_c1_g1_i1	G
	Zinc finger protein 182	Gvel_44834_c8_g7_i1	A
	Zinc finger protein 271	Gvel_80211_c1_g1_i1	A

Table B.3 continued

Function	Description	Contig ID	Pattern
	Zinc finger protein 330 homolog	Gvel_79200_c1_g1_i2	B
	Zinc finger protein 391	Gvel_70172_c3_g6_i1	D
	Zinc finger protein 425	Gvel_23073_c0_g1_i1	H
	Zinc finger protein 436	Gvel_67274_c0_g1_i2	A
	Zinc finger protein 436	Gvel_4482_c0_g1_i2	D
	Zinc finger protein 583	Gvel_30226_c5_g12_i2	A
	Zinc finger protein 652	Gvel_64997_c0_g1_i1	A
	Zinc finger protein 658	Gvel_47218_c0_g1_i1	A
	Zinc finger protein 706	Gvel_88016_c0_g1_i1	C
	Zinc finger protein basonuclin-2	Gvel_78940_c0_g1_i1	H
	Zinc finger protein jing homolog	Gvel_41632_c0_g1_i1	E
	Zinc finger protein ush	Gvel_24964_c0_g1_i1	H
	Zinc finger protein Xfin	Gvel_44802_c0_g1_i1	B
Response to Stress	Forkhead box protein L2	Gvel_41282_c0_g1_i2	H
	Metal regulatory transcription factor 1	Gvel_51888_c0_g1_i1	D
	Homeodomain-interacting protein kinase 2	Gvel_46976_c1_g1_i1	B
	Homeodomain-interacting protein kinase 2	Gvel_46976_c1_g1_i2	H
Cell Cycle/Apotosis	LIM domain-containing protein jub	Gvel_63855_c0_g1_i2	H
	Max-binding protein MNT	Gvel_54552_c0_g1_i1	E
	HMG box-containing protein 1	Gvel_80210_c0_g4_i2	A
	Transcription factor kayak	Gvel_55714_c0_g1_i1	G
	Transcription factor kayak	Gvel_55714_c0_g1_i3	H
	Transcriptional repressor CTCF	Gvel_84806_c0_g1_i1	D
	Zinc finger HIT domain-containing protein 1	Gvel_82020_c0_g1_i1	C
	Homeodomain-interacting protein kinase 2	Gvel_46976_c1_g1_i1	B
	Homeodomain-interacting protein kinase 2	Gvel_46976_c1_g1_i2	H
RNA Processing	ATP-dependent RNA helicase DHX8	Gvel_62756_c0_g1_i1	G
	ATP-dependent RNA helicase p62	Gvel_49169_c0_g1_i1	C
	ATP-dependent RNA helicase p62	Gvel_49169_c0_g1_i2	D
	Box C/D snoRNA protein 1	Gvel_63966_c0_g1_i2	D
	LIM and calponin homology domains-containing protein 1	Gvel_82488_c0_g1_i3	H
	Peptidylprolyl isomerase domain and WD repeat-containing protein 1	Gvel_80573_c0_g1_i1	C
	Probable ATP-dependent RNA helicase DDX5	Gvel_82013_c0_g1_i3	C
	Probable ATP-dependent RNA helicase DDX17	Gvel_37175_c0_g1_i2	E
	Probable ATP-dependent RNA helicase DDX46	Gvel_81996_c0_g3_i1	E
	Probable ATP-dependent RNA helicase DHX35	Gvel_32073_c0_g1_i1	E
	WD repeat-containing protein 36	Gvel_63749_c0_g1_i2	D
	WD repeat-containing protein 37	Gvel_62586_c0_g1_i1	E
	Zinc finger CCCH domain-containing protein 13	Gvel_15051_c0_g1_i1	D
	Zinc finger protein 36, C3H1 type-like 1	Gvel_35319_c0_g1_i1	D

Table B.3 continued

Function	Description	Contig ID	Pattern
Ubiquitination	Ankyrin repeat and SOCS box protein 8	Gvel_70174_c8_g9_i1	B
	Ankyrin repeat and SOCS box protein 8	Gvel_70174_c8_g9_i2	C
	CCR4-NOT transcription complex subunit 4	Gvel_35911_c1_g1_i3	A
	F-box only protein 9	Gvel_36722_c0_g1_i2	B
	F-box/LRR-repeat protein 7	Gvel_46226_c0_g1_i1	A
	F-box/WD repeat-containing protein 1A	Gvel_41154_c0_g1_i3	B
	F-box/WD repeat-containing protein 4	Gvel_68222_c0_g2_i1	B
	RING-box protein 1A	Gvel_4127_c0_g1_i1	C
	SPRY domain-containing SOCS box protein 3	Gvel_54345_c0_g1_i4	A
	WD repeat-containing protein 11	Gvel_25027_c0_g1_i3	B
Other	Glutamate-rich WD repeat-containing protein 1	Gvel_84817_c0_g1_i1	C
	Probable ATP-dependent RNA helicase DDX28	Gvel_23096_c0_g1_i1	D
	Protein cycle	Gvel_46912_c0_g1_i2	E
	Signal transducer and activator of transcription 5B	Gvel_23665_c0_g1_i4	C
Putative signalling transduction genes[c]			
General	Homeodomain-interacting protein kinase 2	Gvel_46976_c1_g1_i2	H
	Homeodomain-interacting protein kinase 2	Gvel_46976_c1_g1_i1	B
	Serine/threonine-protein phosphatase 5	Gvel_61181_c0_g1_i1	A
	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	Gvel_48735_c0_g2_i1	E
Metabolism	5'-AMP-activated protein kinase subunit gamma-2	Gvel_61474_c0_g1_i1	E
	Adiponectin receptor protein	Gvel_46799_c0_g1_i1	B
	FGGY carbohydrate kinase domain-containing protein	Gvel_51886_c0_g2_i1	C
	Tyrosine-protein phosphatase non-receptor type 23	Gvel_85672_c0_g1_i2	D
	Scavenger receptor class B member 1	Gvel_70834_c0_g1_i1	B
	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	Gvel_46757_c0_g1_i2	A
	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	Gvel_45193_c0_g1_i1	H
Insulin signalling	3-phosphoinositide-dependent protein kinase 1	Gvel_8541_c0_g1_i1	G
	3-phosphoinositide-dependent protein kinase 1	Gvel_85409_c0_g1_i1	E
Cell cycle/Apoptosis	CDK-activating kinase assembly factor MAT1	Gvel_21437_c0_g1_i1	C
	Dual specificity protein phosphatase 10	Gvel_34643_c0_g1_i1	H
	Death-associated protein kinase 1	Gvel_79256_c0_g1_i1	A
	Kinase D-interacting substrate of 220 kDa	Gvel_44786_c3_g1_i1	E
	Kinase D-interacting substrate of 220 kDa	Gvel_70232_c1_g1_i1	A
	Mitogen-activated protein kinase 14A	Gvel_17318_c0_g1_i2	B
	NUAK family SNF1-like kinase 1	Gvel_37649_c0_g1_i1	B
	Ras GTPase-activating protein 1	Gvel_17341_c0_g1_i2	B
	Transforming growth factor-beta receptor-associated protein 1	Gvel_47003_c0_g1_i1	A
	Serine/threonine-protein phosphatase 1 regulatory subunit 10	Gvel_19509_c1_g1_i2	C
Cytoskeleton	EGFR kinase substrate 8-like protein 2	Gvel_43838_c0_g1_i1	B

Table B.3 continued

Function	Description	Contig ID	Pattern
	Ras GTPase-activating protein 1	Gvel_17341_c0_g1_i2	B
	Rho GTPase-activating protein 100F	Gvel_4601_c0_g1_i1	H
	Serine/threonine-protein kinase OSR1	Gvel_48744_c1_g1_i1	E
	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	Gvel_46757_c0_g1_i2	A
	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	Gvel_45193_c0_g1_i1	H
	Tyrosine-protein kinase Src64B	Gvel_81960_c0_g1_i1	B
Stress response	G-protein coupled receptor Mth	Gvel_35842_c0_g1_i1	H
	G-protein coupled receptor Mth2	Gvel_27887_c0_g1_i1	E
	Stress-activated protein kinase JNK	Gvel_49939_c0_g1_i1	A
	Serine/threonine-protein kinase OSR1	Gvel_48744_c1_g1_i1	E
RNA processing	Serine/threonine-protein kinase Doa	Gvel_62201_c0_g3_i2	H
	Serine/threonine-protein kinase Doa	Gvel_62201_c0_g2_i1	D
	Serine/threonine-protein kinase SMG1	Gvel_19473_c0_g2_i2	C
cAMP signalling	Adenylate cyclase type 5	Gvel_28980_c0_g4_i4	D
	Metabotropic glutamate receptor 3	Gvel_3867_c0_g1_i1	A
	Protein kinase DC2	Gvel_84502_c0_g1_i1	H
Inositol signalling	Inositol 1,4,5-trisphosphate receptor	Gvel_41178_c0_g4_i1	B
	Inositol-trisphosphate 3-kinase A	Gvel_87759_c0_g1_i1	H
	Phosphatidylinositol 5-phosphate 4-kinase type-2 alpha	Gvel_53190_c0_g1_i1	B
	Phosphatidylinositol phosphatase PTPRQ	Gvel_80174_c5_g1_i1	A
	Tyrosine-protein phosphatase non-receptor type 13	Gvel_70163_c1_g1_i1	B
Immune	Inhibitor of nuclear factor kappa-B kinase subunit alpha	Gvel_31546_c0_g1_i1	A
	Interleukin-1 receptor-associated kinase 4	Gvel_62503_c0_g1_i1	C
	Protein toll	Gvel_60975_c0_g1_i1	E
	Serine/threonine-protein kinase RIO3	Gvel_73695_c0_g1_i1	G
	Src kinase-associated phosphoprotein 2-A	Gvel_88116_c0_g1_i1	A
Vesicles	ADP-ribosylation factor GTPase-activating protein 1	Gvel_27359_c0_g1_i1	A
	Low-density lipoprotein receptor-related protein 1B	Gvel_65167_c0_g1_i1	B
	Prolow-density lipoprotein receptor-related protein 1	Gvel_78586_c0_g1_i1	B
Autophagy	Phosphoinositide 3-kinase regulatory subunit 4	Gvel_64764_c0_g1_i1	D
	Rab3 GTPase-activating protein non-catalytic subunit	Gvel_51317_c0_g2_i2	A
Other	Serine/threonine-protein kinase Sgk3	Gvel_84125_c0_g1_i2	B
	Tankyrase	Gvel_41304_c0_g1_i1	E
	UMP-CMP kinase 2, mitochondrial	Gvel_32063_c0_g1_i1	C

[a]Genes with homology to C-type lectins (homologous to fish antifreeze proteins), Pfam domain PF00059. Two putative sialic acid synthase (homologous to fish antifreeze proteins) with Pfam domain PF08666 were not differentially expressed;
[b]Gene names that include: ‘box,’ ‘DNA-binding,’ ‘homeodomain,’ ‘LIM domain,’ ‘RNA’ (excluding polymerases, RNA binding proteins, tRNA), ,‘transcription,’ ‘WD repeat,’ ‘zinc;’
[c]Gene names that include: ‘cyclase,’ ‘G-protein,’ ‘GTPase,’ ‘kinase,’ ‘phosphatase’ (excluding biochemical pathway kinases and phosphatases), ‘receptor’ (excluding organelle level receptors, PPAR signalling),.

Table B.4. Selected genes of interest that were abundant, but not differentially expressed, in *Gryllus veletis* fat body during acclimation. '--NA--' indicates the transcript is unannotated.

Description	Contig ID	Transcript count[a]
Transferrin	Gvel_70215_c1_g7_i1	1,094,891
Transferrin	Gvel_70215_c1_g4_i1	680,004
--NA--	Gvel_37243_c0_g2_i1	657,095
Putative uncharacterized protein ART2	Gvel_53547_c0_g1_i1	642,521
Phosphoenolpyruvate carboxykinase [GTP]	Gvel_47855_c0_g2_i1	641,599
Acyl-CoA Delta(11) desaturase	Gvel_54359_c1_g1_i2	493,723
Carboxypeptidase N subunit 2	Gvel_61067_c0_g1_i1	444,731
--NA--	Gvel_31776_c0_g1_i1	370,799
Elongation factor 2	Gvel_9392_c0_g1_i1	358,856
Cytochrome P450 4C1	Gvel_66551_c6_g2_i1	352,700
--NA--	Gvel_70215_c1_g5_i1	313,522
Heat shock 70 kDa protein cognate 4	Gvel_34771_c0_g1_i1	253,490
Hemolymph lipopolysaccharide-binding protein	Gvel_57983_c0_g1_i1	232,161
3-ketoacyl-CoA thiolase, mitochondrial	Gvel_62183_c0_g2_i1	214,886
Myosin heavy chain, muscle	Gvel_62487_c0_g1_i1	180,653
--NA--	Gvel_32829_c0_g1_i1	161,287
Probable phospholipid hydroperoxide glutathione peroxidase	Gvel_65566_c0_g1_i1	144,136
Probable medium-chain specific acyl-CoA dehydrogenase	Gvel_18079_c0_g1_i1	137,974
--NA--	Gvel_35884_c1_g1_i1	134,723
Delta(24)-sterol reductase	Gvel_84826_c0_g1_i1	133,590
--NA--	Gvel_75329_c0_g1_i1	124,759
--NA--	Gvel_52219_c0_g1_i1	124,207
Clavesin-1	Gvel_51746_c0_g1_i1	123,962
Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	Gvel_82643_c0_g2_i1	123,713
Long-chain-fatty-acid--CoA ligase 5	Gvel_3925_c0_g1_i1	121,244
Bifunctional trehalose-6-phosphate synthase/phosphatase	Gvel_54376_c0_g1_i1	119,317
ATP-binding cassette sub-family G member 1	Gvel_44596_c0_g2_i1	115,246
Very low-density lipoprotein receptor	Gvel_82464_c0_g1_i1	115,013
Glutathione peroxidase	Gvel_12774_c0_g1_i1	113,014
Catalase	Gvel_54149_c0_g1_i1	111,760
40S ribosomal protein S2	Gvel_87148_c0_g1_i1	104,344
Long-chain-fatty-acid--CoA ligase 3	Gvel_57805_c1_g1_i1	101,293
--NA--	Gvel_24150_c0_g1_i1	100,799

[a]Sum of transcript read counts across three biological replicates of acclimated crickets; each biological replicate includes fat body RNA from five freeze-tolerant *G. veletis* (acclimated for six weeks).

B.2 Supplementary Figures

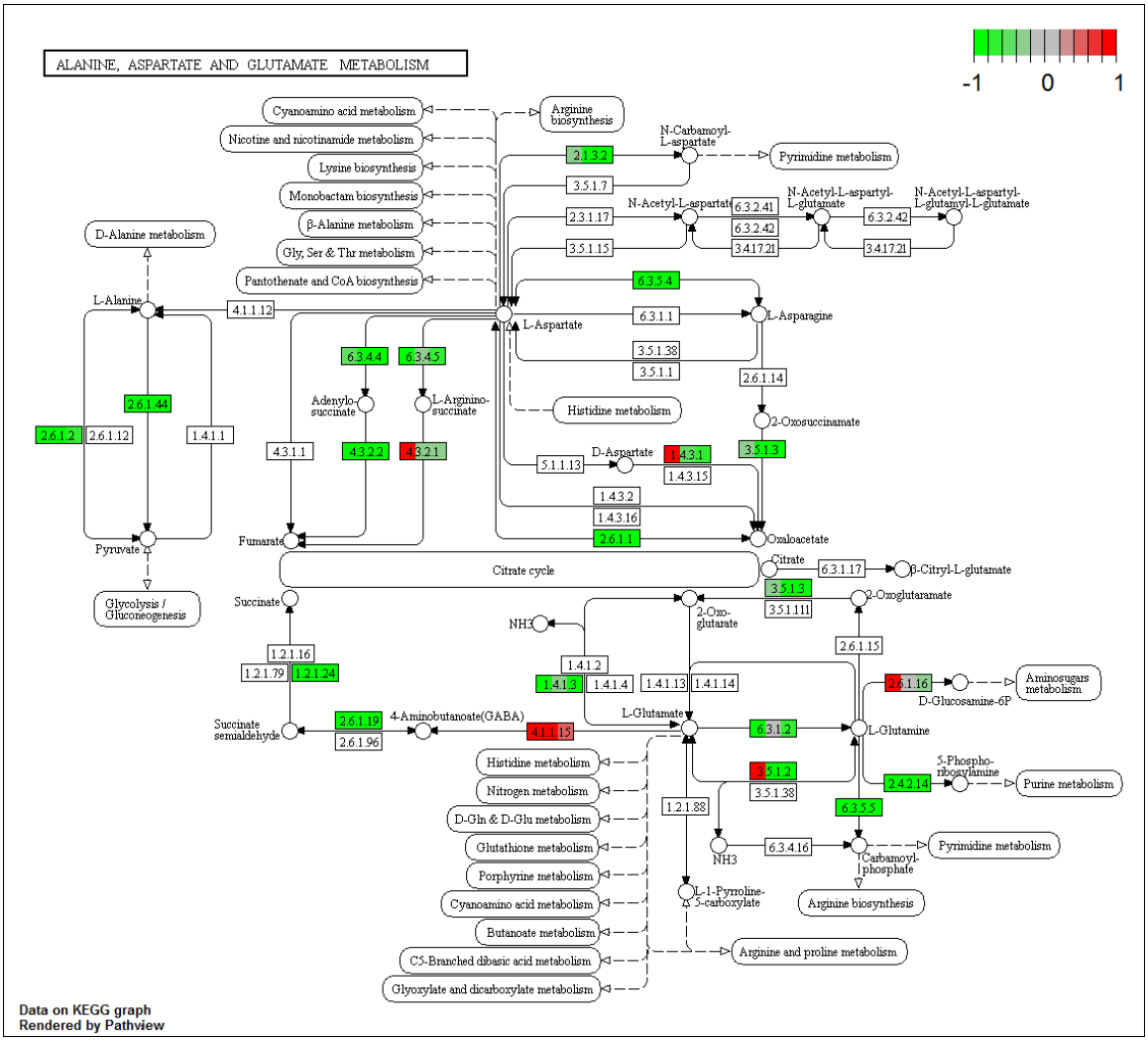

Figure B.1. Differential gene expression of the 'alanine, aspartate and glutamate metabolism' KEGG pathway components in *Gryllus veletis* acclimated for three weeks relative to zero week controls. Each pathway component contains three colour bars indicating the three biological replicates of crickets acclimated for three weeks compared to the mean expression of control crickets (week zero), with red indicating increased expression, and green indicating decreased expression. 2.6.1.1 - aspartate transaminase; 2.6.1.2 - alanine transaminase; 2.6.1.44 - alanine-glyoxylate transaminase; 6.3.5.4 - asparagine synthase. For a complete description of each pathway component, see the KEGG 'alanine, aspartate and glutamate metabolism' reference pathway (http://www.genome.jp/kegg-bin/show_pathway?ko00250).

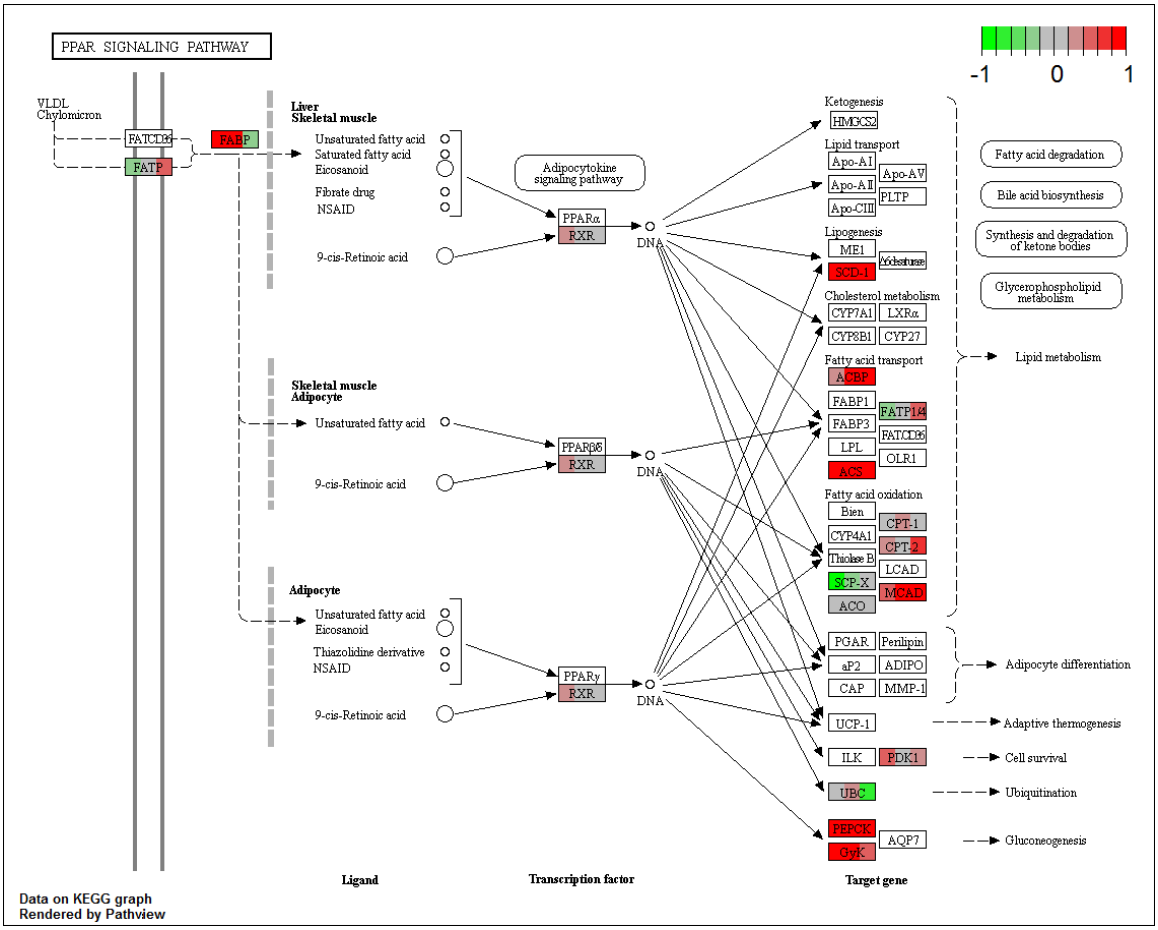

Figure B.2. Differential gene expression of the 'PPAR signaling' KEGG pathway components in *Gryllus veletis* acclimated for six weeks relative to zero week controls. Each pathway component contains three colour bars indicating the three biological replicates of crickets acclimated for six weeks compared to the mean expression of control crickets (week zero), with red indicating increased expression, and green indicating decreased expression. GyK, glycerol kinase; MCAD, medium chain acyl-CoA dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; SCD, stearoyl-CoA desaturase (SCD). For a complete description of each pathway component, see the KEGG 'PPAR signaling pathway' reference pathway (http://www.genome.jp/kegg-bin/show_pathway?ko03320).

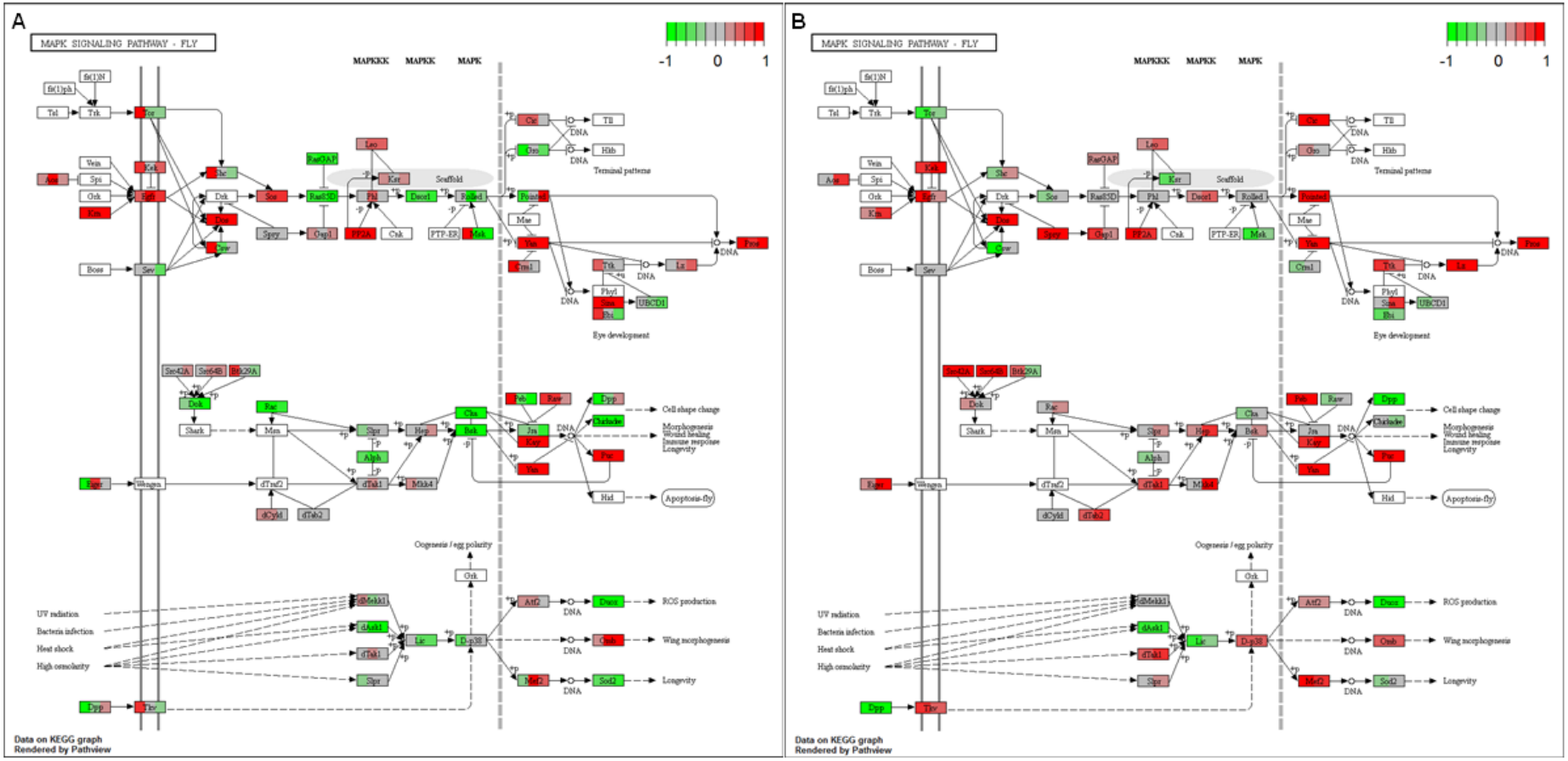

Figure B.3. Differential gene expression of the 'MAPK signaling' KEGG pathway components in *Gryllus veletis* (A) acclimated or (B) maintained under control conditions for six weeks relative to zero week controls. Each pathway component contains three colour bars indicating the three biological replicates of crickets under acclimation or control conditions for six weeks compared to the mean expression of control crickets (week zero), with red indicating increased expression, and green indicating decreased expression. RasGAP, Ras GTPase activating protein. For a complete description of each pathway component, see the KEGG 'pentose phosphate pathway' reference pathway (http://www.genome.jp/kegg-bin/show_pathway?ko04013).

B.3 Supplementary Code

The code used for transcriptome assembly and gene expression analysis is included below. The platform for analysis is indicated in square parentheses at the end of each platform. Anything following "#" is a comment, not code.

B.3.1 Quality control, assembly and annotation of transcriptome

B.3.1.1 Trimming and filtering libraries [Sharcnet]

```
##REFERENCE MATERIAL##
https://cutadapt.readthedocs.io/en/stable/index.html

##INSTALLATION##
ssh jtoxopeu@orca.sharcnet.ca
cd /work
pip install --user --upgrade cutadapt
~/.local/bin/cutadapt --help

##TRIMMING and FILTERING – do for each library (JT1 through JT16)
~/.local/bin/cutadapt -a AGATCGGAAGAGCACACGTCTGAACTCCAGTCA -A
AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT -m 15 -o JT14_R1filtered.fastq
-p JT14_R2filtered.fastq *JT14_R1.fastq *JT14_R2.fastq
```

B.3.1.2 Converting FASTQ libraries to FASTA libraries [Sharcnet]

```
##REFERENCE MATERIAL##
http://emboss.open-bio.org/rel/rel6/apps/seqret.html
ftp://emboss.open-bio.org/pub/EMBOSS/

##INSTALLATION of EMBOSS ###
ssh jtoxopeu@iqaluk.sharcnet.ca
cd /scratch/jtoxopeu
wget ftp://emboss.open-bio.org/pub/EMBOSS/EMBOSS-6.6.0.tar.gz
tar xpzf EMBOSS-6.6.0.tar.gz
cd EMBOSS-6.6.0
./configure
make

##FASTQ to FASTA## – do for each library (JT1 through JT16)
cd /work/jtoxopeu/Gveletis2016
/scratch/jtoxopeu/EMBOSS-6.6.0/emboss/seqret -sequence JT1_R1filtered.fastq -outseq
JT1_R1.fasta
```

B.3.1.3 Merging FASTA files for transcriptome assembly [Sharcnet]

```
ssh jtoxopeu@orca.sharcnet.ca
cd /work/jtoxopeu/Gveletis2016/Fasta
tmux new -s mergeR1
cat *R1.fasta > mergeR1.fasta
ctrlB+D ## to exit tmux
tmux a -t mergeR1 ## to reenter tmux
cat *R2.fasta > mergeR2.fasta
```

B.3.1.4 Assembling transcriptome with Trinity [Sharcnet]

```
##REFERENCE MATERIAL##
https://github.com/trinityrnaseq/trinityrnaseq/releases
https://www.sharcnet.ca/help/index.php/TRINITY

##INSTALLATION ##
ssh jtoxopeu@iqaluk.sharcnet.ca
cd /scratch/jtoxopeu
wget https://github.com/trinityrnaseq/trinityrnaseq/archive/v2.2.0.tar.gz
module unload mkl openmpi intel
tar xpzf v2.2.0.tar.gz
cd trinityrnaseq-2.2.0/
cp -a Makefile Makefile.orig (see diff below for edits)
cp -a Trinity Trinity.orig (see diff below for edits)
module unload mkl openmpi intel
make TRINITY_COMPILER=gnu 2>&1 | tee make.out

##TEST ##
cd sample_data/test_Trinity_Assembly
./runMe.sh

##RUN ##
export PATH=/usr/lib/jvm/java-1.7.0-openjdk.x86_64/bin:$PATH
export _JAVA_OPTIONS="Xmx10G
cd /scratch/$USER/trinityrnaseq-2.2.0
./Trinity --seqType fa --left /scratch/jtoxopeu/mergeR1.fasta --right
/scratch/jtoxopeu/mergeR2.fasta --min_contig_length 200 --CPU 1 --max_memory 20G
```

B.3.1.5 Transcriptome summary statistics [R]

#Run summary analysis on the Trinity file in R. Thi scripts is to calculate N50, average and mean length of #contigs, etc from a Fasta file

```
##install packages if needed
source("http://bioconductor.org/biocLite.R")
biocLite("Biostrings")

##choose files
in_f <- "ref_seq.fasta"

##change fasta file name to yours out_
f <- "Fasta_info.txt"

##you can change out put file name
library(Biostrings)
fasta <- readDNAStringSet(in_f, format="fasta")
Total_length <- sum(width(fasta))      ##total contigs length
Number_of_contigs <- length(fasta)     ##number of contigs
Average_length <- mean(width(fasta))   ##average length of contigs
Median_length <- median(width(fasta))  ##Median
Max_length <- max(width(fasta))        ##Maximum length
Min_length <- min(width(fasta))        ##min

##calculating N50
sorted_length <- rev(sort(width(fasta)))
N50 <- sorted_length[cumsum(sorted_length) >= Total_length/2][1]

##GC contents
hoge <- alphabetFrequency(fasta)
CG <- rowSums(hoge[,2:3])
ACGT <- rowSums(hoge[,1:4])
GC_content <- sum(CG)/sum(ACGT)

##save all info
tmp <- NULL tmp <- rbind(tmp, c("Total length (bp)", Total_length))
tmp <- rbind(tmp, c("Number of contigs", Number_of_contigs))
tmp <- rbind(tmp, c("Average length", Average_length))
tmp <- rbind(tmp, c("Median length", Median_length))
tmp <- rbind(tmp, c("Max length", Max_length))
tmp <- rbind(tmp, c("Min length", Min_length))
tmp <- rbind(tmp, c("N50", N50))
tmp <- rbind(tmp, c("GC content", GC_content))
write.table(tmp, out_f, sep="\t", append=F, quote=F, row.names=F)
```

B.3.1.6 Transcriptome completeness with BUSCO [Sharcnet]

```
##INSTALLATION##
ssh jtoxopeu@iqaluk.sharcnet.ca
cd /scratch/jtoxopeu

wget http://busco.ezlab.org/files/BUSCO_v1.22.tar.gz
tar xpzf BUSCO_v1.22.tar.gz
cd ./BUSCO_v1.22

wget http://busco.ezlab.org/files/arthropoda_buscos.tar.gz
tar xpzf arthropoda_buscos.tar.gz


##RUN ##
module list
module avail

module unload intel/12.1.3
module load gcc/4.9.2 python/gcc/2.7.8
module load blast/2.2.28+
module load perl/5.22.1

##copy hmmer directory from Trinotate directory into BUSCO directory

export PATH=$PATH:/scratch/jtoxopeu/BUSCO_v1.22/hmmer-3.1b2/binaries

wget ftp://emboss.open-bio.org/pub/EMBOSS/EMBOSS-6.6.0.tar.gz
tar xpzf  EMBOSS-6.6.0.tar.gz
cd EMBOSS-6.6.0
./configure
make
make install
cd ..

export PATH=$PATH:/scratch/jtoxopeu/BUSCO_v1.22/EMBOSS-6.6.0/scripts

[jtoxopeu@iqaluk BUSCO_v1.22]$ python BUSCO_v1.22.py -o BUSCO-test -in
/scratch/jtoxopeu/trinityrnaseq-2.0.6/trinity_out_dir/Trinity.fasta -l
/scratch/jtoxopeu/BUSCO_v1.22/arthropoda  -m trans
```

B.3.1.7 Transcriptome annotation with Trinotate [Sharcnet]

```
##REFERENCE MATERIAL##
https://trinotate.github.io/

### INSTALLATION of all the programs ### (do only once)
##Trinotate

ssh jtoxopeu@iqaluk.sharcnet.ca

cd /scratch/jtoxopeu
wget https://github.com/Trinotate/Trinotate/archive/v3.0.1.tar.gz
tar xpzf v3.0.1.tar.gz
cd Trinotate-3.0.1/sample_data
./runMe.sh

cd /scratch/jtoxopeu/Trinotate-3.0.1

##TransDecoder
wget https://github.com/TransDecoder/TransDecoder/archive/v3.0.0.tar.gz
tar xpzf v3.0.0.tar.gz
cd TransDecoder-3.0.0
module unload mkl openmpi intel
make
cd sample_data/simple_transcriptome_target
./runMe.sh

##sqlite
wget http://www.sqlite.org/2016/sqlite-tools-linux-x86-3140200.zip
unzip sqlite-tools-linux-x86-3140200.zip
## rename folder to "sqlite"

##NCBI BLAST+
module load blast/2.2.28+

##HMMER
wget http://eddylab.org/software/hmmer3/3.1b2/hmmer-3.1b2-linux-intel-x86_64.tar.gz
tar xpzf hmmer-3.1b2-linux-intel-x86_64.tar.gz
## rename folder to "HMMER"

### DATABASES ###
##SwissProt
wget
https://data.broadinstitute.org/Trinity/Trinotate_v3_RESOURCES/uniprot_sprot.pep.gz
gunzip uniprot_sprot.pep.gz
makeblastdb -in uniprot_sprot.pep -dbtype prot
```

```
##Pfam
wget https://data.broadinstitute.org/Trinity/Trinotate_v3_RESOURCES/Pfam-A.hmm.gz
gunzip Pfam-A.hmm.gz
HMMER/binaries/hmmpress Pfam-A.hmm

### ANALYSIS ###

## 1. FILES NEEDED ##
##Trinity.fasta (in my case, TrinityGvel.fasta)
##Trinity.faseta.transdecoder.pep
cd TransDecoder-3.0.0
./TransDecoder.LongOrfs -t /work/jtoxopeu/Gveletis2016/TrinityGvel.fasta
##Use file: TransDecoder-3.0.0/TrinityGvel.fasta.transdecoder_dir/longest_orfs.pep for
Pfam and/or BlastP searches

## 2. BLAST HOMOLOGIES ##
cd /scratch/jtoxopeu/Trinotate-3.0.1
module load blast/2.2.28+

blastx -query /work/jtoxopeu/Gveletis2016/TrinityGvel.fasta -db uniprot_sprot.pep -
num_threads 8 -max_target_seqs 1 -outfmt 6 > blastx.outfmt6

blastp -query TransDecoder-3.0.0/TrinityGvel.fasta.transdecoder_dir/longest_orfs.pep -
db uniprot_sprot.pep -num_threads 8 -max_target_seqs 1 -outfmt 6 > blastp.outfmt6
[here]
## 3. HMMER ##
HMMER/binaries/hmmscan --cpu 8 --domtblout TrinotatePFAM.out Pfam-A.hmm
TransDecoder-3.0.0/TrinityGvel.fasta.transdecoder_dir/longest_orfs.pep > pfam.log

### LOADING RESULTS into DATABASE ###

## 1. Trinotate SQLite database
wget
"https://data.broadinstitute.org/Trinity/Trinotate_v3_RESOURCES/Trinotate_v3.sqlite.g
z" -O Trinotate.sqlite.gz
gunzip Trinotate.sqlite.gz

## 2. Load transcripts & coding regions
/scratch/jtoxopeu/trinityrnaseq-
2.0.6/util/support_scripts/get_Trinity_gene_to_trans_map.pl
/work/jtoxopeu/Gveletis2016/TrinityGvel.fasta > TrinityGvel.fasta.gene_trans_map

./Trinotate Trinotate.sqlite init --gene_trans_map TrinityGvel.fasta.gene_trans_map --
transcript_fasta /work/jtoxopeu/Gveletis2016/TrinityGvel.fasta --transdecoder_pep
TransDecoder-3.0.0/TrinityGvel.fasta.transdecoder_dir/longest_orfs.pep
```

```
## 3. Load BLAST homologies
./Trinotate Trinotate.sqlite LOAD_swissprot_blastp blastp.outfmt6
./Trinotate Trinotate.sqlite LOAD_swissprot_blastx blastx.outfmt6

## 4. Load Pfam domain entries
./Trinotate Trinotate.sqlite LOAD_pfam TrinotatePFAM.out

### ANNOTATION OUTPUT ###
./Trinotate Trinotate.sqlite report -E 0.001 > trinotate_annotation_report.xls
## -E is the e-value (in this case 10^-3) as a cutoff for BLAST results
## can also add the option --pfam_cutoff <string>
```

B.3.2 Differential gene expression

B.3.2.1 Mapping each library to the assembled transcriptome, and determining transcript read counts [Sharcnet]

```
### INSTALLING BOWTIE ###
cd /work/jtoxopeu/bowtie
wget https://sourceforge.net/projects/bowtie-bio/files/bowtie2/2.2.9/bowtie2-2.2.9-linux-x86_64.zip/download
unzip download

### BOWTIE-2 ###
./bowtie2-2.2.9/bowtie2-build –f /work/jtoxopeu/Gveletis2016/TrinityGvel.fasta btout
./bowtie2-2.2.9/bowtie2 -f -x btout -1 /work/jtoxopeu/Gveletis2016/Fasta/JT1_R1.fasta -2 /work/jtoxopeu/Gveletis2016/Fasta/JT1_R2.fasta --sensitive -S JT1.sam
#repeat for each fasta file

sort -k 3,3 -k 4,4n JT1.sam > JT1.sorted.sam
#repeat for each sam file

### INSTALLING CUFFLINKS ###
cd /work/jtoxopeuwget http://cole-trapnell-lab.github.io/cufflinks/assets/downloads/cufflinks-2.2.1.Linux_x86_64.tar.gz
tar xpzf cufflinks-2.2.1.Linux_x86_64.tar.gz

### CUFFLINKS ###
cd /work/jtoxopeu/bowtie
/work/jtoxopeu/cufflinks-2.2.1/cufflinks JT1.sorted.sam -o JT1cuff
#repeat for each sorted.sam file

#create file called "assemblylist.txt" in the bowtie directory, which contains the location of each transcripts.gtf file on separate lines
```

```
export PATH=$PATH:/work/jtoxopeu/cufflinks-2.2.1.Linux_x86_64/
/work/jtoxopeu/cufflinks-2.2.1/cuffmerge -o cuffmergeGvel assemblylist.txt

### INSTALLING HTSEQ ###
cd /work/jtoxopeu
wget
https://pypi.python.org/packages/3c/6e/f8dc3500933e036993645c3f854c4351c9028b180
c6dcececde944022992/HTSeq-
0.6.1p1.tar.gz#md5=c44d7b256281a8a53b6fe5beaeddd31c
tar xpzf  HTSeq-0.6.1p1.tar.gz
cd /work/jtoxopeu/HTSeq-0.6.1p1/
module load python/intel/2.7.8
python setup.py install --user

### HTSEQ ###
## http://www-huber.embl.de/HTSeq/doc/count.html#count
cd /work/jtoxopeu/bowtie
module load python/intel/2.7.8
./work/jtoxopeu/HTSeq-0.6.1p1/scripts/htseq-count JT1.sam merged.gtf --stranded=no -o
JT1 > JT1.txt
#repeat for each sam file
```

B.3.2.2 Normalizing read counts with edgeR [R]

```
###NORMALIZING READ COUNTS###

biocLite("edgeR")
library(edgeR)

#Creating data matrix
targets<-readTargets("Targets.txt", row.names=1)
x<-read.delim("Variance-C1 (not filtered or normalized).txt", header=TRUE,
row.names=1, stringsAsFactors=FALSE)
y<-DGEList(counts=x, group=targets$Treatment)
colnames(y) <- targets$Label
dim(y)

#Filtering such that there are >1 cpm in at least 8 (half) of the 16 columns in each row:
yields 17474 genes
keep <- rowSums(cpm(y)>1) >=8
y <- y[keep,]
dim(y)

#Recalculating the library sizes
y$samples$lib.size <- colSums(y$counts)
```

```
#Normalizing
y <- calcNormFactors(y)
y$samples

plotMDS(y)
y <- estimateCommonDisp(y, verbose=TRUE)
y <- estimateTagwiseDisp(y)
plotBCV(y)

y$pseudo.counts
write.table(y$pseudo.counts, "Variance-C1 (filtered and normalized).txt", sep="\t")
```

B.3.2.3 Clustering differential gene expression with maSigPro [R]

```
source("https://bioconductor.org/biocLite.R")
biocLite("maSigPro")
library(maSigPro)

###maSigPro###

edesign<-read.table(file="edesign2.txt", header=TRUE, row.names=1) #edesign2 is a
table of the experimental design
edesignm<-as.matrix(edesign)
data<-read.table(file="Variance-C1 (filtered and normalized).txt",
header=TRUE,row.names=1)
datam<-as.matrix(data)
str(datam)
library(MASS)
dm <- make.design.matrix(edesign)
dmp <- p.vector(datam, dm, counts=TRUE, Q = 0.05, MT.adjust = "BH", min.obs = 10)

dmp$i
dmp$alfa
dmp$SELEC

dmt <- T.fit(dmp)
get<-get.siggenes(dmt, vars="all")
get$summary
see.genes(get$sig.genes, k = 9) #Assigns the genes into one of 6 patterns

names(get)
names(get$sig.genes)

cluster=see.genes(get$sig.genes,k=9) #Exporting the results
write.table(cluster$cut, file="Cluster k9.txt")
write.table(get$sig.genes$sig.profiles, file="Sig profiles k9.txt")
```

```
write.table(get$sig.genes$sig.pvalues, file="Sig pvalues k9.txt")
write.table(get$sig.genes$summary, file="Sig summary k9.txt")
write.table(get$sig.genes$coefficients, file="Sig coefficients k9.txt")
write.table(get$sig.genes$group.coeffs, file="Sig group coefficients k9.txt")
write.table(get$sig.genes$g, file="Sig g k9.txt")
write.table(get$sig.genes$groups.vector, file="Sig groups vector k9.txt")
```

B.3.2.4 GOSeq analysis [R]

```
## read in fasta file with transcript IDs
library("seqinr")
my.data <- read.fasta("TrinityGvel.fasta", seqtype="DNA", as.string=TRUE)
head(my.data)

seqID <- names(my.data)
head(seqID)
lengths <- nchar(my.data)
head(lengths)

write.table(seqID, file="assayedgenes.txt", quote=FALSE, sep = "\t")
write.table(lengths, file="genelengths.txt", quote=FALSE, sep = "\t")

library("rtracklayer")
library("goseq")
## read in all gene names (gene.vector)
assayed.genes <- read.table("assayedgenes.txt", sep = "\t")
gene.vector <- as.character(assayed.genes[,1]) ##character vecotr of all gene names
str(gene.vector)

## read in names of differentially expressed genes (de.genes)
gene.clusters <- read.csv("degenes.csv")
head(gene.clusters)
str(gene.clusters)

#if want to distinguish between up and downrregulated genes, choose 1:
gene.clusters <- gene.clusters[gene.clusters$A6_log2FC>0,] #for working with
upregulated genes (can modify cutoff as desired)
gene.clusters <- gene.clusters[gene.clusters$A6_log2FC<0,] #for working with
downregulated genes
#(can skip this step though, if you just want do look at all DEGs together)

## make character vector of differentially expressed gene names
de.gene.names <- gene.clusters[,1]
de.gene.names <- as.character(de.gene.names)

## make numeric vector of DEGs (1) and non-DEGs (0)
```

```
logical.assays.genes <- gene.vector %in% de.gene.names ##TRUE for all instrances of
DEG
de.genes <- 1*logical.assays.genes
str(de.genes)
table(de.genes)

##fitting the probability weighting function (PWF)
gene.lengths <- read.table("genelengths.txt", sep = "\t" )
gene.lengths <- as.numeric(gene.lengths[,1])
head(gene.lengths)
pwf <- nullp(DEgenes=de.genes, bias.data=gene.lengths)
rownames(pwf) <- gene.vector ##putting gene names as row names
head(pwf)


#importing GO terms for genes
GOs <- read.csv("GOdb-clusteredgenes.csv")
GOs <- GOs[,1:2]
head(GOs)
str(GOs)
GOdb <- as.list(as.character(GOs[,2]))
names(GOdb) <- as.character(GOs[,1])
head(GOdb)

##running GOSeq
output <- goseq(pwf, gene2cat=GOs)
head(output)
write.table(output, file="GOSeqoutput_downregulated.txt", quote=FALSE, sep = "\t")
```

B.3.2.5 KEGG Pathway analysis [R]

```
### gage with no prior DE list input
source("https://bioconductor.org/biocLite.R")
biocLite("pathview")
biocLite("gage")
library(pathview)
library(gage)

data(kegg.gs)

#Loading the count data for a given two-treatment comparison
count <- read.table(file="Counts U3vU6.txt", header=TRUE, row.names=1)
cnts <- as.matrix(count)

#Setting the KEGG species list to generic
kg.ko=kegg.gsets("ko")
```

```
kegg.gs=kg.ko$kg.sets[kg.ko$sigmet.idx]

#This is the new annotation ref library
YRWA <- read.csv("KEGG ref (proper).csv", header=FALSE,
colClasses=c("character","factor"), sep=",")

#Parsing in the library, and adding up all the KEGG term counts
D2 <- as.matrix(YRWA)
cnts <- as.matrix(cnts)
cnts.data <- mol.sum(cnts, id.map= D2)
head(cnts.data)

cnts=cnts.data
sel.rn=rowSums(cnts) !=0
cnts=cnts[sel.rn,]

libsizes=colSums(cnts)
size.factor=libsizes/exp(mean(log(libsizes)))
cnts.norm=t(t(cnts)/size.factor)
range(cnts.norm)
cnts.norm=log2(cnts.norm+8)
range(cnts.norm)

##Referencing the treatments --> Ref = control columns (1-3), samp = treatment columns
(4-6)
cnts.kegg.p <- gage(cnts.norm, gsets = kegg.gs, ref = 1:3, samp = 4:6, compare
="unpaired")
cnts.d= cnts.norm[, 4:6]-rowMeans(cnts.norm[, 1:3])

###Making the heat map
library(ggplot2)
kegg.sig<-sigGeneSet(cnts.kegg.p, cutoff =0.1, outname="A3vA6 - KEGG",pdf.size =
c(9,13), margins = c(6,20))
kegg.sig

##Making the pathways. Done manually for each pathway of interest (e.g. ko04260)
pv.out <- pathview(gene.data = cnts.d, pathway.id = "04260", species = "ko", out.suffix =
"ko.data", kegg.native = T)

#Yields text files with q values. The P.val is the global P-value and q.val is the
corresponding FDR q-value.

cnts.kegg.esg.up <- esset.grp(cnts.kegg.p$greater, cnts.norm, gsets = kegg.gs, ref = 1:3,
samp = 4:6, test4up = TRUE, output = TRUE, outname = "U3vU6 - Upregulated
pathways", make.plot = TRUE, compare='upaired')
```

```
cnts.kegg.esg.dn <- esset.grp(cnts.kegg.p$less, cnts.norm, gsets = kegg.gs, ref = 1:3,
samp = 4:6, test4up = FALSE, output = TRUE, outname = "CvU3 - Downregulated
pathways", make.plot = TRUE, compare='upaired')
cnts.kegg.esg.2d <- esset.grp(cnts.kegg.2d.p$greater, cnts.norm, gsets = kegg.gs, ref =
1:3, samp = 4:6, test4up = TRUE, output = TRUE, outname = "gse16873.kegg.2d",
make.plot = TRUE)
```

Appendix C: Chapter 4 supplementary material

C.1 Sequence Alignments

The following sequences are from the *Gryllus veletis* transcriptome. Regions complementary to qPCR primers are outlined, and primers for dsRNA templates are highlighted in the colours indicated prior to each sequence. Regions that are underlined were confirmed by sequencing qPCR products. Regions that are highlighted in grey were confirmed by sequencing templates for dsRNA synthesis. Sequencing products were aligned to transcriptome sequence using ClustalOmega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

C.1.1 Elongation factor 1β

```
>Elongation factor 1 beta
GGCCCGTTTCCCAGCCTCCTCCGTTCTTCCTCTTTCAAGTAGCTCACAGCTGAGTAGGGT    60
TATTGTCAGTTGTTCGAAAATAATTGTGAAATTGTACCTATAAACGCTCAATATGGTTGT    120
TGGAGACTTAAAAACTGATCAGGGAATTAAGTCCCTAAACGAATACCTTGCGGATCGAAG    180
CTATGTCTCAGGGTACGAACCTTCACAAGCTGATATAAGTGTCTTCGAAAGTATTTCCAA    240
AGTGCCTAGTGCCAATACCCCTCATGTTTTACGCTGGTATAACCACATCAAATCTTTCGA    300
TGGAGCTGAGCGT                                                   313
```

C.1.2 Ribosomal protein l18

```
>Ribosomal protein l18
GTCCAAGATGGGAATCGATATCTGCCATAAAAATGACCGCAAGGTTCGGCGAACCGAACC    60
TAAATCGCAGGATGTGTATTTACGTTTACTTGTTAAGCTCTATCGCTTTTTGGCCAGGAG    120
AACGCGTAATAAGTTCAATAAGATAGTGATGAAGAGGCTTTTTATGTCTAGGATTAATAG    180
ACCAGCTATTTCACTGGCCCGCATTTCTCGTTTCATGAAGAAACCCAATAGGGAGAAGTT    240
GACGGCTGTGGTCGTTGGTACTGTTACTGACGATCAGAGGATTTACACTGTTCCGAAGTT    300
GAAGATTTGTGCATTGCGATTCACGGAGAAAGCTCGAGCACGTATTTTGAGGAATGGAGG    360
ACAGATCATGACATTTGATCAGTTGGCTGTAAAGCACCCGTTGGGGAAGAAAACTGTTCT    420
TATGCAAGGCCGCAGAAATGCTCGTGAAGCTTGCAAACATTTTGGCCGTGCACCAGGTGT    480
GCCTCACAGTCACACTAAGCCTTATGTACGTTCCAAGGGACGCAAATTTGAGCGTGCTCG    540
TGGTCGAAGGCGTAGCTGTGGTTACAAGAAGTAAGCTGATATTGATGTTATTGGTTGTAA    600
CCATTTGCTTTGGTAAATAAAAAATTATATCTACATACATGTTCATTATTTTATGGTAGA    660
TATGGCCACACCTTCACTCCGTCAGTGTTAAACTTTCATAAAAATGTTGTATTTGGGAAG    720
AAATTAAATCATATTTGTATTCATTCAAAAAAAAA                             755
```

C.1.3 Ferritin

dsRNA primers are highlighted in green (ds*Ferritin*.1) or blue (ds*Ferritin*.2).

```
>Ferritin
CAATGTAAGTTTTTATTTGTACATGAAAATGAAAAATACATAACTGAACATAATTAAAAT    60
CCTCTACTCCTTGACAACTTCGATTTCCTCTTAAAGGCACAAAATTCTAACACAAGTCAA    120
CATTTCATATATTTTACAAATTCTTTTTCAAGTACTCATCAAGCAGGAACACCTGGAGCC    180
CAACTGATCTCTGGGGTGATTCAGGATCAATTTCAACAAGGCGCTTGAGCTCTGTAGTGT    240
ACCCTACAAGCTCTCTGATGGTCTTTGCATGCTTGTGCATGAATGTGTCCTCTACAAATG    300
CTCCCATCTCAGGATCATGCTCATTAGCAACCTTGTAAATGCTGTGGGCTTCTTCAGCAA    360
GTTTCTTCTGAATGTCAACAGCTTTAGACAAACTCTCAACTTCATTCAATGCAAGTGGCT    420
CTCCCTGGAAAATTGTTGATCGGTCTTCTTTTTCTGAATCATAGTATGGTTTCAGGTCAA    480
TCTTCTCACCTCTCAGGGTCATGTATTTGATTACATCAATAGCATCATCCCATGCAGTGT    540
CCGACAATTTACGATACAGCTTCTTGAAGCCATCTCGTTCCATTTTGTATCCTCCAAAGT    600
GACTCGACATCATCAAATACTGCAAACTACGTGAAATATGCATGTTGAAGTAATCATTTA    660
GTCTGTTCATGTAGTTTGCTGCCTTGCCAAATTTTGCATCACAGTTGGAAAAAGTCACAT    720
CTGGGCTGGTGGGATGACATGCTTCTTTAGCATCCTTGTAGCAATGCTCACTAGCAGCCA    780
CGGCAATAAGACACACAAAAACTGCGAACCACTTCATGACGAACCCGAGTGAAGATGAGC    840
AATAAATAGACCTTCGATCTGATCTATTGCCCAGTTTCACCGGAACAATACGAACCAAGT    900
TACCCTGGTCACAAGTGCTTCATTCGATAACGAGCAGACGTTCGTGATTCGTCACTGGCC    1020
GCTTTATTCTTTGCCTCTCAGAATAGGTTGCACTGCACGCGATAGCCTGATGGAGCTGGA    1140
GTAGTGGTGATTATATTAGAGGGAGTTCGGCGTATTTCAGTTTAGCTTGAGTTGGTTCGC    1200
CTTCTGCATCAGTGTGTGTAAAGGCGATCACTCCCTCAAGCATTCCTGAACTTTTCTTTT    1260
GTGTTTAGTGGTGATTGTTGAGTTGTTTATTCGGCCTTACGATATAAGGAACTGGGATTG    1320
TTTCTGCTGTGTTTTTCTGCTGCCTGGCCCTCGCAACTGCTACTCAGTACAGTTGCAAAG    1440
CCCAGGCAGTGCAATTTCCCGCTTTCAAGAGTATGGAAAATGAATGCATTGCCCTAATGA    1500
AACATCAAGTTCAGAAAGAGCTTAATGCAGCACTTACTTATTTGGCCATGGCAGCTCATT    1560
TTTCTGAAGATCAAGTGAACCGACCAGGTTTTGCAAAGATGTTCTTTGAGAGTGCCAATG    1620
AAGAGCGCGAACATGCCATCAAATTTATAGAATACCTTCTGATGCGTGGTGAACTTGAAA    1680
CTGACATCAAAGATCTCATTAGTGATCCTTACCCAGTAAGAGGACTGACTTGGGACTCTG    1740
GTCTTGCCGCTCTGAAGAATGCTTTGGCTTTGGAAGTGAATGTAACTAAGAGTATCAATG    1800
AGATAATTAAGAAGTGTGAAAATCCTGATGTGGGAGAAAATGATTATCATCTTGTCGACT    1860
ACCTGACTGGAGAGTTCTTGGATGAACAATATAAAGGACAAAGGGAATTGGCTGGCCAAA    1920
TTTCAACCCTGGGCAAAATGACAAAATCACTGGGAGACCTTGGAGAATATATGTTCGACA    1980
AGAAACTTCTTGATGGCGAGCTGCCAGTTTTAGGCTAGATAATGGGAGTTTTGATTGTGA    2040
AATATTTGTTGAATGTAGAAGTTTGTTGGTAGTTAATGTCAAAGAAATTAAATATTTGAT    2100
ATTGGTTTGAACTTGTACAGTAATTGTCTTCATATTAGAGCCTGCTGTTGAGGAATGTCA    2160
TATGTTGTAATATATGGATAAAGATAAAATAAACTTGATTTTTAATTATTTAAAAAAAAA    2220
```

C.1.4 Heat shock protein 70

dsRNA primers are highlighted in green (ds*HSP70*).

```
>HSP70
AAAAAATTGTTATAGAGTATTGTTCTCGTTGCAATAAGGCATGCGTGCATTCACAATGAA    60
ATATATATTACGTGCTAAACATATCTGAAAACCACAGTTAACAGTCATTTTATTTTTCTG    120
ATGTCTGTCACTAACATTGCCAGCGTCCCCAATTAAACACTGTTCTTAAATTAAAATTGA    180
AAATTGCTTTTTCTCTACACGAGTCTCTAGAACAATCGTATTTGTTGCTCTTTTCTTTCC    240
ATTTATTTATTTATATACTATTCAATTTATTTTCTAAACCAAGAGTAACACGAGCCTTAA    300
TACCCATTTGTAGGCACTCAGTCAAAGTAACCAGTTTCACTAGAATCTGTAAGAATACAC    360
TTGTCGCTTCAACCACAAGTCGTAATTAACTGCGAAATGTATACGTATAACACTAACAAG    420
AATTCTGCTTGCTTGACATCGTACTACCTCCCAAATTCACAAGCCACCAAATATAAAAAT    480
AGACATTTTTCCCCTCTTTATAACTTGCATCAACCATCTTACGTTGTTTTAATACAATAA    540
TGCACTCCCCAAACAGTCGATGTTCCTATAAGATTCTTACGTTGCGCTGATATTCTTCGC    600
TTTTTAATTTTTACCTGTTATTTTAACATTGAACTACTTGTATTTCTTCCAACACTACCA    660
ATTTTCTTTTAGGTGTTAAAAACGCTTTTGTTGTGTATTTTTCACTCGGTTGAAGTAAAA    720
TTGAAGCTCAAATTACCAAACGTTTAACTGATTGTGATCACTCTCGTAAGAAAAAATGGC    780
TACAAACCATAGTTTCGTTGTTATCATTCGGAATATATTTCACCTACCAGTCTCATTGCA    840
ATAATTAATAACACATACATACTTGTTTTCAACCACAATGAAATACGTGCTCAACATCTA    900
TAACATTTATACCTCACACACATATCTCGATACTCCCTAATTCAATACTTTTCTTCAATA    960
ATAATTGATTTTTTTCTTCCCCCCCCTTAATATTAGTTTGTTGTAATGGGTGTTCTTTCC    1020
AGAATTTACTCATTTATTGCAGTTCAATCTTATTATTCAATGAGAAATACGACCATTTAT    1080
ACCCATGTACAGTCAAAGGAATATTTCACCTCATTCCATCAGAACCGCTTTTCTTCCCCA    1140
CGACCTCAAAAGTATCATTAACTGCTCTACGTATGTGTATACCACTAATATATTACTCGA    1200
TCGATTTTATAGCTCTCTAGCTAACTGATACCCGACGACCAAATATCCAAATTGAAACTT    1260
TCTTTTTTGTTTGCTTCTCTTACACGAAACCACTACCACTACCTTAACACTTTATAATAC    1320
AACAAAAATTCCACAAAATGTCGCTGTTCCTCTACGATTTTACTTTTTGCATGATACTTA    1380
GCTTCTTTATTTTTTACCGTTACTTAAAATTAACTCCTTTAGCAAAATTTTATCCAAATC    1440
ACAAATTTTAATTTTGTACTATAAACCGCTTTATTACACTATTTCATACTCCGTCGATGT    1500
AATAACAAACCTCACACCAACGTTCACACCAAATACACCGTAATACCAAACCTCACTCAA    1560
ACATTCAATTGTCTTTACTTGACGTAAGAGAAAATGGCTACACACCGTTGGTCCATTATC    1620
TATCATTCGTGACATTTCACGTATTTATCTCATTGCAATAAGGAATAACACTCGTATGCG    1680
TTCACTCACAACGAAATATGTCTAATCTAAGAAAGCATATAAACCCATAACTAAGTCGTC    1740
ATTTTGTCCCTAAACGTCTGACACTAATATTGCCCTACTCTCCAATCAAATACGTTTCTT    1800
ATTTATTAACAATTGAAATTTTTGGCAATGTTTATCTCCTTAAGTCTCTTGGATCGTCGT    1860
ATTCGACATTTCTCGTTGTTACAATGAATCCCACCATCAATACCCATGTTTTGAAATCTG    1920
ACGAAGTAATCGCCATCTCCCGAACTGCTTTTGTTCGCTATAACCACGAAAATCTCAATG    1980
CGCTATGTACGCGTATTCTACTAACCAGAATACTCGCTCGATTATGTAGTTCTCTAGCTC    2040
```

CTAGTTAATATCAAAATAAGACACTTATTCTTTTTTCTGACTGGTATCTCAAATCAATAT 2100
CTTACACCATTTTAATACAATATTGCATTCCACAAACAGTCTATGACCATGTAGTATTTT 2160
ATCTTTCGTTACGCTGATATTCTTTACTTCTACATATTTTACCTGTTTAATTGATTGGCA 2220
TTACTCGTATAAGATAATAGCTGCACACCATGGTTCAGTTACATCCATCATTCGGGCAAC 2280
AATTTCCCCTAATGATCTCATTGCAATAATAAATAACACATACGTAGACTTCGTTTCCAC 2340
CCTATGTTTCTCCATTAGTCTCTTACACAAAGTAATTTGACTCGCATTCCATTCCTGTTA 2400
TTAATTGAATTTATTCAATGAAAAATACGATCCTCAATACCCATGTTCTGAGCAGTCAAT 2460
TAAAGTAATCAGTTTCACCGAAAACTAAAAGAAACTCTTTTCATCGCTACAACCACAAGC 2520
TTTTATTTAACTGCTATAATACATATGCGTACACCACTAATTAGAACACCTCACGCTCGA 2580
CTAAGCTCTAAGAGTCGACACATTTAACGACTAACAAACATTACAATACACTTTATTCCC 2640
TTTTCTAATTCGCATCTCAAATCAATTATCTTACGCCTTTTTAAATATAAGAATGCATTC 2700
GACAAACACTATCTCTAAGGTTTTATGTCATTTGTTTTGTTAAGATTCCTCGTTGTTTTT 2760
TAGCACTACTGACAATAACTTGTTTGCAGCTTAATTAACACTCCACATTTTTCCCTTTGA 2820
TCCATAAAACCATTTATTACACTGCATATCTTACTCGCCGGAAGTAATCTCAAACCTCCA 2880
CATCACCAAACGTTGATTGATCATCTTTACTTGCGTAAAATTAATATCTACACAACATTG 2940
TTCCTTCATATCCATCATCATAACTAATACAGAAGACGTGCGTTGCATCTGAATTAAATA 3000
TTTACTACGTGATTATCACGGGGCTACGATAGCTGAAACCCACAGTTAGTCATGTTGCGT 3060
TCCTCGACGATTGTCACTAACATTGCCATACTCTCCAATCAATCATTTGTCTTATTTAAT 3120
TAAAATTGAAATTTATAATTACTATTGAAAATTATTTTCACTTGTTATGTAACTTGTATT 3180
CTGTTACTTCTTTTATTACTTACTTTTACTTCTGTTATTCCTTTTAGCACACAACTACCA 3240
TCACTACCCAATTTCCTTTTGTTCTATAAAACACTTTTGTTACACTGTATCGTTGAAGAA 3300
ATCAAATCTCACAACACGTTCAAATAGCATTACTCGCGTAAGATAAATTGGCTACAAACC 3360
AGTATTCCCTGATCTTCATTCAAAACACAGAACATACACTTACGTAGACTTGCGTATAAG 3420
ATTTTATAGATCTTCCTTTTATCAAAATTCTATTAACACTACCGATTTTATTTTTGTTGT 3480
ATAAATCGATTTTTATAACACTATCTTACTCCGTCAAACCTCACACCAGTTTAATTGATT 3540
GTCTTTGTTCGTCCCAAAAAAATTGTTATAGAGTATTGTTCTCGTTGCAATAAGGCATGC 3600
GTGCATTCACAATGAAATATATATTACGTGCTAAACATATCTGAAAACCACAGTTAACAG 3660
TCATTTTGTTTCTAAACGTTTCACTAACATTGCCACACTCCCCGATCACTTTTCTTGTTC 3720
TATTAAAACTGACATTTTTCTCTAACTGAAACTTAAACAATCGGGAGAAATTCGAGCCTA 3780
AATTCCCATTTGTTTGAACGTAAGCAGTTTCACCGGAAACTATAAGAATTGATTTTTTTT 3840
CGTAACCGCAAGTCTCAATGAACTGTTGTTATACCTATGCATATACCTCTAATACGAATA 3900
CAGTCTGCTCGACTTTATACCTCTCAAGCTTCCAGTTACACGACTACCAAATATAAAAAA 3960
AAGACATTTATCTCTTTGTACCTCATCTGAAATCAACCCATCTTACGCTGTTTCTATACA 4020
ATAATGCACTCCACAAGCAGTCAATGTTCCTATAAGATTTTACCTGTATTTTCCTTATGT 4080
TGCCCTGATATTCTTTGCTTTTTAATTTTTACCTGCTATTGCAACATTAACTACTTTTAG 4140
CAAATTTCTTCCAACACTACCCATTTTCTAAAACGCTTTTGTTACACTGTATTTCTTACT 4200
CGGTTGAAGTAATATCAATTCTTTAAGTACCAAACGTTCAATCATCTGTCATTACTCCCG 4260
GAAGATTTTATGACTACACACCATTGTTTCGTTATATCATTCGGGACTATAAATTTCATC 4320

TACTGGTCTCATTGCAATAATTAATACTTTACTTATCTTATCTTTACTTATTGTAGTTTT 4380
AGCTAACCTGAATCTATAAGAACTGCTTTTCCTACTTACAACCTCAAAAGTATCAATTAA 4440
CTACTCTACGTATGCGTATACCAGTAATAAGAGAACTACTCGCTTGACTTTGTAGCTCTC 4500
GATCTCACAGAAACCTAACGACCAAATATCCAAAAAGAAACTTTAAGATACTTCAGAAAC 4560
ATTTTTAATTATATTCTAACTAGCATAAAACGAATACCATAACATTTTTAATACAACAAT 4620
GCATTCCATAAAATGCCGCTGTTCCTTGAAGATTTCGCTTTCTTTCTCATATCAAACATC 4680
ACACCACCAAACGTTTAATTGATTAAGTCCAGTGTTCGTCTTAAAGAATGGCAACACTTT 4740
TCGTCCAGTTGTCTATCGTCGTGTCAATACAGTTCACTTCTAATTAAATAAGGAATAACA 4800
CTTGCATAGACTTGCGTTCAATCATAATGAAAATTTACGTGGTCAATTCAGAGCTAAGGC 4860
TGCTTATAAAACCCATCACTGTCCCTCCACGACAATAACATTGCCATCCTCTCCCATCAA 4920
ATACTTTCTTAGTCAATTACAATTTCAATTTTTGTCAGTTCAGTCTCTTAAGTACCATCT 4980
TATTCGACTTATTTCTCGTTGTTTATTTACTGACGCAGTTTCTATCGTACTCAATTCTAC 5040
AACCAAACACCATAAAAAATATCCATTAATTGCCATACGTATGGGTATACCATTAATAAG 5100
AACACTACTCTCAGTACTTTGTAGCTATAGCACGTAATTACAGGAGGAAAAATTCTAAAT 5160
ATGCCGATTCCCTCTTCCAACTCGTACCTCAATTAATTATCTAGCCTTATTTAATACAAT 5220
AATGAATGCCATTGTTCCTCTGGGGTGATATTCATTTAATTTACGTTCCGCTAATACGCG 5280
TTGCTTCTTTTAGGTTACTTCTGGTATTGCAACTTTTTGCACTTTTCAATTCATTTTCCC 5340
TCGCTACACCTTTGTTTCACCCACCACTATATCCAACATTCGGGAAAATACAGTTCCATC 5400
AATTGCAGTAAGTGATTACACATTAAGACTTGCGTTTAACCACAATGAAGTTCTAAGGTA 5460
AGACAGCTGAAATCCACATTGAACTAGCATACATTTCAATACAACTCTCACTAACTCTCA 5520
AATCAGACTTCTTTTTCAATTACAAATTCAATTACACGTTTCTTTCGAAAGATTTAAGTT 5580
ATCTTACTATGTTTTGATATTCTTTGTTGTTTTTGCGTTAGTTGTGTCAACACTTACTCT 5640
TGTTACTTTTAATTTAACTCCGCTCATTTTCCGTATTAACACTTTTTAATACAGTATTTC 5700
TTACTCAGTCGAAGTAATGTCTGAAATCGCCTAACAATCAATGCTTTTCTTTCAACAACA 5760
TATGTGAGTAAGAATACTCCCCCCTCGACAGTACTAAAGCTCACTGATACCCGACGGCCA 5820
ATATTAAACCAGACATGTTTTTCTAAATCGCATTAAAAACAATTACCATAAATATTTTTT 5880
ATAACATTATTAACATAATGCATTCGACAGTCTGTTCCTACATACTTTTATCCACATTTG 5940
TGCTGACATTCTTTAATGTTGTTGGTTTTTTTTTTTTTTTTTTTTTT 5988

C.1.5 Pyrroline-5-carboxylate reductase

dsRNA primers are highlighted in green (ds*P5CR*).

```
>P5CR
GTGGATGTTTATTCAATATCTCTGAGAACAATTTCACTTCTCCGTAGTACGAAAAAATCA    60
ATTAGTTACTCTTGAGTGATACGTTTGCAGTTACAGAATTGTTTATGAACGCAGGAAGTC    120
ACGTGAAAAAATAGTAGTTGAGAAACTTCCACTCATTCGAACGTATTTCTTGATAAACGA    180
TTTTTGTCACTCACATCTTTACAAGAGTTGAGCCTTCACTCACTGAATGTCTTATCTGAC    240
AGCTTAAATTGTAGTCTGACGGGTGATAGGGCTAATCCTCTTTAACTGATATATTCCAGG    300
CAGTCCGGGTTTCTGCTGTACTATTTTAGTAACTGCGGAAATATATTTTGACACTTATCG    360
CGACTGGAAAGATTTCTCAGGGCGCGACCTTTGATTTCAGGTCTTAGCAAATCGTCCGCG    420
GCACGATGGAAATGTGTCAATGAAGAATGTTCTTAAAGAGCATTTCAATGATATGGCAAC    480
TCTGCCGTAGATTGAAGTGACCTCTCGGTTAGCATTGCGTATCAGTCCGTTCTTCTTTCT    540
TTAGCATGGATAGAAATCACACAGTCTTCTCCAGACGTCATCATGATGAAAATCGGTTTC    600
CTCGGAGCCGGGAAAATGGCACAGGCCTTAGCAAAAGGCTTTATTGCTGCAGGTTTAACA    660
AAAGGAGAAAATATCGTAGCAAGTTGTGCACCAAAAGATGTAGACTGTATTAAGGCCTTT    720
GAGGAAATTGGAGCATCTGCAATACCGAATAATACAGTTCCTGTTCAAAAGTCTGATGTT    780
ATTTTCCTGGCTGTGAAACCTACTGTAGTTCCAGATGTATTGAAAGATATATCAGCATGT    840
ATAACAACACATCACTTACTTTTATCAATTGCCATGGGTGTAAATATTGAGGATATAGAA    900
AAGAACTTACCACCATTATCAAGAGTGGTGAGAATAATGCCAAACACCCCAGCAATGGTA    960
AGAAGTGCAGCTTCAGTATTTTCTTGTGGATCCAACACTCTACCAGAAGATACAATAACA    1020
ACACAGAAACTTCTTGAAGCTGTGGGATCTTGCCATGAAGTTTCCGAATCAATTTTAGAT    1080
CCTATTACTGCTCTCAGTGGTTCTGGGCCAGCTTATCTTTATGTTATAATTGAGGCAATA    1140
GCAGATGGTGGAGTGAAAATGGGTTTACCCAGAGATCTGGCCTATCAGTTAGCAGCTCAA    1200
ACAGTATTAGGTGCTGGGAAAATGGTACTGGAAACCAAATTGCATCCAGGACAATTAAAG    1260
GATGATGTCACATCTCCTGCAGGTTCTACTGCAGCAGGTCTACACTACCTAGAGAAATGT    1320
AAGATACGTTCAGCACTCATTGGAGCGATAGAAGTTGCAACAAAACGATGTCAGGAAATG    1380
AACAATATGTCATAGCATTTAAGTTATTTATTGTGCCTTTTAGTATAAATGTAAGATATG    1440
TATAGTAGACAAAAATAGTTTGTATATTTTTTAATGTTCTTCGATTTTAAAAACATGCAA    1500
TAACTAAAGATAATTAAATTGTATAAAATTTCAAATACGAAA                      1542
```

C.1.6 Supervillin

dsRNA primers are highlighted in green (ds*Supervillin*.1), blue (ds*Supervillin*.2), pink (ds*Supervillin*.3), or yellow (ds*Supervillin*.4).

```
>Supervillin
ATTTGCTTGGTGTAACTTAAATATTATATTAGTTATACAAATTAATCTGTTATATACAAA    60
AAATTAAGAAATAGTATACAAGCCACCTGCCAAAAATCTACAATTCTTTACAGATTATCT    120
GCTGTACAAAATAACTGAAACAAAACCAGCAAAACACTGACAAAGAGCAAAAAATGGAAG    180
TTTACCTCAATAGTGAGAAGCTAATTTTTACAAGAATGAAAAATGTTCCTCCCATTTTGT    240
GCTAAGACACAAGCTCACAACTAAAACCAATGTTGACTTTGTGGAATACCCATTCTTTTG    300
TTAACTGTGAATCCCACAAAGATACATCGGAAAACAGACACATGACTACACTCTTTAATG    360
AAAAGAAGGACGATTCAGGTAAGTAAATACATAATTATGTTCAGAAAAATATGCCAAGAA    420
ATTGGCAGGGGAATCGCGTCACAATGCATCTTCAAAGATCTCTGGAAATACATATTATGT    480
ACAAAATATTACAAAAGCAGGACACTCAAGCAAAAGGAATAAGGCAACACTGGCATTTAC    540
ATCACACTTTCAAAAACATTTTATCTCATTTGAAGTTATGAAACCACAATATCACAAACA    600
TAACAGAAGAATATAATAGAAAGAATGGACTAGAAGCTTCAAAAAGGACTGATTCCTTTT    660
GTTTTCTTCACACAAGTTTAGCTTGTCTTCAAATCTGACATCACAAGAAAGGCACAGGAT    720
TGCACAAGTAAATGCCACAATAAAATGGCATGGCACATAGAATCACCTGCCTTTTCAGCA    780
CAAAGAACAAAATGCAATTATTAATGAACAAGTAGGAACATATGAATATTACATTGCCAA    840
TTAAAAAGAGTTTATTTTATACAAGATAAGTGAAAATAGCTATAAGAGGCAACAATCACA    900
AGAATGTGTTAGCAGGGTAATATGACAACAGCTCCTTCAAATGCATTTTTATCTGCAAGT    960
TCTTGAACTGTCTTGAAAGCGGAAGCATTGCCTTTTAGACTGGAAACCTTCCTATTTTTA    1020
TACATCAAATTCCTTATATCATATATTCACATACAACAAACCTACAATTCTAGTAACAAT    1080
ATCACAATGTTATAGTACACCAATTTTTTTTAAATAGTAACTGAACAGGGAAAATGAAAC    1140
GTGTGATATTCATTTAGATGAGCCTTGCATTTTCTTTGGGCAAGCTTGCAGCTAATGAAA    1200
ATACATTAATATTAGTACATAGTTTTAGGGTAAGGGCAGTTCAGTTATTTCTCTCATAAA    1260
CAAGATATATATTTCAAAAAGCTATCAATTAGAAATAATTCTATATCAAACATTGATTTG    1320
TCTTGCTGAGATATTCAAAATGTCCATGTACACATTTAGGTCACAGTATAAACAACTTGT    1380
CATTTAAAGAAATGCAAGTAAATTGGAAGAGAGGGAATATCACAAGAAAACAGACTTATA    1440
ATTCAATGCAAATTGTACTAAATATAAAAAACAAGAAAGACTTTTGCTTTCTTTCTAAAA    1500
GCCCATTTGCATTTTTTAAACAAGCTTTCATTTTAGCATAGTTTCTTAATGATTTATTCT    1560
TTTTTAAATTTCTGAGTTCCCTAAATTTGGTTGTCTTGCAATCCTTAGTGGATTTATTTG    1620
GTTTAGGTATGTATTTATTTTAATTCCCAATGAATTCTACTAGAAATTGATAATTTCTTC    1680
CTCATGAGTAAATGATTCCCAAAAAATTTAATTATATTAAAAAAAGTTTGAGGAAATAAC    1740
AAACAATTTATACACATTGAAAGATACTGTAATTAAAAATTATTTATGTATAAAACATTC    1800
TTCAGTACAAAGAGTTTTTAAATGTGATGAATAATGCAGGTAAAGAGGACCAACAGTGTT    1860
AACTGCAGGTTTCTCAAAAAGTGGTAACAATGAAAATATGTCCAGTGAAATAATTCACAT    1920
GATTATTTCACACAAAAATAAATATCTAGTTTCATTCTCCCATTTGCTCCATTTAGAATA    1980
AACCAACTTCCTTTTTAATGTTTGTCTGTTTCCAAGATGGTAACTCCATAAACTCTTCTT    2040
```

TACTCATACCCAGCAGTTCTTGGAAATGATGAGGCTCAAGATACAGTTCAAGGCGAGTAG 2100
GATCCACACCATCAGGTAATGGTCTCTGAAGAAGTTGAGCTGGTGGATATGTTGAGCGGG 2160
TGAGACGAGCCAATTCCATTTCCACCTCTAGTACCTCTCCAGGCTTTTTCCCATCTCTAA 2220
TATTAATTTCAGCAATATCATCTCGATCTGTCCACATTGGGAAGAGGTTTGTAAATTGTA 2280
GAGGTTCCAAGCCAGCCCACACCAGGAACGCTGGCACATTAGTTGATGCTTTGTGGTCGG 2340
TATGAGAAAGATGCCAATAATCTAAAGCAGTCTGCATTGCAGCTCTGCGTTCTGCTTGCC 2400
ATCTGACTGCTCCAGAACCAGTTTGAATACTAGAATCTAAACTCTGGTCCTCAGAGTCAT 2460
AGGGCCACCATCCTTGCCAAAGCCACAACTCAGTATCATTGTCCAATAGAAAGAGAGCTG 2520
GCTGACTAGCACTATATAAATCACTCTGTAGAAAAGGGTATGCCACAGTCTCTCCTATTT 2580
GACGGTAAGGGCCAAGTATTTCAGTTGCTGTGAAGTTTCCAGATACACTACTGAAGTGGA 2640
AAAGACGTGGAGAGTGGAGCAATGGTTTAAGATGGTTTGTCAGCGATACATACAAATGTC 2700
TGTTACTGCCTCCAAGACCTTTCATAAATTCTTTGCTTTCTTCACCCTCGTGTTCTTCAT 2760
GAATTGTGATTGCCACTCCACTCTTAATTCCATATTCTGGAGCTTTACTTGTCTTTAATT 2820
TTTCTGCTGCTTTGCGAGTGACCTTGCAGGTATTTTGACTAGCTTTGGCACCATACCACA 2880
CATACAAAACTCCTGTGCTTGTATTAAGAAGTACCAATCCAGCTCTACTTCGGAAGTGTC 2940
TCGTACTACATGCTACTTGTACAAGCACGGCTTCTTCAACTCTTTCGCCACGGCAAAGGA 3000
ACAATTTCCAGGATTCTTCATGCTTGCTTTCAGTTGTAGGCTCTGATTCTCGATGCCCTG 3060
AATGAACTGTCATACCACCATCAAAAAGACGCAAGAATGCAGGGGGCTCTGTCCCTTCTA 3120
CTGCCCTTACTTGAGGCCCATGCTCACGGTCTAGTTCCACAGTAAGCAATGCTGCTGCAC 3180
CTTGCTCATTAACCGATGCATTCTGGCCCTGCCAGAAAAAGTATGCACATCGATCACGTC 3240
CGACTACAGAATGTCGAGATGGTTGACCACTGCTTAATTCACGACCTGTGACTGTTACAG 3300
AATACTGCCATCGTACTACATAACTGTCCTCTGAATGGAACTGCCCTACAGAAATTGATG 3360
GAAGTTGGGATGATTCATACTCCTGAATATGCCAGACAGAAACACCAATTGTTGAAATTT 3420
CATAATGTCTGTGAGTCTCTTCATCAAAAAATTGATTTCCACGCCCTAAATGTGTATTTT 3480
CAAGTACAAGATCTGGGTCGTTTGTATTTGGTTTCAACATTTCCTTTGCATCACAGGGTT 3540
TTACATCTATAGTGGTTTCAGTCTGTTTCTCATTTTCTTCAGATCCTTTAGTACGAATAA 3600
CTCTTGAGAAATCAGGCCAATCCAGAAATTTTTCTCGGAAAAGGATGGTTTCCATGTGTT 3660
GAGTGACACGAGCCAAGAGAGACCAGTTAGGTCTATTTGATCCACTCGCCAAGACTGTAT 3720
CATCATTTTCAACACTTCGAGAGCCTAATGATGATGAAACATTCAGAGGACACACATCAC 3780
ATTCAGAATAATCGTAGCCTTCATCCCACAGTTCTCGGGCTAGTCTCTGAGCTTGTCGGC 3840
GAGCTTCTACAGGTGCGTTTTTCCCATTCCATACATACAATTCAGATCCAAAGTCAAATA 3900
CAAGTATCTTGGCAGGGTCCAACATACTAATGCGAGGTATGCTTCCCCAGAATTCTTCCA 3960
TAGGCACTAGTTCTTCTCCTTGTACTTCATATATCATGTTAGTGGCTATCACAGAGGTTT 4020
CAAAGAGTTCATCTTCATCGGGATGTCCAGCATTAATGGGCACAGTTTTTGAATCAGAAC 4080
CCAATAATTTCCAGAAACGTTCTTGTTGTCTCAAGGAGCAAGTGGGCTTTTCTTCACTGA 4140
TAGTAGTCACCTGACTGGCACTTGTACAGCCTAAGTCTTTGTGCTGTAATATGTGCAAAG 4200
CAACTTCTGCACCTCTTGATCGTTCAATTACATTTGAGTAACGCCCAACCCAGTTGAAAA 4260
CCTCTGTTGGAGTAACAAGTACATAGTTGTCTCCAGAATTGATACTACTGGCTACTGGTT 4320

GTACTAATCTTGTCTGAACATGTCTTCTTCCTTTAATTAGCAAGAGCATAAGATCCTGAT 4380
AAGGTAACATTGCTGATGAATTGGCAACAGCCTTTTTCAACTGAACAGCTGTAAAATCCT 4440
CTTTACTGGCAAGTCCTGCAAGTGCTTCTACAGCCAAGGAAGAATGTTTTGCCAATTGTT 4500
CCACTCGTAATCTCTTCAATTCTTTATCTGCTACTCCAATTTTCACTTCTTCATATTCAT 4560
CTCTCAAATCTGTTCGTGCTTGTAATGATTTAATAGGGTTGCGAGATGTAAATTTGCGAC 4620
GTTGTATTTTGACAGAGCGCTTTTGAACAAGTAAACTAGTAGTTTGACTAACAATGTTAT 4680
CAAGATCTGTATCATTTATTTCAAGCCTTTGTTGTTGTAGTTGCAATTTTTCTGTACTGA 4740
TGCTACTGTAAAATTGAGTAAATGTTTCATCATCGGGTCTTGGAATAGCAACTTTAGCAG 4800
TAGGTGATGATTCTTCAGTTTGGACACCATTCTCATCGCCTTCAGGTGCTGAGACACTGC 4860
GTTTCAGTGTGACAAGAGGTTCTTTTTGGGGCGATGATGGAGTTGATTGGGCTTCTTTAA 4920
GCTGGGCCGTCTTGGCGCGGAAGCGCAACGGGCGCGGCTTCTTCTTCTGGCGGTCCCCGG 4980
CGCCCTCCACGGAGAGCCGCGCCATGAGCGGCGACAGGAACTCCGGCGCTGGCCCCGCCC 5040
CCGCCCTGCGCTCGGCCGAGGCGGCGTCGGCGTCGGCGTCGGCCCTGGCGCGGGCGCGCG 5100
CGAGGGCCGGGGAGGCGCGCAGGTCG 5126

C.1.7 Trehalose transporter Tret-1

dsRNA primers are highlighted in green (ds*Tret-1*.1), blue (ds *Tret-1*.2), or pink (ds *Tret-1*.3).

```
>Tret-1
TGCGCTACTTACGTTTTTATATATTGAGAGTACAATAATACTACCCTACAATATTAGGAA    60
TATATTTTATTTCTCGTGAAGAGAGAAGAAAAAAGGGTCTGCTCTCATAAGTTATAATAA    120
TATTGTGCCTCGCATGTGAAGCGATTGTTTAAAATTATTAGGATGGAGTGAACATATGTA    180
CACATATTAAAAACAGCAATCCATATATTTTAGAATTATATTTGGATACTACTTAAAGTA    240
TGCACAACGCAAAACACGCAATCTCATCGTCACTACTATTTAGCACTGATTTATATCACT    300
CATACATTCTTTCTCACAATTATTAACAGATTATAAAAAGATAACAATTTTAGGAATGTA    360
ATGGCAATATTTTAAATACAATAAATATTGACTCAATTTCTCAAACATATAAAAGTTTAC    420
TTACATTAATTATTCTATTCTTTTCTATACAATACGCAACTATTTGTACATTAAATAAAT    480
AGTAAAAAAGAAAATACATTTGGTATTGTGGGTGTGGTTGCGTTCGAATGAAGGGGGATG    540
TGCTGCAGCTCCCACCGCCTCCCGTGCTGAGGCCGCGCTGCGCGTGCAGGGCAGGGCGGA    600
TGCGTTCTTGCACTCAGCTGCGCCCCCACGAAAACACCGCAACATGGCACTCGAGAGGTT    660
GACGCCACTTCTCTTAAGCGTCCTCTTATCGCAACTTCGTTTATCGGCGTGGAGCCTCTG    720
AACTATGTTGAATTTGCTTGTTGTTGTTTTTGTTGCGGATTGGGAATTGAGGAGAAGATT    780
AATATCTTCAGATTATCAGATCTAACTCTTCCACATCCTTCGGTATACCGATACATCTCA    840
AATCTAAAGGAAAAAATAAAGAGTTCGAGCCAACTAATACTTTAACATAACGAGATTCGA    900
GTAACCGAGAGAAATAGGCCGACGTTTAGAAAAGTTGGACATTTAGAAAGGAGAATTTGA    960
CTGCTGGAACCACTATCTGCCACTCCTCATCTAGTTACAAACGCGCATTATTATTTTGAC    1020
GCAAATGTATCTGTACGTTGTGATGAGCTCTCCCTCTCCCCCTTTTCTCGTTGTCTGTCT    1080
CGCTCTCTTCCTTTCCTTTTCTCTGCCTCTCTCGCAAAATATTTGGAGAAAAATAATCGC    1140
TGTACTGATAGAGCAGAAGAGCCCAAATAACGGGTACTAATGGTTTCTTTAAAACAGGTT    1200
CTTTATGAAACGAGAAGGAGAGTAAAGAGAATTGGAGATATCGAGGAGCTCAAGGCCACA    1260
ACGTTCAAGTGTACTGCTGTTAGAGTCGAGGAGCGCTGTCAGAAGCTGGTGCACATCCTG    1320
TGCTCGGACTGCGAGCGAGTGATGGCTGGTTATGGGCAGCGCAAGCGCAGTGGGAGTCGG    1380
GACCAGCCCAGCCCCTCCATGGCCCCTCCTCTGTCTCAGTCTTTGCGTTCGCACCGGGCG    1440
AGACGCTATCTGCAGCACTACACGGCCATAGGCATAGGTTTGATGTTCGTGATCGAGCTC    1500
ATCCTCCGAACCTTGCCATTGAAGTTCTTCTCGATGGCCTCGAGACTCCTGCCCTGCGTT    1560
TCGGGCACACAGAAGATAACGAAGAACAAGCTGGTGAAGCAGACGACGCCGAACATCCAA    1620
AAGGCGCCGTACCCACCCAACGCTTCCTCGAGGTCGGTGAACGTCTTCGTGACGACGAAG    1680
GTGCACGCCCAGTTGAAAGCGGTGGCCAACGAAGCAGCGGGACCACGAATTTTGGCTGGA    1740
AAGATTTCGCCCAGCATGAGCCAGGGAATGGGCCCAAAGCCGAGGGAAAAGCCTATAACG    1800
AAGACGACGAAGCTGAGCAGCGGCAACCAGCCGTACTCGGACACGTCCACTTCAATGCTG    1860
CGCAAGTAGAAGAAGGTGCCCAGCGCGGCGAGGGAGACAGTCATGGCGATGGCGGAGATG    1920
CCCAGCAGCACGCGCCGGCCCAGCCGGTCGATGAGCACTGTGGCCAGGAAGGTGGAGCCC    1980
```

AGATTCACGATGCCCACCACGATGGTGGAGAGATTCTCGTCGATGGTGCTGCCGGCGCCC 2040
TTGAAGATCCTGACAGTGTAAAAGATGACTGCGTTGATTCCACTGAGCTGCTGAAAGAAC 2100
ATGAGGCCGATGGAGATGAAGATGGGCTTGAGGTTGCCTTTGTTGAAGAGCTCGCCGAAG 2160
GAGACGGGCGCGTTCTTGATCGCGTCGCGGTGGTTCTTCTCGATCTCGGACAGCTCCTCG 2220
CTCACGTCCACGTGCTGGCCGCCGCGCAGCCACTTCAGCGACTTCGACGCCTCCTCGTGC 2280
TTTTCTTTGGAGATGTACCATCGAGGAGTCTCCGGAATGAACAGAGTGCAGAGGAGGAAG 2340
GGCACCGGAATCATGGCGCCGAAGAAAGCTAAGTTCGACCAATTGAGGTACTTGCCCGCC 2400
AGGAAGCAGACCAAGATTCCGATGTTGCCTAGTGTTGTGGAGGTGAGGCCCAGCATGCCC 2460
CGCACCTCGGGCTGAATGGTCTCGCCCATGTAGACGGGCAGGCTGAGGGACGTGACGCCC 2520
ACGCAGAAGCCCGAGATGAAGCGGCCCGCGTACAGCCAGCCCACGTTGCTCGCGAAGAAG 2580
ATGACAAGCCACGCAATGGCGAAAGACGGCGCGGTGAGGAGGATGGTAGTCCTGCGGCCC 2640
CGCCACTCGATGAGGAAGCCGCCCGCTATCCCTCCGAACAGCGCCGCCGCCGGCATCAGG 2700
CTGCCCACCCACGAGCCCTGCGACGGAGTGACTGAGAGCGTGCTGTTGGGCTCCGCCATG 2760
GACACGAGCGCGGGCGACGTGTAGGCGGAGGAGAAGCCCACGAGCAGCGAGCCCAGCGCC 2820
ACCGCCACCGACGCGAACATCTGTCGGCCGAGGCCCTGCAGTGGCTGCGGCTGCAGCGGC 2880
TGCGAGCCGAAGCTCTGCTTGGGCGTCCCGTGGAGCAGCTCCTCGGTGACGACCGCCAGC 2940
TCCTGGCCCTTCGTCATGGCGGCGCCGCGCAGCCACTTGTATGGAGATAAGGAAACCGGA 3000
GCTGGCTTCCCAGAGCGTCTTTAGTCTTTCGCCGCTGAAGATCTACTGAATGTGAGGCAG 3060
CATTTTGAGATAGTAATCAAGGTTGTTCCAGTTTGATTGCCTGTTTGGAGTGATGCGCGC 3120
GGTCGACACTTTATTCTTCCGCTTCCTAGTGACGCGTCAAAACACTGCGTCGTCGACCAG 3180
CAGTCCGCACGTCCCGCTG 3199

Appendix D: Reprint permissions

RE: Permission to reprint article as a chapter in PhD dissertation

Wiley Global Permissions

Tue 2018-07-03 9:44 AM

To: Jantina Toxopeus

Dear Jantina,

Thank you for your email.

Permission is granted for you to use the material requested for your thesis/dissertation subject to the usual acknowledgements (author, title of material, title of book/journal, ourselves as publisher) and on the understanding that you will reapply for permission if you wish to distribute or publish your thesis/dissertation commercially.

You should also duplicate the copyright notice that appears in the Wiley publication in your use of the Material. Permission is granted solely for use in conjunction with the thesis, and the material may not be posted online separately.

Any third-party material is expressly excluded from this permission. If any material appears within the article with credit to another source, authorisation from that source must be obtained.

Many thanks,

Rights Assistant
John Wiley & Sons Ltd

WILEY

Biological Reviews

Published by Wiley on behalf of Cambridge Philosophical Society (the "Owner")

COPYRIGHT TRANSFER AGREEMENT

Date: April 20, 2018

Contributor name: Jantina Toxopeus

Contributor address:

Manuscript number: BRV-01-2018-0014.R2

Re: Manuscript entitled Mechanisms underlying insect freeze tolerance (the "Contribution")

for publication in Biological Reviews (the "Journal")

published by John Wiley & Sons Ltd ("Wiley")

Dear Contributor(s):

Thank you for submitting your Contribution for publication. In order to expedite the editing and publishing process and enable the Owner to disseminate your Contribution to the fullest extent, we need to have this Copyright Transfer Agreement executed. If the Contribution is not accepted for publication, or if the Contribution is subsequently rejected, this Agreement shall be null and void.
Publication cannot proceed without a signed copy of this Agreement.

A. COPYRIGHT

1. The Contributor assigns to the Owner, during the full term of copyright and any extensions or renewals, all copyright in and to the Contribution, and all rights therein, including but not limited to the right to publish, republish, transmit, sell, distribute and otherwise use the Contribution in whole or in part in electronic and print editions of the Journal and in derivative works throughout the world, in all languages and in all media of expression now known or later developed, and to license or permit others to do so. For the avoidance of doubt, "Contribution" is defined to only include the article submitted by the Contributor for publication in the Journal and does not extend to any supporting information submitted with or referred to in the Contribution ("Supporting Information"). To the extent that any Supporting Information is submitted to the Journal for online hosting, the Owner is granted a perpetual, non-exclusive license to host and disseminate this Supporting Information for this purpose.

2. Reproduction, posting, transmission or other distribution or use of the final Contribution in whole or in part in any medium by the Contributor as permitted by this Agreement requires a citation to the Journal suitable in form and content as follows: (Title of Article, Contributor, Journal Title and Volume/Issue, Copyright © [year], copyright owner as specified in the Journal, Publisher). Links to the final article on the publisher website are encouraged where appropriate.

B. RETAINED RIGHTS

Notwithstanding the above, the Contributor or, if applicable, the Contributor's employer, retains all proprietary rights other than copyright, such as patent rights, in any process, procedure or article of manufacture described in the Contribution.

C. PERMITTED USES BY CONTRIBUTOR

1. Submitted Version. The Owner licenses back the following rights to the Contributor in the version of the Contribution as originally submitted for publication (the "Submitted Version"):

> **a.** The right to self-archive the Submitted Version on the Contributor's personal website, place in a not for profit subject-based preprint server or repository or in a Scholarly Collaboration Network (SCN) which has signed up to the STM article sharing principles [http://www.stm-assoc.org/stm-consultations/scn-consultation-2015/] ("Compliant SCNs"), or in the Contributor's company/ institutional repository or archive. This right extends to both intranets and the Internet. The Contributor may replace the Submitted Version with the Accepted Version, after any relevant embargo period as set out in paragraph C.2(a) below has elapsed. The Contributor may wish to add a note about acceptance by the Journal and upon publication it is recommended that Contributors add a Digital Object Identifier (DOI) link back to the Final Published Version.
>
> **b.** The right to transmit, print and share copies of the Submitted Version with colleagues, including via Compliant SCNs, provided that there is no systematic distribution of the Submitted Version, e.g. posting on a listserve, network (including SCNs which have not signed up to the STM sharing principles) or automated delivery.

2. Accepted Version. The Owner licenses back the following rights to the Contributor in the version of the Contribution that has been peer-reviewed and accepted for publication, but not final (the "Accepted Version"):

> **a.** The right to self-archive the Accepted Version on the Contributor's personal website, in the Contributor's company/institutional repository or archive, in Compliant SCNs, and in not for profit subject-based repositories such as PubMed Central, subject to an embargo period of 12 months for scientific, technical and medical (STM) journals and 24 months for social science and humanities (SSH) journals following publication of the Final Published Version. There are separate arrangements with certain funding agencies governing reuse of the Accepted Version as set forth at the following website: https://authorservices.wiley.com/author-resources/Journal-Authors/licensing-open-access/open-access/func . The Contributor may not update the Accepted Version or replace it with the Final Published Version. The Accepted Version posted must contain a legend as follows: This is the accepted version of the following article: FULL CITE, which has been published in final form at [Link to final article]. This article may be used for non-commercial purposes in accordance with the Wiley Self-Archiving Policy [https://authorservices.wiley.com/author-resources/Journal-Authors/licensing-open-access/open-access/self-].

b. The right to transmit, print and share copies of the Accepted Version with colleagues, including via Compliant SCNs (in private research groups only before the embargo and publicly after), provided that there is no systematic distribution of the Accepted Version, e.g. posting on a listserve, network (including SCNs which have not signed up to the STM sharing principles) or automated delivery.

3. Final Published Version. The Owner hereby licenses back to the Contributor the following rights with respect to the final published version of the Contribution (the "Final Published Version"):

a. Copies for colleagues. The personal right of the Contributor only to send or transmit individual copies of the Final Published Version in any format to colleagues upon their specific request, and to share copies in private sharing groups in Compliant SCNs, provided no fee is charged, and further provided that there is no systematic external or public distribution of the Final Published Version, e.g. posting on a listserve, network or automated delivery.

b. Re-use in other publications. The right to re-use the Final Published Version or parts thereof for any publication authored or edited by the Contributor (excluding journal articles) where such re-used material constitutes less than half of the total material in such publication. In such case, any modifications must be accurately noted.

c. Teaching duties. The right to include the Final Published Version in teaching or training duties at the Contributor's institution/place of employment including in course packs, e-reserves, presentation at professional conferences, in-house training, or distance learning. The Final Published Version may not be used in seminars outside of normal teaching obligations (e.g. commercial seminars). Electronic posting of the Final Published Version in connection with teaching/training at the Contributor's company/institution is permitted subject to the implementation of reasonable access control mechanisms, such as user name and password. Posting the Final Published Version on the open Internet is not permitted.

d. Oral presentations. The right to make oral presentations based on the Final Published Version.

4. Article Abstracts, Figures, Tables, Artwork and Selected Text (up to 250 words).

a. Contributors may re-use unmodified abstracts for any non-commercial purpose. For online uses of the abstracts, the Owner encourages but does not require linking back to the Final Published Version.

b. Contributors may re-use figures, tables, artwork, and selected text up to 250 words from their Contributions, provided the following conditions are met:

(i) Full and accurate credit must be given to the Final Published Version.

(ii) Modifications to the figures and tables must be noted. Otherwise, no changes may be made.

(iii) The re-use may not be made for direct commercial purposes, or for financial consideration to the Contributor.

(iv) Nothing herein will permit dual publication in violation of journal ethical practices.

D. CONTRIBUTIONS OWNED BY EMPLOYER

1. If the Contribution was written by the Contributor in the course of the Contributor's employment (as a "work-made-for-hire" in the course of employment), the Contribution is owned by the company/institution which must execute this Agreement (in addition to the Contributor's signature). In such case, the company/institution

hereby assigns to the Owner, during the full term of copyright, all copyright in and to the Contribution for the full term of copyright throughout the world as specified in paragraph A above.

For company/institution-owned work, signatures cannot be collected electronically and so instead please print off this Agreement, ask the appropriate person in your company/institution to sign the Agreement as well as yourself in the space provided below, and upload the signed Agreement to the Wiley Author Services Dashboard. For production editor contact details, please visit the Journal's online author guidelines.

2. In addition to the rights specified as retained in paragraph B above and the rights granted back to the Contributor pursuant to paragraph C above, the Owner hereby grants back, without charge, to such company/institution, its subsidiaries and divisions, the right to make copies of and distribute the Final Published Version internally in print format or electronically on the Company's internal network. Copies so used may not be resold or distributed externally. However, the company/institution may include information and text from the Final Published Version as part of an information package included with software or other products offered for sale or license or included in patent applications. Posting of the Final Published Version by the company/institution on a public access website may only be done with written permission, and payment of any applicable fee(s). Also, upon payment of the applicable reprint fee, the company/institution may distribute print copies of the Final Published Version externally.

E. GOVERNMENT CONTRACTS

In the case of a Contribution prepared under U.S. Government contract or grant, the U.S. Government may reproduce, without charge, all or portions of the Contribution and may authorize others to do so, for official U.S. Government purposes only, if the U.S. Government contract or grant so requires. (U.S. Government, U.K. Government, and other government employees: see notes at end.)

F. COPYRIGHT NOTICE

The Contributor and the company/institution agree that any and all copies of the Final Published Version or any part thereof distributed or posted by them in print or electronic format as permitted herein will include the notice of copyright as stipulated in the Journal and a full citation to the Journal.

G. CONTRIBUTOR'S REPRESENTATIONS

The Contributor represents that the Contribution is the Contributor's original work, all individuals identified as Contributors actually contributed to the Contribution, and all individuals who contributed are included. If the Contribution was prepared jointly, the Contributor has informed the co-Contributors of the terms of this Agreement and has obtained their written permission to execute this Agreement on their behalf. The Contribution is submitted only to this Journal and has not been published before, has not been included in another manuscript, and is not currently under consideration or accepted for publication elsewhere. If excerpts from copyrighted works owned by third parties are included, the Contributor shall obtain written permission from the copyright owners for all uses as set forth in the standard permissions form or the Journal's Author Guidelines, and show credit to the sources in the Contribution. The Contributor also warrants that the Contribution and any submitted Supporting Information contains no libelous or unlawful statements, does not infringe upon the rights (including without limitation the copyright, patent or trademark rights) or the privacy of others, or contain material or instructions that might cause harm or injury and only utilize data that has been obtained in accordance with applicable legal requirements and Journal policies. The Contributor further warrants that there are no conflicts of interest relating to the Contribution, except as disclosed. Accordingly, the Contributor represents that the following information shall be clearly identified on the title page of the Contribution: (1) all financial and material support for the research and work; (2)

any financial interests the Contributor or any co-Contributors may have in companies or other entities that have an interest in the information in the Contribution or any submitted Supporting Information (e.g., grants, advisory boards, employment, consultancies, contracts, honoraria, royalties, expert testimony, partnerships, or stock ownership); and (3) indication of no such financial interests if appropriate.

H. USE OF INFORMATION

The Contributor acknowledges that, during the term of this Agreement and thereafter, the Owner (and Wiley where Wiley is not the Owner) may process the Contributor's personal data, including storing or transferring data outside of the country of the Contributor's residence, in order to process transactions related to this Agreement and to communicate with the Contributor. By entering into this Agreement, the Contributor agrees to the processing of the Contributor's personal data (and, where applicable, confirms that the Contributor has obtained the permission from all other contributors to process their personal data). Wiley shall comply with all applicable laws, statutes and regulations relating to data protection and privacy and shall process such personal data in accordance with Wiley's Privacy Policy located at: https://www.wiley.com/en-us/privacy.

[X] I agree to the COPYRIGHT TRANSFER AGREEMENT as shown above, consent to execution and delivery of the Copyright Transfer Agreement electronically and agree that an electronic signature shall be given the same legal force as a handwritten signature, and have obtained written permission from all other contributors to execute this Agreement on their behalf.

Contributor's signature (type name here): Jantina Toxopeus

Date: April 20, 2018

SELECT FROM OPTIONS BELOW:

[X] Contributor-owned work

[ ] U.S. Government work

Note to U.S. Government Employees
*A contribution prepared by a U.S. federal government employee as part of the employee's official duties, or which is an official U.S. government publication, is called a "U.S. government work", and is in the public domain in the United States. If the Contribution was not prepared as part of the employee's duties or is not an official U.S. government publication, it is not a U.S. government work. If **all** authors are U.S. government employees, there is no copyright to transfer and Paragraph A.1 will not apply. Contributor acknowledges that the Contribution will be published in the United States and other countries. Please sign the form to confirm Contributor Representations. If at least one author is **not** a U.S. government employee, then the non-government author should also sign the form, indicating transfer of those rights which that author has and selecting the appropriate additional ownership selection option. If more than one author is not a U.S. government employee, one may sign on behalf of the others.*

[ ] **U.K. Government work (Crown Copyright)**

Note to U.K. Government Employees

For Crown Copyright this form cannot be completed electronically and should be printed off, signed in the Contributor's signatures section above by the appropriately authorised individual and uploaded to the Wiley Author Services Dashboard. For production editor contact details please visit the Journal's online author guidelines. *The rights in a contribution prepared by an employee of a UK government department, agency or other Crown body as part of his/her official duties, or which is an official government publication, belong to the Crown and must be made available under the terms of the Open Government Licence. Contributors must ensure they comply with departmental regulations and submit the appropriate authorisation to publish. If your status as a government employee legally prevents you from signing this Agreement, please contact the Journal production editor. If this selection does not apply to at least one author in the group, this author should also sign the form, indicating transfer of those rights which that author has and selecting the appropriate additional ownership selection option. If this applies to more than one author, one may sign on behalf of the others.*

[ ] **Other**

Including Other Government work or Non-Governmental Organisation work

Note to Non-U.S., Non-U.K. Government Employees or Non-Governmental Organisation Employees

For Other Government or Non-Governmental Organisation work this form cannot be completed electronically and should be printed off, signed in the Contributor's signatures section above by the appropriately authorised individual and uploaded to the Wiley Author Services Dashboard. For production editor contact details please visit the Journal's online author guidelines. *If you are employed by the Australian Government, the World Bank, the World Health Organization, the International Monetary Fund, the European Atomic Energy Community, the Jet Propulsion Laboratory at California Institute of Technology, the Asian Development Bank, the Bank of International Settlements, or are a Canadian Government civil servant, please download a copy of the license agreement from https://authorservices.wiley.com/author-resources/Journal-Authors/licensing-open-access/licensing/licensing-inf and upload the form to the Wiley Author Services Dashboard. If your status as a government or non-governmental organisation employee legally prevents you from signing this Agreement, please contact the Journal production editor. If this selection does not apply to at least one author in the group, this author should also sign the form, indicating transfer of those rights which that author has and selecting the appropriate additional ownership selection option. If this applies to more than one author, one may sign on behalf of the others.*

Name of Government/Non-Governmental Organisation:

_____

[ ] **Company/institution owned work (made for hire in the course of employment)**

For "work made for hire" this form cannot be completed electronically and should be printed off, signed and uploaded to the Wiley Author Services Dashboard. For production editor contact details please visit the Journal's online author guidelines. *If you are an employee of Amgen, please download a copy of the company addendum from https://authorservices.wiley.com/author-resources/Journal-Authors/licensing-open-access/licensing/licensing-inf and return your signed license agreement along with the addendum. If this selection does not apply to at least*

one author in the group, this author should also sign the form, indicating transfer of those rights which that author has and selecting the appropriate additional ownership selection option. If this applies to more than one author, one may sign on behalf of the others.

Name of Company/Institution:

_____

Authorized Signature of Employer: _____

Date: _____

Signature of Employee: _____

Date: _____

Curriculum Vitae

Name: Jantina Toxopeus

Post-secondary Education and Degrees:

St. Francis Xavier University
Antigonish, Nova Scotia, Canada
2007-2011 B.Sc. (Hons) Biology

Dalhousie University
Halifax, Nova Scotia, Canada
2011-2014 M.Sc. Biology

The University of Western Ontario
London, Ontario, Canada
2014-2018 Ph.D. Biology

Select Honours and Awards:

St. Francis Xavier University President's Scholarship, 2007
Natural Sciences and Engineering Research Council of Canada (NSERC) Undergraduate Student Research Award, 2009, 2010
NSERC Julie Payette Award, 2011
Dalhousie University Killam Memorial Scholarship, 2012
NSERC Canada Graduate Scholarship-Doctoral, 2014
Company of Biologists JEB Travelling Fellowship, 2014
Orthopterists' Society Theodore J. Cohn Research Grant, 2015
Province of Ontario Graduate Scholarship (OGS), 2017

Related Work Experience:

Teaching Assistant
St. Francis Xavier University
2008-2011

Teaching Assistant, Course Instructor
Dalhousie University
2011-2014

Course Instructor
Acadia University
2013

Teaching Assistant
University of Western Ontario
2015-2017

Instructor, Teaching Assistant Training Program
Western University Centre for Teaching and Learning
2015-2018

Publications:

Toxopeus, J. and Sinclair, B.J. (2018) Mechanisms underlying insect freeze tolerance. *Biological Reviews*, 10.1111/brv.12425.

Newman, C.E., **Toxopeus, J.**, Udaka, H., Ahn, S., Martynowicz, D.M., Graether, S.P., Sinclair, B.J. and Percival-Smith, A. (2017) CRISPR-induced null alleles show that Frost protects *Drosophila melanogaster* reproduction after cold exposure. *Journal of Experimental Biology*, **220**, 3344-3354.

Des Marteaux, L.E., McKinnon, A.H, Udaka, H., **Toxopeus, J.** and Sinclair, B.J. (2017) Effects of cold-acclimation on gene expression in Fall field cricket (*Gryllus pennsylvanicus*) ionoregulatory tissues. *BMC Genomics*, **18**,357.

Andrushchenko, I.V., Taylor, B.R., **Toxopeus, J.** and Wilson, E. (2017) Congregations of the leaf-shredding insect *Lepidostoma togatum* mediate exceptionally rapid mass loss from leaf litter in Nova Scotia rivers. *Hydrobiologia*, **78**,245-265.

Toxopeus, J., Lebenzon, J.E., McKinnon, A.H and Sinclair B.J. (2016) Freeze tolerance of *Cyphoderris montrosa* (Orthoptera: Prophalangopsidae). *The Canadian Entomologist*, **148**, 668-672.

Toxopeus, J., Jakobs, R., Ferguson, L.V., Gariepy, T.D. and Sinclair, B.J. (2016) Reproductive arrest and stress resistance in winter-acclimated *Drosophila suzukii*. *Journal of Insect Physiology*, **89**, 37-51.

Toxopeus, J., Warner, A.H. and MacRae, T.H. (2014) Group 1 LEA proteins contribute to the desiccation and freeze tolerance of *Artemia franciscana* embryos. *Cell Stress & Chaperones*, **19**, 939-948.

King, A.M., **Toxopeus, J.** and MacRae, T.H. (2014) Artemin, a diapause-specific chaperone, contributes to stress tolerance of *Artemia* cysts and influences their release from females. *Journal of Experimental Biology*, **217**, 1719-1724.

King, A.M., **Toxopeus, J.** and MacRae, T.H. (2013) Functional differentiation of small heat shock proteins in diapause-destined *Artemia* embryos. *FEBS Journal*, **280**, 4761-4772.

Toxopeus, J., Kozera, C.J., O'Leary, S.J.B. and Garbary, D.J. (2011) A reclassification of *Mycophycias ascophylli* (Ascomycota) based on nuclear large ribosomal subunit DNA sequences. *Botanica Marina*, **54**, 325-334.