LOW TEMPERATURE STRESS IN THE ALFALFA LEAFCUTTING BEE, *MEGACHILE ROTUNDATA*

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Program:
Zoology

March 2017

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Dramatic changes in ambient temperature can have a significant impact on insect physiology throughout development. The accumulations of the deleterious physiological effects throughout low temperature exposure are collectively known as chill injury. The mechanisms underpinning the downstream physiological consequences of chill injury such as oxidative stress, perturbations in ion homeostasis, and changes in metabolism have yet to be elucidated. Brief, daily pulses of increased temperatures have been shown to repair and/or protect against the continued accumulation of chill injury, leading to an increase in survival across several insect taxa. Until recently, no transcriptomic-level assessments of gene expression during low temperature stress had been conducted. In this document I present a comparison of low-temperature stress response mechanisms across life stages in the alfalfa leafcutting bee, *Megachile rotundata.* RNA-seq, qPCR and oxidative stress assays were used to determine the physiological effects of low temperature exposure on two life stages: one adapted for low-temperature exposure and one that is not. Differential expression analysis revealed distinct gene expression profiles between life stages. The lack of overlap in expression profiles suggests different mechanisms are driving the response. Furthermore, an overlap in the functional classes of differentially expressed transcripts suggest that the response may be physiologically robust, even though the response is variable at the level of gene expression. Gene expression suggests oxidative stress may be a critical component in chill injury response and recovery. Antioxidant activity and lipid peroxidation, a common proxy for oxidative stress, were assessed in both life stages. *M. rotundata's* ability to cope with an induced oxidative stress did not vary between treatments in either life stage. Furthermore, a lack of statistical differences between treatments in

lipid peroxidative do not support the hypothesis that the benefits of fluctuating temperatures are, in part, due to reduction in oxidative stress.

ACKNOWLEDGMENTS

First and foremost, I would like to express my appreciation and gratitude to my advisor, Dr. Julia Bowsher, for shaping who I have become as a scientist throughout my undergraduate and graduate studies. Her consistent support for her students and enthusiasm for research, teaching, and mentorship have been critical to my success and are all qualities that I will strive for throughout my career.

I would also like to acknowledge the rest of my advisory committee, Drs. George Yocum, Kendra Greenlee, and Jason Harmon for their support and guidance throughout my doctoral studies. Your passion for science has been an inspiration throughout my time at NDSU and I am forever grateful. I'll never forget George's written comprehensive exam question asking me to come up with three alternative hypotheses to my first chapter, knowing full well that it took me six months of pouring over RNA-seq data to come up with the first one. You all expected a lot out of me and I appreciate that. In a similar vein, I'd also like to thank my fellow lab members past and present. Dacotah Melicher, Bryan Helm, Sean Nash, Kally Kvidera, Garret Slater, Bodini Herath, Paige Nash, Tanner Ferderer and many others: you have all left an impact on me throughout the years; I truly appreciate it.

Additionally I owe a debt of gratitude to those who provided support that were not directly associated with the Bowsher lab. Marnie Larson from the USDA-ARS provided a wealth technical support and training, these projects would not have been possible without her expertise and patience. Furthermore, I would like to express my gratitude to members of the USDA/NDSU insect physiology working group. This group has provided an enormous amount of feedback on a variety of projects and provided a platform for thoughtful discussion.

The work presented in this dissertation would not have been possible without significant financial contributions. The NDSU GraSUS Fellowship provided me with financial support for my first two years of study and yielded valuable teaching experience at the K-12 level. The USDA-ARS provided financial assistance in the form of several research assistantships and funding for most consumables and sequencing in all three data chapters. The NDSU College of Science and Mathematics provided several travel grants to assist in travel funds to attend conferences. The Department of Biological Sciences provided support in the form of the Sheila Kath scholarship.

Finally, I'd like to thank my family, especially my parents Hal and Linda, sister Molly, brother Andy, and my incredibly patient girlfriend Laura for their continued support as I spent an entire decade studying at NDSU. Their words of wisdom and guidance throughout this whole process have been critical to my success and completion of this final degree.

DEDICATION

This dissertation is dedicated to my grandfather, Donald (Josh) L. Rawlings.

I wish you were here for this.

If you put truth above your own desires
And value those as friends who feel the same,
If you take pride in things that you've accomplishes
And when you're wrong, stand up and take the blame....

If you can understand your limitations
And not waste time on tasks beyond your scope,
But take the future as a brand-new challenge
That you can meet with confidence and hope...

If you can listen to those who would advise you
and then judge for yourself just what is right,
If you can keep in touch with all about you
And settle differences without a fight...

If you can find delight in simple pleasures
And see the rainbow, not the falling rain,
If you can lose and never give up trying,
Believing that there's nothing done in vain...

If you can firmly stand by your convictions
And not let others set your goals for you,
If you can be as practical as needed
And still remember sometimes dreams come true...

If you can live the life that you believe in
And trust your judgment and maturity,
Then you'll be happy and successful
And the worthwhile person you are meant to be.

- E. M. Gerus

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LIST OF ABBREVIATIONS

ApoD.....Apolipoprotein D

ANOVA.....Analysis of variation

cDNA.....Complimentary DNA

Cat.....Catalase

CM.....Cell membrane

CNRQ.....Calibrated normalized relative quantity

CPY.....Cytochrome P450

DGD.....Development, growth, and differentiation

DNA.....Deoxyribonucleic acid

FTR.....Fluctuating thermal regime

GO.....Gene ontology

GPx.....Glutathione peroxidase

GST.....Glutathione s-transferase

IH.....Ion homeostasis

ILE.....Implicated in life expectancy

IMC.....Integral membrane component

IR.....Immune response

MT.....Membrane transport

NA.....Neural activity

NOS.....Nitric oxide synthase

NUPR1.....Nuclear protein 1-like

qPCR.....Quantitative real-time polymerase chain reaction

RA .....Receptor activity

SOD .....Superoxide dismutase

STR .....Static thermal regime

TS .....Transmembrane signaling

RNA .....Ribonucleic acid

RNA-seq .....High throughput mRNA sequencing

ROS.....Reactive oxygen species

TAC.....Total antioxidant capacity

TBARS.....Thiobarbituric acid reactive substances

Vg.....Vitellogenin

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CHAPTER 1: INTRODUCTION

Chill injury

Exposure to low, suboptimal temperatures can have significant, often detrimental effects on all organisms, especially ectotherms. The extent to which low temperatures can affect an ectotherm depends on the intensity and duration of the stressor as well as the animal's ability to cope with low temperatures. In insects, a common strategy to survive harsh environmental conditions such as winter is diapause: a physiological state characterized by decreased metabolic rate and resistance to temperature stress (Danks, 2006). This resilience to low-temperature stress is imperative for optimal development and can have significant effects on the quality of the adult if winters are abnormally harsh. With climate change threatening to increase temperature variance (Easterling et al., 2000; Hansen et al., 2012), insects will likely face increased bouts of unpredictable, low temperature stress throughout their life (Vasseur et al., 2014). Furthermore, those that reside in colder, more temperate regions may be increasingly affected if overall warmer ambient temperatures decrease snow cover (Bale and Hayward, 2010). With this, it is important to understand an insect's ability to cope with low temperature stress at a physiological level across all stages of development.

The physiological mechanisms driving responses to cold temperatures in insects are diverse, but, generally, the discussion of cold tolerance can be divided into two different components: freezing and chilling. Freezing, characterized by extracellular ice formation, is relatively well characterized at the physiological level (Boardman et al., 2011; Doelling et al., 2014; Findsen et al., 2013; Marshall and Sinclair, 2012; Philip et al., 2011). This field has focused on freeze avoidance through supercooling and freeze tolerance using antifreeze proteins and ice nucleating proteins, respectively (Costanzo and Lee, 2013). Chilling in insects is defined

as cooling sufficient to induce damaging effects or even death in living organisms, in the absence of freezing within the organism (Hayward et al., 2014). These damaging physiological effects of relative low temperature exposure are collectively known as chill injury. The primary symptoms of chill injury at the organismal level are loss of coordinated movements and locomotion, suggesting damage to the neuromuscular system. Currently, the responses and consequences of chilling at the physiological level are poorly understood, but perturbations in ion homeostasis, including an influx of potassium and decrease of sodium and magnesium in the hemolymph of chill-injured insects have been observed (Kostal, 2004; Kostál et al., 2006; Kostál et al., 2007). Additionally, prolonged chilling is associated with increased oxidative stress that may lead to damage (Joanisse and Storey, 1998), but the mechanism responsible for an increase in oxidative stress is unclear. These physiological responses to chilling may help to explain loss of neuromuscular function seen in chill injured insects (Lee, 2010). These observed responses have been hypothesized to be downstream consequences of membrane phase transitions (Hayward et al., 2014; Lee, 2010). While membrane phase transitions are likely to occur under low temperature exposure and could easily explain collapses in ion gradients observed in chill-injured insects, empirical evidence of this in an insect has yet to be observed.

The effects of chilling on an insect are heavily context-dependent. This dependence is both intra- and interspecific. For example, temperatures that are perceived as "cold" for an insect species in the tropics are different than those residing in more temperate regions. This interspecific variation in absolute cold tolerance may result in different mechanisms driving low-temperature stress response across species. Direct evidence of mechanistic differences are lacking, but global assessments of cold tolerance and stress responses at the "–omics" level could provide evidence for variable mechanisms across taxa and geographic location. Additionally,

cold tolerance may vary significantly throughout the development of an insect leading to intraspecific variation in responses to temperature stress. Interestingly, cold tolerance can even be variable throughout a single developmental stage in insects. For example, prior exposure to relative, moderate cold (termed "hardening") can subsequently increase an insect's ability to cope with more severe temperature stress (Coulson and Bale, 1990; Danks, 2005; Lee, 2010; Teets and Denlinger, 2013). These factors are critical to consider when extrapolating mechanistic responses to low temperature stress across species, or even within developmental stages of the same species.

Insect storage and low temperature stress

Some agriculturally important bee species are subjected to winter storage protocols for use in the following spring or summer field seasons. There are several metrics to consider when designing storage protocols for insects such as thermoperiod (Miyazaki et al., 2011) and photoperiod (Neven, 2013) to help maintain circadian and circannual cycles and thus, bee quality. Optimal insect development is highly dependent upon the temperature regimes experienced during both development and dormancy (Yocum et al., 2010). Classically, agriculturally relevant insects have been stored under constant temperatures, especially during overwintering dormancy periods when they are stored at temperatures significantly below their developmental threshold, the temperature at which the organism will begin its developmental progression. This storage protocol is known as a static thermal regime (STR).

While rearing insects under an STR protocol can be accomplished with relative ease, an STR protocol is not an ecologically accurate representation of the thermal environment that the organism has adapted to endure during dormancy. Exposure to these static, low temperatures throughout development can led to chill injury (Lee, 2010). Within the last decade, a growing

literature has emerged in favor of a fluctuating thermal regime (FTR), consisting of daily, periodic increases in temperature (Budejovice et al., 2004; Colinet et al., 2006; Rinehart et al., 2011; Yocum et al,. 2012). The implementation of these regimes has lead to increased survival, increased quality, and even extended life expectancy (Renault et al., 2004; Rinehart et al., 2011). This paradigmatic shift towards an FTR protocol represents a compromise between evolutionarily and ecologically accurate temperature conditions and the ease of storage and cost effectiveness that an STR protocol provides. A wide variety of temperature fluctuations have been used, but ensuring that the periodic increases in temperature remain below the insect's developmental threshold is critical to the success of these rearing protocols. Even with the diversity of treatments that have been used, the daily increases in temperature associated with FTR have provided beneficial effects across taxa, including Diptera (Chen and Denlinger, 1992; Koštál et al., 2016; Leopold et al., 1998; Marshall and Sinclair, 2012), Coleoptera (Renault et al., 2004), Collembola (Nedvěd et al., 1998), Hemiptera (Kostál et al., 2007), Hymenoptera (Colinet et al., 2006; Rinehart et al., 2013), Lepidoptera (Boardman et al., 2011), and Orthoptera (Jing et al., 2005). The beneficial effects of FTR exposure across taxonomic boundaries suggest that there may be a conserved mechanism driving the response.

An understanding of the biological mechanisms driving the beneficial effect of FTR exposure, relative to STR, is in its infancy, but empirical support is growing for the hypothesis that the periodic increases in temperature during chilling in the FTR protocol allow for the repair of damage caused by chill injury (Hayward et al., 2014). Alternatively, it is possible that FTR provides a type of protective effect against further damage, but evidence of this is vastly outweighed by the former (Colinet et al., 2015). FTR exposure during chilling has been shown to restore ion gradients (Kostál et al., 2006; Kostál et al., 2007), cause shifts in metabolic profiles

(Koštál et al., 2016; Lalouette et al., 2011), and decrease oxidative stress levels (Lalouette et al., 2011). With the increasing demand for efficient alternative pollinators, development of alternative storage protocols and an increased understanding of the basic, biological principles that drive these beneficial effects will allow for the ability to manipulate storage time, leading to greater flexibility in timing of emergence, and increased insect quality, allowing for the pollination of a wider variety of crops.

Megachile rotundata

Since its introduction in the mid 1940s, the Alfalfa Leafcutting Bee, *Megachile rotundata,* has become the most intensively managed solitary bee in North America and has emerged as the primary pollinator of alfalfa seed (*Mendicago sativa)*, one the of largest agricultural feed crops in the United States (Pitts-Singer and Cane, 2011). Alfalfa crop yield increases from 450 to 1,300 kg hectare^{-1} when *M. rotundata* are introduced (Pitts-Singer and Cane, 2011). With their potential to pollinate onion seed, carrot, hybrid canola, legumes, lowbush blueberry, annual clovers, and glasshouse crops and their development as an emerging alternative pollinator (Pitts-Singer and Cane, 2011), their proper storage and management is highly important. An improved understanding of the physiological responses to various rearing protocols and their effect on adult performance will allow for increased control in matching emergence time to peak floral bloom of crops.

Adult *M. rotundata* generally emerge during late summer. Soon after emergence, the females mate once and begin provisioning brood cells (Pitts-Singer and Cane, 2011). These brood cells are generally constructed in previously existing holes above ground and are partitioned using disk-shaped leaf pieces cut using the female's mandibles (Klostermeyer, 1969). This holometabolous insect experiences a rapid development with embryogenesis spanning 2-3

days. Embryogenesis is followed by four larval instars, where the immature individuals consume a liquid diet, and a fifth instar where it eats the entire nutritional provision provided by the female during construction of the brood cell. The fifth instar larvae then defecate and enter a diapause state as prepupae (Michener, 2007). This diapause state, characterized by enhanced stress tolerance, entails molecularly, physiologically, and morphologically important changes (MacRae, 2010). These individuals generally diapause over winter and resume development as temperatures warm in late spring or early summer (Pitts-Singer and Cane, 2011). Alternatively, this facultatively bivoltine species may forgo diapause, yielding a second generation before late summer (Krunic, 1972). The environmental cues and mechanisms that control diapause initiation and bivoltinism are not well understood, but photoperiod (Kemp and Bosch, 2001), extreme environmental stresses (Pankiw et al., 1980), and poor larval nutrition (Parker & Tepedino, 1982) have been suggested.

Megachile rotundata as a model for low temperature stress physiology

Megachile rotundata provides a unique system to take an integrative approach to study low temperature stress across multiple levels of organization. Because *M. rotundata* is such a valuable alternative pollinator, we have extensive knowledge about how to rear these animals throughout development and ample data about how different rearing practices can affect survival and adult performance (Bennett et al., 2013; Pitts-Singer and James, 2009; Rinehart et al., 2011; Trostle and Torchio, 1994; Yocum et al., 2005; Yocum et al., 2010; Yocum et al., 2011). Importantly, knowledge about this animal's cold tolerance and physiological responses to cold stress has increased in recent years (Bennett et al., 2013; Kemp et al., 2004; O'Neill et al., 2011; Rinehart et al., 2013; Yocum et al., 2006). Additionally, *M. rotundata* has a sequenced genome, allowing for the assessment of changes in gene expression when looking at low temperature

stress responses, including the comparison of STR and FTR. The inclusion of gene expression data with what is already known about these responses at the physiological level yields *M. rotundata* as a valuable model for integrative physiology to understand these responses across multiple levels of biological organization.

The benefits of FTR against standard rearing practices are present in *M. rotundata* in both life stages where it has been implemented so far. This phenomenon allows for me to examine whether or not FTR rearing protocols will exhibit similar reparative/protective mechanisms across life stages in *M. rotundata*. Currently, our understanding of the benefits of FTR exposure in chill-injured insects is an amalgam of discrete studies in diverse taxa of insects. Evidence for a conserved mechanism driving these responses across life stages in *M. rotundata* would suggest that the similarities that have been observed at the physiological level in other species are likely a result of similar mechanisms. However, if there is a lack of evidence to support the hypothesis that FTR acts using a conserved mechanism across taxa, which is the current paradigm, the field's ability to extrapolate experimental observations across taxonomic boundries will be significantly diminished.

Broader Impacts: The pollination crisis

With three quarters of the world's food crops depending on pollination by animals, mainly insects (Tylianakis, 2013), pollinator survival and diversity is of paramount importance. The Western honeybee (*Apis mellifera*) is the single most valuable pollinator to agricultural systems (Williams et al., 2010). Because of their ease of maintenance and transportability, some estimates suggest that the honeybee is responsible for 80 percent of the food crops pollinated by insects (USDA-ARS). A lack of pollinator diversity has arisen due to heavy dependence on the honeybee for the pollination of our food crops.

Over the past decade a phenomenon termed Colony Collapse Disorder (CCD), characterized by the rapid, unexplained disappearance of 30-90 % of adult honeybees, has created a pollination crisis (Vanengelsdorp et al., 2009). During the winters of 2006-2007 and 2007-2008, the United States experiences significant bee morbidities (VanEngelsdorp, 2007; 2008). Colonies affected during this period were characterized by a common set of symptoms including the rapid loss of adult worker bees, a noticeable lack of dead workers both within and surrounding affected hives, and delayed invasion of hive pests (Cox-Foster et al., 2007).

Colony collapse disorder has been attributed to a number of different causes including pathogens (Rosenkranz et al., 2010), parasites (Core et al., 2012), poor management (Anderson et al., 2011), environmental stressors such as neonicotinoid pesticides (Cresswell et al., 2012) and anthropogenically induced climate change (Schweiger et al., 2010), which may be acting singly or in combination to cause CCD (Rogers, 2007). With significant decreases in honeybee populations and sub-optimal pollination of some crops, the demand for alternative pollinators has increased dramatically over the recent decade (Davidson et al., 2010). With CCD plaguing honeybee populations, farming with alternative pollinators as been suggested as a more reliable option to the current agricultural pollination practices (Christmann and Aw-Hassan, 2012). An increased understanding of physiological responses to low temperatures will allow managers to dramatically improve management protocols for alternative pollinators, decreasing pressure on the currently decreasing honeybee populations and increasing the economic benefit of more efficient alternative pollinators.

Objectives

My research goal was to characterize the physiological mechanisms responsible for the beneficial effects of FTR exposure across life stages in *M. rotundata*. First, characterized the

transcriptional profiles of FTR-reared individuals relative to those reared under a normal STR protocol during an extended overwintering period. Second, assessed changes in gene expression between STR and FTR protocols during a weeklong low temperature stress in developing pupae, a life stage that likely has little capacity for cold tolerance. Finally, used the knowledge I gained from the gene expression data in objectives 1 and 2 to assess responses at the physiological level.

Objective 1: Identify transcripts involved in FTR response in prepupae

M. rotundata exposed to an FTR protocol consisting of a one hour pulse of 20°C each day experience a significant increase in survival during extended overwintering than those reared under the standard, constant 6°C. The first objective of this dissertation is to characterize the gene expression profiles of bees reared under both thermal treatments. Post-diapause quiescent prepupae were sampled at two different time points, before and after mortality began to diverge between treatment groups, and transcriptome profiling was performed using high-throughput mRNA sequencing (RNA-seq). Differential expression analysis between groups was used to identify specific identities and functional classes of transcripts to generate hypotheses associated with the specific mechanisms involved in driving the beneficial effect of FTR and their association with chill injury. This data provides the first description of the transcriptional components that drive the differences in life expectancy between individuals reared under FTR and STR protocols in any species.

Objective 2: Identify transcripts involved in FTR response in pupae

In addition to post-diapause quiescent prepupae (Objective 1), FTR exposure also provides a protective effect when *M. rotundata* are exposed to low temperature stress during pupal development. In agricultural practices, when the emergence of these bees will not by synchronized with peak floral bloom, managers frequently drop ambient temperatures to

constant, low temperatures to delay emergence. This practice results in diminished flight capacity and behavioral abnormalities in emerged adults. Interestingly, treatment with a similar FTR protocol to Objective 1 can diminish these sublethal effects. The second objective will be to assess gene expression patterns after seven days of low temperature treatment to compare STR and FTR rearing protocols. I hypothesize that, developing prepupae have a diminished capacity for low temperature stress, and predict that they will exhibit vastly different gene expression profiles to those observed in Objective 1.

Objective 3: Integrate gene expression data with physiological response across life stages

Chill injury is characterized by a variety of downstream physiological responses such as metabolic imbalance, disruptions in ion homeostasis, and oxidative stress. Two previous assessments of transcriptomic-level responses to FTR, relative to constant chilling, in the Alfalfa Leafcutting Bee, *Megachile rotundata* show little overlap in expression profiles across life stages, suggesting discrete mechanisms driving the beneficial effects of FTR across developmental stages (Objectives 1 and 2). Although gene expression across life stages is highly variable, several functional classes of differentially expressed transcripts were still shared across life stages, including those that suggest an oxidative stress response. For this objective, I will present assessments of antioxidant gene expression, total antioxidant activity, and lipid peroxidation between FTR- and STR-exposed individuals in both prepupae exposed to an extended overwintering and pupa exposed to a cold snap during spring development. The combination of these four assays will seek to answer two questions: 1) Do individuals within each treatment vary in their ability to combat an oxidative stressor? and 2) Does FTR diminish damage caused by oxidative stress in either life stage?

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CHAPTER 2: TRANSCRIPTIONAL RESPONSES TO FTR IN PREPUPAE[1]

Abstract

The transcriptional responses of insects to long-term, ecologically relevant temperature stress are poorly understood. Long-term low temperature exposure, commonly referred to as chilling, can lead to physiological effects collectively known as chill injury. Periodically increasing temperatures during long-term chilling has been shown to increase survival in many insects. However, the transcripts responsible for this increase in survival have never been characterized. Here we present the first transcriptome-level analysis of increased longevity under fluctuating temperatures during chilling. Overwintering post-diapause quiescent alfalfa leafcutting bees, *Megachile rotundata,* were exposed to a constant temperature of 6°C, or 6°C with a daily fluctuation to 20°C. RNA was collected at two different time points, before and after mortality rates began to diverge between temperature treatments. Expression analysis identified differentially regulated transcripts between pairwise comparisons of both treatments and time points. Transcripts functioning in ion homeostasis, metabolic pathways, and oxidative stress response were up-regulated in individuals exposed to periodic temperature fluctuations during chilling. The differential expression of these transcripts provide support for the hypotheses that fluctuating temperatures protect against chill injury by reducing oxidative stress and returning ion concentrations and metabolic function to more favorable levels. Additionally, exposure to

[1] The material in this chapter was co-authored by Alex S. Torson, George D. Yocum, Joseph P. Rinehart, William P. Kemp, and Julia H. Bowsher and published in the Journal of Experimental Biology in April 2015 under the title "Transcriptional responses to fluctuating thermal regimes underpinning differences in survival in the solitary bee *Megachile rotundata*" and adapted here with permission. Alex S. Torson had primary responsibility for all of the methods described in this chapter including processing and analysis of RNA-seq data and qPCR validation. Alex S. Torson drafted and revised all versions of this chapter. Julia H. Bowsher proofread the text written by Alex S. Torson.

fluctuating temperatures lead to increased expression of transcripts functioning in immune response and neurogenesis, providing evidence for additional mechanisms associated with increased survival during chilling in *M. rotundata*.

Introduction

Insects have evolved the ability to cope with harsh, long-term environmental conditions by using an array of physiological and behavioral responses. One of the most common defenses against unfavorable environmental conditions is the physiological state known as diapause, a life-history strategy characterized by diminished metabolic activity and a hiatus in development. While diapause offers a level of protection against seasonal stress, such as cold winter temperatures, exposure can still take a physiological toll when temperatures are abnormally low or when winter lasts for a long time (Hayward et al., 2014; Renault et al., 2004; Teets and Denlinger, 2013).

Exposure to low temperatures, also known as chilling, can have a wide range of physiological effects (Teets and Denlinger, 2013). The physiological effects associated with long-term chilling, such as exposure to winter conditions, are known as indirect chill injuries and have been associated with gradual failure of homeostatic processes (Lee, 2010). The downstream consequences of this phenomenon are likely complex, but the disruption of ion homeostasis and metabolic imbalance are likely outcomes (Kostal, 2004; Kostál et al., 2006). Additionally, chill injury has been associated with the occurrence of oxidative stress (Lalouette et al., 2011; Rojas and Leopold, 1996).

While the accumulation of chill injuries can have a significant effect on the well being of the insect, periodically increasing temperatures during long-term chilling can allow for an increase in survival (Colinet et al., 2006; Coulson and Bale, 1996; Rinehart et al., 2011; Rinehart

et al., 2013). These periodic increases in temperature, commonly known as fluctuating thermal regimes (FTR), have been implicated in the repair of, or protection against, chill injury during exposure to low temperatures and have been observed across insect life stages and taxa (Kostál et al., 2007; Renault et al., 2004). Despite the common physiological mechanism associated with chill injury and its repair, few studies have measured the chill injury response at the level of gene expression (Hayward et al., 2014).

The alfalfa leafcutting bee, *Megachile rotundata,* widely used in alfalfa seed production agroecosystems, provides an excellent model for assessing changes in gene expression during chilling. Adult *M. rotundata* generally emerge during early summer. Soon after emergence, the females mate once and begin provisioning brood cells (Pitts-Singer and Cane, 2011). Larvae develop within their brood cells until the fifth instar. At this point, they defecate and enter diapause as prepupae and remain dormant through the winter (Michener, 2007). Development resumes as temperatures warm in late spring or early summer; bees pupate and later emerge as adults (Pitts-Singer and Cane, 2011). Diapause in *M. rotundata* often terminates before harsh environmental conditions end and is followed by a physiological state known as post-diapause quiescence (Yocum et al., 2005; Yocum et al., 2006). While quiescent individuals are responsive to warmer temperatures, they maintain the cold tolerance characteristic of diapause (Hayward et al., 2005). Quiescent individuals may be aroused to continue development, but will stay quiescent until more favorable conditions arise (Kostál, 2006).

The harsh winter temperatures experienced by *M. rotundata* during diapause and post-diapause quiescence have the potential to cause chill injury in both natural populations and commercial-use bees. In managed populations, diapausing individuals are typically overwintered at a constant temperature of 4-6°C, (Pitts-Singer and Cane, 2011). Individuals reared under

constant, low, temperatures over long periods of time likely experience an accumulation of chill injuries, eventually leading to an increase in mortality (Kostal, 2004; Kostál et al., 2007). When exposed to daily fluctuations to a warmer temperature (20°C) during chilling, *M. rotundata* shows a dramatic decrease in mortality, and this decrease is maintained for many months (Rinehart et al., 2013). After extended exposure to fluctuating temperatures, emerging adults have no significant differences in quality compared to control (Bennett et al., 2013; Rinehart et al., 2013). This result suggests fluctuating temperatures provide a protective effect during chilling, alleviating sub-lethal effects associated with long-term low constant temperature exposure. We hypothesized that (1) long-term exposure to low temperatures causes chill injury and that subsequent exposure to fluctuating temperatures provides a protective effect by repairing and/or diminishing the effect of chill injury and that (2) this protective effect is a result of a decrease in oxidative stress and a return to more favorable ion concentrations and metabolic rates.

In this study, we screened for the transcripts responsible for the differences in survival between individuals reared under either constant or fluctuating temperatures. RNA samples extracted from post-diapause quiescent prepupae were harvested from individuals reared under both temperature treatments before and after mortality rates began to diverge. Treatment and time specific transcriptome profiles were assessed using RNA-seq on the Illumina platform. We predicted (1) that a larger quantity of transcripts would be differentially expressed in late-sampled individuals, after mortality diverged and (2) that transcripts functioning in a stress response (i.e. oxidative stress pathways) and those functioning in diminishing other sub-lethal effects associated with chill injury would be up-regulated in individuals exposed to fluctuating temperatures.

Results

Transcriptome assembly

Individuals were harvested for RNA-seq at two time points: (1) after 12 months in storage, before mortality rates diverge between temperature treatments and (2) two months later, after mortality had significantly decreased under constant temperatures. (Fig. 2.1A; modified from Rinehart et al., 2013). For simplicity, individuals selected before mortality diverged will be referred to as "early" and those selected after mortality diverged will be referred to as "late" (Fig. 2.1B). Exposure to constant temperatures will be referred to as STR (Static Thermal Regime), and exposure to fluctuating temperatures as FTR (Fluctuating Thermal Regime) in all subsequent figures and tables.

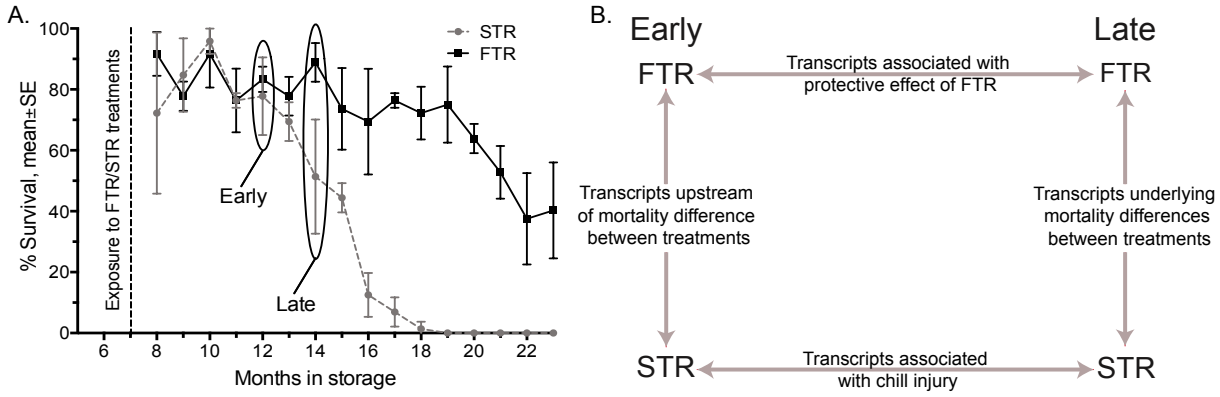

Figure 2.1. Survival rates under temperature treatments and experimental comparisons. (A) Post-diapause quiescent prepupae exposed to fluctuating temperatures (black, FTR) have higher survival than those exposure to constant 6°C (grey, STR; Modified from Rinehart et al., 2013). The fluctuating temperature protocol consisted of 21 hrs at 6°C, with a daily 1 hr pulse at 20°C, and two 1 hr ramps. Survival is defined as successful development and emergence as an adult. RNA seq samples were collected after 12 months in storage (early) and again two months later (late), as indicated by circles. (B) All differential expression pair-wise comparisons and primary goal of each comparison.

Twelve individual RNA-seq libraries (Accession number: SRP047335) consisting of an early and late time point for each treatment with three biological replicates for each were generated, averaging 62,774,914 pair-end reads per library (Table 2.1). Both the percentage of

raw reads mapped and the percentages of reads properly paired with its mate were used as initial quality metrics of the assembly. On average, approximately 80% of the raw reads generated mapped to the *M. rotundata* genome (Accession number: PRJNA66515); of these 88%, on average, could be properly paired with its mate (Table 2.1).

Table 2.1. Assembly statistics.

Biological replicate	Total PE reads	Reads mapped to genome	% of reads mapped	% of reads properly paired	% Singletons
Early_STR_1	64,560,308	51,204,141	79.31%	88.90%	6.86%
Early_STR_2	54,782,127	42,599,350	77.76%	88.99%	7.19%
Early_STR_3	59,506,886	47,458,404	79.75%	88.65%	7.04%
Early_FTR_1	37,978,827	29,176,612	76.82%	86.31%	7.58%
Early_FTR_2	63,582,685	49,534,551	77.91%	88.65%	7.12%
Early_FTR_3	53,545,275	42,999,071	80.30%	88.25%	7.12%
Late_STR_1	70,934,170	59,354,375	83.68%	87.48%	6.80%
Late_STR_2	69,922,574	56,056,522	80.17%	87.88%	6.89%
Late_STR_3	79,473,779	64,792,233	81.53%	88.27%	6.69%
Late_FTR_1	51,322,443	40,532,597	78.98%	87.99%	7.30%
Late_FTR_2	65,924,838	51,883,396	78.70%	88.18%	7.00%
Late_FTR_3	81,765,052	67,250,268	82.25%	86.33%	7.32%
Average:	62,774,914	50,236,793	79.76%	87.99%	7.08%

Raw, paired-end (PE) RNA-seq reads from each biological replicate were mapped to the *M. rotundata* genome. Each biological replicate was RNA from a single individual.

Differential expression analysis

Four pair-wise comparisons were deemed biologically relevant because they either directly compare expression profiles of one treatment at two time points or compare the differences between two treatments in the same time point (Fig. 2.1B). Differential expression analysis was conducted using the Tuxedo protocol (Trapnell et al., 2012). Transcripts with an absolute value greater than or equal to $\log_2 2$, (α=0.05) were selected for downstream analysis. With this conservative significance threshold, 287 differentially expressed genes were identified among the four biologically relevant comparisons. We confirmed 79 comparisons (both differentially expressed and not) from the expression analysis using quantitative real-time PCR

(qPCR; Table A.1). RNA samples assessed for qPCR validation originated from different individuals than the ones subjected to RNA-seq, providing an independent confirmation of expression differences.

In differential expression analysis, multiple transcripts may represent a single gene locus. The Tuxedo protocol averages expression values for each transcript from a gene to give the fold change value for the gene (i.e. each gene is an average of all transcripts at that locus). To include the identities of each individual transcript in downstream analyses (319 among all four biologically-relevant comparisons), each transcript was treated as a unique entity for analysis and is included in all following iterations and numerical representations of the data.

Quantification of differentially expressed transcripts

The expression profiles show a clear asymmetry in the distribution of differentially expressed transcripts among comparisons (Fig. 2.2). The largest number of differentially expressed transcripts came from the comparison of individuals exposed to either constant or fluctuating temperatures *before* mortality diverged (Fig. 2.2A). We had predicted that the greatest number of differentially expressed transcripts would occur after mortality diverged because of the marked difference in mortality at that time. However, only 63 transcripts were differentially expressed at the later time point. The larger number of differentially expressed transcripts in the early comparison (217) indicates that expression profiles are changing months before observable changes in bee quality.

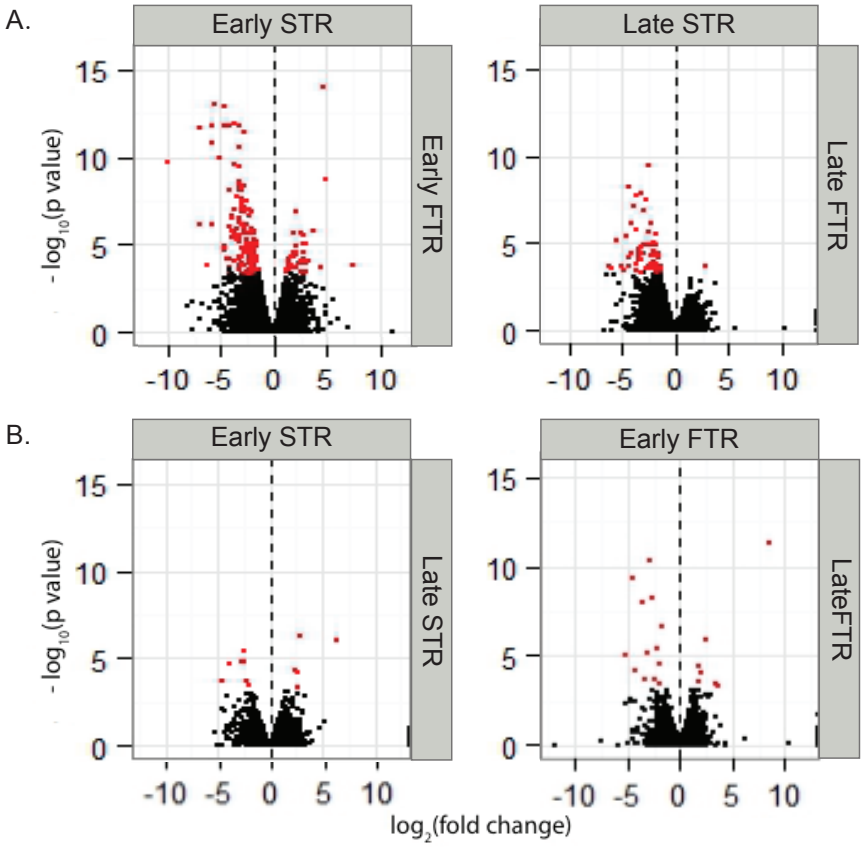

Figure 2.2. Differential expression profiles between temperature treatments. Transcripts were plotted by fold change (log_2) in expression and the log of the p-value for each comparison. Red points indicate transcripts that have significantly different expression between samples. Statistical significance was set at $log_2 2$, (α=0.05). (A) Within-time point comparisons (B) Within-treatment comparisons.

Few differentially expressed transcripts were identified for the within-treatment comparisons (i.e. Early STR vs. Late STR) for both constant and fluctuating temperatures (12 and 22 transcripts respectively; Fig. 2.2B). The low number of differentially expressed transcripts indicates that the profiles of samples sequenced in each treatment over the two dates are relatively static between the time points sampled. This stasis could be due to either the emergence of large-scale differences before the time-points sampled, or relatively few transcripts regulating the physiological response to the temperature treatments.

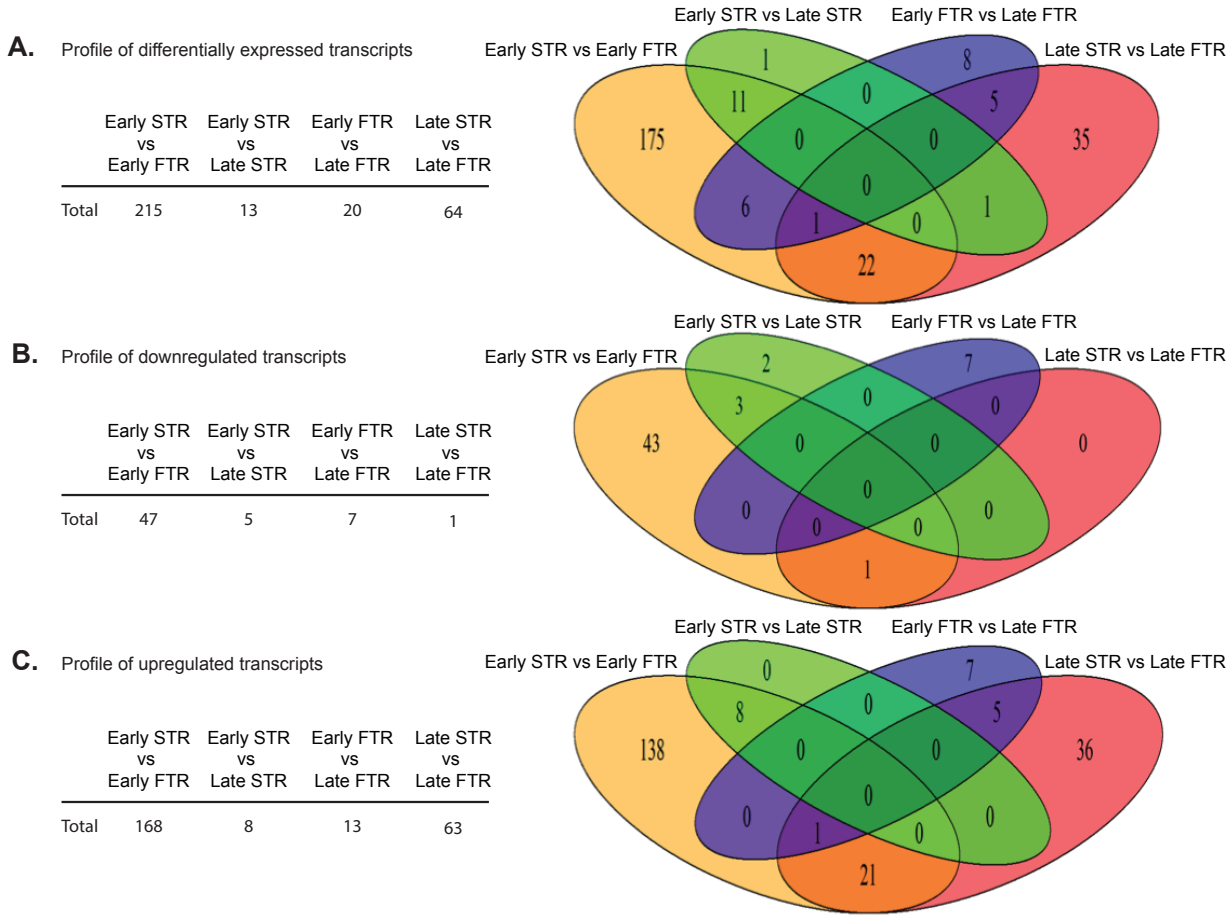

Figure 2.3. Overlap of differentially expressed transcript profiles among biologically relevant comparisons (A) Overall profile of differentially expressed transcripts, (B) profile of down-regulated transcripts, (C) profile of up-regulated transcripts. The direction of up- or down-regulation is relative to the second treatment in each comparison (e.g. in the Early STR vs. Early FTR comparison, up- or down-regulation is relative to Early FTR). When looking at interactions between comparisons, the same holds true; the interaction between, for example, up-regulated transcripts in Early STR vs. Early FTR and Late STR vs. Late FTR identifies transcripts that are up-regulated in both Early FTR and Late FTR. The numbers of transcripts in (B) and (C) do not necessarily add up to the totals shown in (A) because directionality of regulation is not taken into consideration in (A) and a transcript shared in that comparison may be up-regulated in one comparison and down-regulated in the other. The tables to the left of each Venn diagram indicate total number of differentially expressed transcripts in each comparison.

Venn Diagrams were used to identify differentially expressed transcripts among the four biologically relevant comparisons that were unique either temperature treatments or time points sampled (Fig. 2.3). Differentially expressed transcripts in Early STR vs. Early FTR, the

comparison with highest distribution of differentially expressed transcripts (67.8%), shared 23 of its 215 differentially expressed transcripts with the Late STR vs. Late FTR comparison (Fig. 2.3A). These 23 transcripts (22 up-regulated, 1 down-regulated) are specific to individuals reared under fluctuating temperatures before and after mortality diverged (Fig. 2.3A). Because the direction of regulation is relative to the second treatment in each comparison, an FTR-specific up-regulated transcript is also a down-regulated STR-specific transcript.

Gene ontology

A gene ontology (GO) analysis, conducted using Blast2GO (Conesa et al., 2005), revealed an abundance of transcripts functioning in oxidative stress, various metabolic functions, ion homeostasis, neurogenesis immune response, and several functioning in growth and development (Table 2.2; A.2). These transcripts are up-regulated before mortality diverged between temperature treatments. An ontological analysis of the 63 differentially expressed transcripts after mortality diverged (Late STR vs. Late FTR) shows an up-regulation of transcripts functioning in similar GO classes to the earlier comparison (Early STR vs. Early FTR). Twenty-three of the 63 differentially expressed transcripts (36.5%) in late-sampled individuals were also differentially expressed in early-sampled individuals (Table A.3).

Discussion

Chilling can be deadly to insects, but periodic warm pulses during chilling can partially alleviate the damage of chill injury. The gene expression changes associated with the protective effects of these warm pulses are unknown. Assessing gene expression changes during chilling will provide validation of the already-established physiological responses and may elucidate additional mechanisms that have not been previously identified. Here we present the first transcriptome-level analysis of increased survival under fluctuating temperatures during chilling.

Megachile rotundata were exposed to either a constant, low temperature (6°C) or the same low temperature with a daily fluctuation in temperature to 20°C. When reared under these conditions, individuals exposed to daily temperature fluctuations show a marked increase in survival, suggesting some type of physiological benefit to periodic deviations away from constant, low temperature exposure. We hypothesized that prolonged exposure to a constant temperature leads to indirect chill injury in *M. rotundata*, and further, that the increase in survival in bees that were exposed to fluctuations in temperature during post-diapause quiescence would be the result of a decrease in the physiological stressors associated with chill injury.

First, we predicted that more transcripts would be differentially expressed in late-sampled individuals, after mortality diverged. However, differential expression analysis reveals an opposite trend; the greatest quantity of differentially expressed transcripts occurred in early-sampled individuals, before any significant differences in mortality were observed. This result suggests that sub-lethal effects accumulate *prior* to significant mortality in the constant-temperature-reared population. The relatively small within-treatment changes in expression profiles between the two time points, in both FTR and STR, also support the hypothesis that the physiological mechanisms responsible for counteracting the accumulation of sub-lethal effects are set into motion earlier in extended post-diapause quiescence. Additional RNA-seq sampling or extensive quantitative real-time PCR (qPCR), over a longer developmental interval, starting farther upstream from this analysis, would be necessary to validate this hypothesis.

Our second prediction was that transcripts functioning in a stress response and those functioning in diminishing other sub-lethal effects associated with chill injury would be up-regulated as a result of exposure to fluctuating temperatures. We discovered transcripts functioning in ion and metabolic imbalance, which have been hypothesized to result in sub-lethal

effects, and several with antioxidant properties; results in concert with our initial predictions. Interestingly, several other classes of transcripts were up-regulated in individuals reared under constant temperatures, suggesting additional mechanisms for coping with stresses related to long-term low temperature exposure.

Ion and metabolic imbalance resulting from membrane phase transitions

Chill injury and subsequent mortality have been associated with aberrations in ion homeostasis and metabolic imbalance in several insect species (Kostál, 2006; Kostál et al., 2007; Lalouette et al., 2011; Macmillan et al., 2012). Disturbances in ion homeostasis could help to explain some of the large-scale physiological effects seen in chill-injured insects such as abnormal muscle contraction (Yocum et al., 1994) and atypical neuronal function (Hosler et al., 2000).

Our GO analysis revealed that an abundance of transcripts involved in counteracting disruptions of ion homeostasis and energy metabolism (including lipid metabolism; Tables 2.2, A.2), both of which have been implicated as molecular components of chilling injury physiology (Hayward et al., 2014). The return of more favorable concentrations of ions within the organisms exposed to fluctuations in temperature may provide protection against failure of neuromuscular coordination in chill-injured individuals. These results suggest that the up-regulation of these transcripts under exposure to fluctuating temperatures may allow for protection against, or repair of, chill injury and, thus, lead to an increase in survival.

Table 2.2. Gene ontology of FTR transcripts.

Function	Transcript	Fold change	Transcript ID
OS	eater	5.68004	MROT_00005566
OS	cytochrome p450 9e2	5.1244	MROT_00000298
OS	cytochrome p450	4.33939	MROT_00002167
OS	peroxidase	3.77461	MROT_00000781
OS	cytochrome p450 9e2-like	3.66962	MROT_00002409
OS	cytochrome p450 6k1-like	3.09328	MROT_00001509
OS	apolipoprotein d-like	3.07233	MROT_00001974
OS	cytochrome p450 9e2	2.98918	MROT_00006118
OS	probable cytochrome p450 304a1-like	2.95969	MROT_00000630
OS	short-chain dehydrogenase reductase family 16c member 6-like	2.91369	MROT_00005918
OS	vitellogenin	2.82886	MROT_00006912
OS	glutathione s-transferase	1.79271	MROT_00006349
OS	nuclear protein 1-like	1.40276	MROT_00003724
NA	neuroligin- x-linked	6.91741	MROT_00001836
NA	neuroligin- y-linked	6.79666	MROT_00010474
NA	low quality protein: dynein heavy chain axonemal-like	6.14084	MROT_00005641
NA	neuropilin and tolloid-like protein 2	3.98551	MROT_00010652
NA	neuropilin and tolloid-like protein 2-like	3.98551	MROT_00004515
NA	synaptic vesicle glycoprotein 2b-like	3.70427	MROT_00004806
NA	neurexin isoform e	3.63132	MROT_00005162
NA	slit homolog 1	3.30025	MROT_00008863
NA	roundabout-like protein 1	3.25388	MROT_00005643
NA	division abnormally delayed	2.64979	MROT_00001457
NA	vasodilator-stimulated phosphoprotein	2.61012	MROT_00002561
NA	growth differentiation factor partial	2.42894	MROT_00002522
NA	growth differentiation factor 11	2.42894	MROT_00002523
NA	cathepsin l-like	1.82219	MROT_00002252
DGD	multiple epidermal growth factor-like domains 10	5.68004	MROT_00010746
DGD	myosin light chain 2	2.92318	MROT_00005203
DGD	low density lipoprotein	2.33347	MROT_00004517
DGD	juvenile hormone epoxide hydrolase 1-like	2.13856	MROT_00008229
DGD	calponin transgelin	1.97253	MROT_00004619
DGD	growth differentiation factor 8-like	1.82621	MROT_00001549
DGD	actin-related protein 2 3 complex subunit 3-like	1.81918	MROT_00007141
DGD	myosin heavy muscle isoform 1	1.63627	MROT_00007351
DGD	dynein light chain cytoplasmic-like	1.54322	MROT_00009419
IR	melanization-related protein	4.50645	MROT_00000056
IR	protein toll	2.63557	MROT_00000828
IR	gram-negative bacteria-binding protein 1-2	2.50276	MROT_00001750
IR	lysozyme 3-like	2.05522	MROT_00007603
IR	serine protease snake-like	1.79965	MROT_00003557
IH	solute carrier family 41 member 2-like	3.73825	MROT_00002378
IH	transferrin	3.1507	MROT_00006050
IH	cysteine dioxygenase type 1-like	3.07038	MROT_00002818
IH	high affinity camp-specific 3 -cyclic phosphodiesterase 7a	2.79103	MROT_00010152
IH	muscle lim protein mlp84b-like	2.33141	MROT_00005271
IH	ring finger-containing	2.21141	MROT_00009879
IH	ring-h2 finger protein atl80-like	1.95467	MROT_00005615
IH	sparc	1.91621	MROT_00009818
IH	sodium hydrogen exchanger 7-like	1.77648	MROT_00000887

Table 2.2. Gene ontology of FTR transcripts (continued).

Function	Transcript	Fold change	Transcript ID
IH	acidic mammalian chitinase-like	1.75997	MROT_00007455
IH	1-phosphatidylinositol- -bisphosphate phosphodiesterase-like	1.74737	MROT_00001826
IH	inositol -trisphosphate receptor	1.59498	MROT_00009217
ILE	dna fragmentation factor subunit beta-like	2.60731	MROT_00002328
ILE	creb atf bzip transcription	1.63111	MROT_00006656

Gene ontology of transcripts up-regulated in early-sampled individuals exposed to fluctuating temperatures. OS – oxidative stress; NA – neural activity; DGD – development, growth, and differentiation; IR – immune response; IH – ion homeostasis; ILE – implicated in life expectancy. These transcripts are a subset of all differentially expressed transcripts. Fold change values are $\log_2$ transformed.

Neurological benefits of fluctuating temperatures

Traditionally, the most obvious symptom of chill injury has been loss of coordination such as defects in crawling behaviors (Hazell and Bale, 2011). This physiological impact suggests a neurological consequence of chilling. In individuals exposed to fluctuating temperatures during quiescence, a total of 14 transcripts (Table 2.2) functioning in neurological patterning and development were up-regulated in early sampled individuals and either were maintained or up-regulated further after mortality rates began to diverge. The maintenance of these expression profiles through the two time points suggests, again, that these transcripts are likely playing a role over time, even when mortality has already diverged.

The up-regulation of these transcripts in fluctuating temperature-reared individuals suggest one of two mechanisms: either these transcripts are functioning in the repair of damage cause by chill injury or they are functioning in normal neurological development and exposure to constant chilling causes a phenocopy defect, impeding normal developmental processes during this life stage. Phenocopy defects were originally used to describe morphological deformities in *Drosophila* exposed to stressful environmental conditions during development (Goldschmidt, 1935). Phenocopy defects are common under high temperature stress (Mitchell and Lipps, 1978;

Mitchell et al., 1979). The mechanisms responsible for high- and low-temperature stress physiology show some overlap (Yocum et al., 1994), indicating that decreased survival in chill injured individuals may be the result of phenocopy defects. A phenocopy-defect-centric hypothesis would suggest that the suspension of normal developmental processes might be a factor to increased mortality between the two treatments. The blocking of development during the STR treatment may lead additional stresses not previously associated with chill injury. The initiation of transcription associated with developmental processes in the FTR treatment may avoid this phenocopy defect.

Current estimates of the developmental threshold in *M. rotundata* range from 15.7-19°C (Kemp and Bosch, 2000; Whitfield and Richards, 1992). While these estimates, even at the upper bound, are still below the 20°C pulse experienced during the FTR protocol, although only by as little as a degree, it has been shown that it is not possible for *M. rotundata* to complete development at 18°C and show greatly delayed development and increased mortality at 22°C (Kemp and Bosch, 2000). While 20°C is indeed above *M. rotundata's* developmental threshold, the individuals harvested for the RNA-seq assay had not yet moved into the next developmental stage (pupation) and any developmental processes occurring during the exposure to the FTR treatment are not sufficient to lead to gross morphological changes in the animal. Although large-scale morphological changes were not witnessed in *M. rotundata* as a result of the FTR treatment, we recognize that smaller-scale developmental processes may still be occurring.

In addition to those functioning in neurogenesis, the up-regulation of transcripts functioning in other aspects of development, growth, or cell differentiation (Table 2.2) provide additional evidence that fluctuating temperatures may allow for normal development in preparation for pupation and adult development. Conventionally, diapause and post-diapause

quiescence have been thought of developmentally inert, but the idea that these life stages are more developmentally dynamic, have started to gain momentum (Kostál, 2006); our data provide support for this paradigmatic shift. The up-regulation of these developmental transcripts also argues for a role in the prevention of phenocopy defects caused by sub-optimal environmental conditions.

Oxidative stress as a component of chill injury

The low temperatures experienced during chilling may have the capacity to reduce the effectiveness of antioxidant enzymes (Joanisse and Storey, 1996), and thus contribute to chill injury through oxidative stress, causing damage to DNA, protein, and lipid molecules (Monaghan et al., 2009). In adult *Alphitobius diaperinus,* exposure to fluctuating temperatures during chilling have been associated with decreased levels of reactive oxygen species (ROS) when compare to individuals reared under constant low temperatures (Lalouette et al., 2011).

In this study, we found a number of transcripts functioning in oxidation stress response, including *peroxidase*, and *glutathione s-transferase*, and several from the *Cytochrome P450* (*CPY*) superfamily (Table 2.2). These transcripts were up-regulated in early-sampled individuals reared under fluctuating temperatures and maintained in late-sampled individuals. Exposure to environmental stressors (i.e. low temperature stress), has been associated with malfunctioning in mitochondrial respiration and the production of peroxide (Prasad et al., 1994). The up-regulation of *peroxidase* witnessed in *M. rotundata* may serve to diminish the resulting oxidative stress associated with the aforementioned metabolic malfunction. During exposure to low temperatures, the function of antioxidant enzymes, such as those belonging to the CYP superfamily, can be diminished; leading to an increase in ROS present in the cell (Baek and

Skinner, 2003). The increased expression of *CPY* transcripts suggest that exposure to fluctuating temperatures may help in counteracting this decreased efficiency.

Furthermore, other transcripts with antioxidant properties and associations with insect survival were up-regulated when *M. rotundata* were exposed to fluctuating temperatures. *Vitellogenin (Vg),* which has been implicated in caste-specific differences in longevity in honeybees and has antioxidant properties (Corona et al., 2007), and *Apolipoprotein D*, another transcript associated with oxidative stress and survival in *Drosophila* (Walker et al., 2006), were both up-regulated. The up-regulation of these transcripts again support our hypothesis that a reduction of oxidative stress plays a role in increased survival caused by fluctuating temperatures during chilling.

Increased cellular membrane damage via an accumulation of ROS to stressful levels may be associated with damage to mitochondrial membranes and may exacerbate ion and metabolic imbalances that have previously been correlated with chilling injury in other insects. Our results suggest that individuals reared under constant, low temperatures may be experiencing elevated levels of ROS and that a periodic increase in temperature may help alleviate the resulting oxidative stress via up-regulation of antioxidants.

Immune function may play a role in survival during chilling

Several other functional classes of transcripts that have not previously been linked to chill injury, including those functioning in growth and development and immune response, were up-regulated in early-sampled individuals exposed to fluctuating temperatures and were maintained in late-sampled individuals. These additional functional classes provide mechanisms leading to increased survival that have not been previously associated with a chill injury response.

Environmental stressors, such as temperature, have been associated with a decrease in immune function across taxa through trade-offs in life history strategies (Lochmiller and Deerenberg, 2000). For example, temperature stress in *M. rotundata* decreases the immune response to chalkbrood disease, *Ascosphaera aggregata* (Xu and James, 2012). Our study revealed a similar suite of transcripts expressed at increased levels when individuals were exposed to fluctuating temperatures, suggesting that chilling may decrease immune function and may be associated with a decrease in survival. Transcripts such as *Serine protease snake*, *Toll*, and *Melanization protein,* which were differentially expressed in our study (Table 2.2), have been previously implicated in temperature-specific immune responses of *M. rotundata* (Xu and James, 2012). Additionally, increased levels of ROS have been associated with immune function because of free radial production during phagocytosis (Nappi and Vass, 1993), suggesting another cause for the up-regulation of the antioxidants highlighted above. Antioxidants have also been shown to play a role in limiting microbial growth (Broderick et al., 2009), again suggesting another link to increased expression of antioxidants.

Conclusions

Our results suggest that rearing individuals under long-term overwintering conditions can lead to an accumulation of chill injury, damage to neural structures and/or defects in development, and a decrease in immune system function. When individuals are exposed to fluctuating temperatures after an extended period of chilling, insect survival increases dramatically. We have developed a working hypothesis, based on both evidence from the literature and this study, to explain the increased survival associated with fluctuating temperature exposure during chilling in *M. rotundata* (Fig. 2.4).

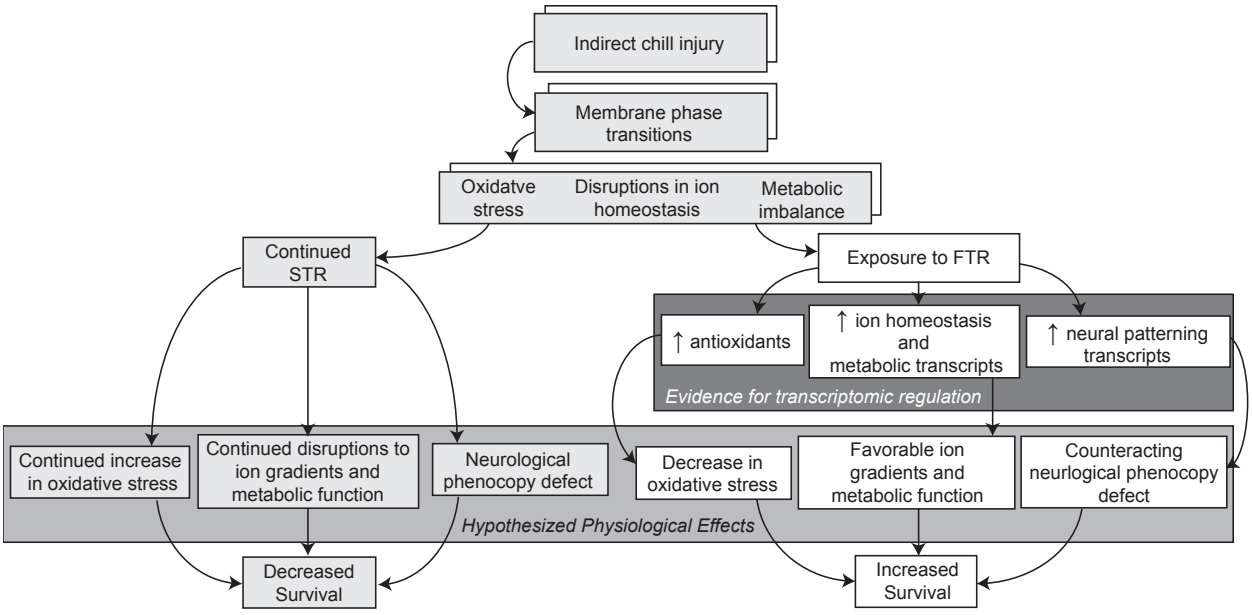

Figure 2.4. Current working hypothesis. Grey boxes indicate exposure to constant, chilled temperatures while white indicates individuals who have been transferred to fluctuating temperatures. Evidence for transcriptional responses to fluctuating temperatures and the hypothesized physiological effects are depicted using boxes.

In this model exposure to fluctuating temperatures activates pathways functioning in oxidative stress response, ion homeostasis, metabolism, and neurogenesis leading to increased survival. This model not only supports current chill injury hypotheses, such as oxidative stress, ion imbalance, and impaired metabolic function by providing evidence for transcriptional responses, but also adds additional mechanisms such as neurological development/repair and increased immune function. While it is clear that exposure to fluctuating temperatures provides an enormous benefit to the organism and the mechanisms behind it are beginning to be understood, it is still ambiguous whether these mechanisms act in a protective fashion, by slowing or stopping the accumulation of chill injury entirely, or by repairing chill injury caused by previous long-term low temperature exposure.

Materials and Methods

Insects

All bees for this project, derived from the 2009 field season, were purchased from JWM Leafcutter, Inc. (Nampa, ID) as loose cell bees and were of Canadian origin. Bees were stored at 6°C under constant darkness upon arrival.

Temperature protocols

Cells containing bees were placed individually into the wells of 24-well culture plates, and housed in Percival model I-30BLL reach-in incubators. In April 2010, after 7 months of storage at constant temperatures, post-diapause quiescent prepupae were placed into a fluctuating temperature treatment or left at standard storage temperatures. The constant temperature regime consisted of a 6°C ± 0.5°C constant temperature with a 15:9 (L:D) photoperiod. Bees reared under temperature fluctuations were exposed to 6°C with a daily warm pulse of 20°C. The warm pulse consisted of a 1 h ramp up to 20°C (0.23°C/ min), a 1 h incubation at 20°C, and a 1 h ramp down, back to 6°C. Peak temperature occurred during the photophase of the 15:9 (L.D.) h light cycle. At monthly intervals, three of the 24-well culture plates were removed from the temperature treatments and place at 29°C to initiate pupal development. Adult emergence rates were used to measure survival (as described in Rinehart et al., 2013).

Library preparation and sequencing

Total RNA was collected from three postdiapause quiescent prepupae reared under either constant or fluctuating temperature treatments at an early and late timepoint in 2010 (12 bees total) using the Invitrogen TRIzol protocol (Carlsbad, CA USA). To ensure that we were assessing response to low temperatures in both treatments, individuals for both treatments were harvested during the cold phase (6°C). RNA was stored as an ethanol precipitate at -80°C until

needed. Samples were then dissolved in RNAase free H_2O and shipped at 1-10 ug total RNA at a concentration of no less than 20 ng/ul on dry ice overnight to University Georgia.

RNA-seq libraries from both constant and fluctuating temperature treatments at both early and late time points in 2010 were prepared using Illumina TruSeq mRNA standard protocol at the University of Georgia Genomics Facility (Athens, GA USA) and sequencing was outsourced to the University of Missouri Columbia DNA Sequencing Core facility (Columbia, MO USA). The RNA samples were sequenced on a HiSeq2000, running HiSeq Control Software (HCS) v1.4.8. Samples were sequenced on two lanes of a HiSeq Flowcell v1.5 and libraries were clustered on a cBot v1.4.36.0 using Illumina's Truseq PE Cluster Kit v2.0 and sequenced using a 200 cycle TruSeq SBS HS v2 kit. The clustered flowcell was sequenced for 206 cycles, broken down into 3 separate reads. The first read was 100 cycles in length, followed by a 6-cycle index read. Following the index read, paired end resynthesis was performed also using Truseq PE Cluster Kit v2.0, which was then followed by another 100 cycles. Image analysis and base calling were performed using the standard Illumina Pipeline consisting of Real time Analysis (RTA) version v1.12.4.2 and Casava v1.8 using the default settings.

Differential expression analysis

Raw sequence data generated from the Illumina HiSeq2000 (Accession: SRP047335) were quality checked using FastQC (Version 0.10.1; Babraham Bioinformatics). The raw reads were aligned to the *M. rotundata* reference genome (Accession number: PRJNA66515) using TopHat (Version 2.0.5). Mapped reads were assembled, with the aid of a GFF (Version 3) annotation file generated using the genome annotation pipeline MAKER (Cantarel et al., 2008), using Cufflinks (Version 2.0.2). The differential expression profiles were analyzed using Cuffdiff (Version 2.0.2) via the iPlant Collaborative Discovery Environment (Stanzione, 2011).

A threshold value of $\log_2 2$, ($\alpha \leq 0.05$) was used to determine significance in the differential expression analysis. The R package cummerbund (Trapnell et al., 2012) was used for downstream analysis and the generation of differential expression figures. The Venn Diagram depicted in Fig. 2.3 was generated using the R package Venn Diagram (Chen and Boutros, 2011) and the Java-based gene ontology enrichment and functional annotation program Blast2GO was used for GO and KEGG analysis (Conesa et al., 2005; Kanehisa et al., 2012).

Quantitative real-time PCR

RNA samples for qPCR were collected from post-diapause quiescent bees during RNA-seq library preparation in one-month intervals between May 2010 and 2011 in the fashion described above. RNA samples were diluted to a concentration of 0.5 μg/μl and then subjected to Invitrogen DNAase I treatment (Carlsbad, CA, USA) followed first strand cDNA synthesis using Invitrogen Super Script III first strand synthesis system for RT-PCR (Carlsbad, CA, USA). Three controls were used for this step: a "no template control" consisting of all enzymatic components except the RNA template, a "negative RT control" lacking reverse transcriptase, and a "positive RT control" using a control RNA template.

Quantative PCR was conducted on the Roche LightCycler 480 (Indianapolis, IN, USA). The FastStart Universal SYBR Green I Master Mix with ROX (Indianapolis, IN, USA) protocol and reagents were used for the qPCR reactions. Primer design for all targets and reference genes was conducted using the Integrated DNA Technologies' (Coralville, IA, USA) IDR program. Biogazelle's qBasePLUS (Ghent, Belgium) was used for the analysis of the qPCR data.

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CHAPTER 3: TRANSCRIPTIONAL RESPONSES TO FTR DURING PUPAL DEVELOPMENT[2]

Abstract

Exposure to stressful low temperatures during development can result in the accumulation of deleterious physiological effects called chill injury. Metabolic imbalances, disruptions in ion homeostasis, and oxidative stress contribute to the increased mortality of chill-injured insects. Interestingly, survival can be significantly increased when chill susceptible insects are exposed to a daily warm-temperature pulse during chilling. We hypothesize that warm pulses allow for the repair of damage associated with chill-injury. Here, we describe transcriptional regulation during exposure to a fluctuating thermal regime (FTR), relative to constant chilled temperatures, during pupal development in the alfalfa leafcutting bee, *Megachile rotundata* using a combination of RNA-seq and qPCR. Pupae were exposed to either a constant, chilled temperature of 6°C, or 6°C with a daily pulse of 20°C for seven days. RNA-seq after experimental treatment revealed differential expression of transcripts involved in construction of cell membranes, oxidation-reduction and various metabolic processes. These mechanisms provide support for shared physiological responses to chill injury across taxa. The large number of differentially expressed transcripts observed on day seven suggests that the initial divergence in expression profiles between the two treatments occurred upstream of the time point sampled. Additionally, the differential expression profiles observed in this study show little overlap with

[2] The material in this chapter was co-authored by Alex S. Torson, George D. Yocum, Joseph P. Rinehart, Sean A. Nash, Kally M. Kvidera, and Julia H. Bowsher and is in review under the title "Physiological responses to fluctuating temperatures are characterized by distinct transcriptional profiles in a solitary bee." Alex S. Torson had primary responsibility for all of the methods described in this chapter including processing and analysis of RNA-seq data and supervision of qPCR validation and time series and analysis of those results. Alex S. Torson drafted and revised all versions of this chapter. Julia H. Bowsher proofread the text written by Alex S. Torson.

those differentially expressed during temperature stress in the diapause state of *M. rotundata*. While the mechanisms governing the physiological response to low-temperature stress are shared, the specific transcripts associated with the response differ between life stages.

Introduction

In ectothermic animals like insects, environmental temperatures play a critical role in the animal's physiology. Exposure to stressful, low temperatures during development can have significant, detrimental effects on adult insects (Bale and Hayward, 2010; Bennett et al., 2013; O'Neill et al., 2011; Whitfield and Richards, 1992; Yocum et al., 1994; Yocum et al., 2006). The degree to which insects respond to these temperature stressors is highly dependent upon the timing, duration, and amplitude of exposure (Colinet et al., 2015). With global climate change threatening to increase temperature variance (Easterling et al., 2000; Hansen et al., 2012), insects will likely be faced with increased exposure to low, seasonally unpredictable temperatures (Vasseur et al., 2014). In chill-susceptible insects, prolonged exposure to low temperatures can lead to the accumulation of deleterious physiological effects called chill injury (Lee, 2010).

Chill injury causes significant increases in mortality (Colinet et al., 2011; Kostál, 2006; Lee, 2010; Renault et al., 2004). The downstream physiological consequences of chill injury are complex but appear to be caused in part by membrane phase transitions (Lee, 2010). Damage to cell membranes results in metabolic imbalances and perturbations in ion homeostasis (Kostal, 2004; Kostál et al., 2006) that have been observed in chill-injured insects. Furthermore, temperature stress is associated with oxidative stress (Lalouette et al., 2011; Rojas and Leopold, 1996) and impairment of neuromuscular system function (Bennett et al., 2015; Yocum et al., 1994); both of which are implicated as downstream consequences of cell membrane damage (Lee, 2010).

The decreased survival and cellular damage associated with chill injury can be mitigated by exposure to transient, daily increases in temperature during chilling (termed fluctuating thermal regimes; FTR). Exposure to these brief, daily temperature increases during chilling results in increases in survival in chill-injured insects (Chen and Denlinger, 1992; Colinet et al., 2006; Colinet et al., 2007; Jing et al., 2005; Kostál et al., 2007; Koštál et al., 2016; Leopold et al., 1998; Marshall and Sinclair, 2012; Renault et al., 2004; Rinehart et al., 2011; Yocum et al., 2012). Exposure to FTR during chilling allows for either the protection against or repair of damage caused by chill injury (Colinet et al., 2015; Lee, 2010). While it is possible that brief, daily exposure to warmer temperatures during chilling allows for a physiological preparation (i.e. protection) for chilling, the hypothesis that exposure to warmer temperatures allows reparative mechanisms to act has more empirical support (Colinet et al., 2006; Kostál et al., 2007; Nedvěd et al., 1998; Torson et al., 2015). FTR repairs chill injury damage in many taxa (Boardman et al., 2013; Jing et al., 2005; Kostál et al., 2007; Leopold et al., 1998; Nedvěd et al., 1998; Renault et al., 2004), but it is unclear whether the mechanisms governing the beneficial effects associated with FTR exposure are conserved.

Gene expression comparisons could reveal whether the response to FTR is conserved across different developmental stages of the same organism, or even across taxa. An RNA-seq study conducted in post-diapause quiescent prepupal *M. rotundata* provided support for the physiological evidence in other chill-injured insects and offered insight into mechanisms driving the reparative effect of FTR versus STR (Torson et al., 2015). Individuals exposed to FTR had increased expression of transcripts functioning in the maintenance of ion homeostasis, response to oxidative stress, and an increase in metabolic function; all congruent with the hypothesis that FTR allows for repair of damage during the warm phase. These transcriptional profiles could

represent a conserved mechanism. In support of a conserved mechanism, FTR exposure is also beneficial during low temperature stress in pupal development in *M. rotundata*, a life stage that has a different physiology from diapause. FTR during cold exposure in pupal development significantly diminished the sublethal effects of chill injury, which occur in pupa exposed to one week of low-temperature stress (Bennett et al., 2015). Given that FTR has similar protective effects in these two life stages, we hypothesized a conserved suite of genes would underlie the response.

The goals of our study were to 1) identify differential gene expression in individuals exposed to FTR protocol during pupal development and 2) determine if expression profiles observed in pupal FTR support a conserved reparative mechanism of FTR across life stages in *M. rotundata*. Developing pupae were exposed to either a constant, low-temperature stress (static thermal regime, STR) or the same low temperature with a daily, one-hour warm-temperature pulse for seven days (FTR). RNA was harvested from bees exposed to either treatment after the warm pulse on day seven. We hypothesized that, as a result of a conserved mechanism driving the reparative effect of FTR across taxa, the exposure to fluctuating temperatures during low temperature stress during this life stage would elicit transcriptional responses similar to prepupal responses observed previously (Torson et al., 2015).

Materials and methods

Insects

M. rotundata were purchased from JWM Leafcutter, Inc. (Nampa, ID), were derived from the 2013 (RNA-seq and validation) and 2014 (qPCR time series) field seasons, arrived in late March in both years (2014 and 2015, respectively) after prepupal overwintering at a constant temperature (4-6°C), and were of Canadian origin. Diapausing prepupae arrived as loose cells

and were housed in Percival model I-30BLL reach-in incubators at 6°C ± 0.5°C under darkness upon arrival.

Temperature protocols

Prepupae were transferred to a Percival model I-30BLL at 29°C to initiate development. To assess the progression of pupal development prior to treatment, bees not destined for experimental treatment were dissected out of their brood cells and placed them in 24-well plates to function as guide plates. For the guide plates, a NaCl solution was used to maintain humidity at 75%. Experimental bees were maintained at 29°C until ~50% of the bees in the guide plates reached a developmental stage characterized by melanization of the eyes, called the "red eye" stage. At the red-eye stage, bees were grouped into one of two treatments: a Fluctuating Thermal Regime (FTR) or Static Thermal Regime (STR; Fig. 3.1). Bees reared under the FTR protocol were exposed to 6°C with a daily warm pulse of 20°C consisting of a 1 h ramp up to 20°C (0.23°C/ min), a 1 h incubation at 20°C, and a 1 h ramp down, back to 6°C for each of the seven days of treatment. Those reared under the STR treatment were maintained at 6°C for the duration of the seven-day treatment.

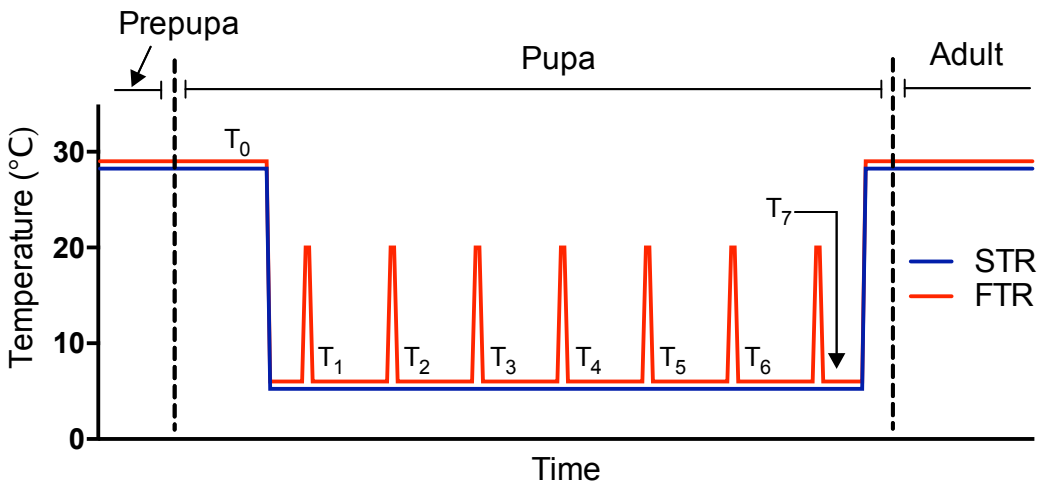

Figure 3.1. Temperature regimes and RNA collection time points. *M. rotundata* were reared at 29°C until "red-eye" pigmentation stage during pupal development. Individuals were exposed to either an STR of 6°C or an FTR of 6°C with a one hour pulse to 20°C with one-hour ramp up and ramp down times. RNA was harvested at T_7 for the RNA-seq and T_0-T_7 for the qPCR time series.

RNA-seq

RNA collection

Total RNA was collected from whole *M. rotundata* pupae exposed to either the FTR or STR treatment (3 biological replicates per treatment; 1 biological replicate is one individual). To ensure the assessment of response to low temperature exposure and not a warm-temperature exposure, individuals from both treatments were harvested for RNA during the cold phase (6°C; Fig. 3.1) and maintained on ice until the animals were sacrificed. Animals were harvested after the cold phase to ensure a measurement of low temperature response in both treatments, and were harvested shortly after in the event that the effect of the warm temperature pulse was transient. Pupae were transferred directly from ice and homogenized in 500 µl of TRIzol using a polypropylene pestle in a nuclease free 1.5 ml microcentrifuge tube. The Invitrogen TRIzol protocol (Carlsbad, CA USA) was followed for RNA extraction. Final product was stored as an ethanol precipitate at -80°C until needed.

RNA-seq library preparation and sequencing

Prior to sequencing, RNA pellets were dissolved in DEPC-treated H_2O and shipped at a concentration of no less than 20 ng/ul (~10ug total RNA) on dry ice overnight to the University of Georgia. Upon arrival to the University of Georgia Genomics Facility (Athens, GA USA), RNA samples from both FTR and STR treatments were checked for quality using an Aligent Bioanalyzer (Santa Clara, CA USA). A total of 4µg of total RNA was used for input in the Illumina Truseq Stranded mRNA kit. Samples were pooled and run on a NextSeq500 using a Version 1 Mid-Output 300 cycle kit with PE150 settings. Demultiplexing and adapter trimming were done by default when data was processed on BaseSpace (Illumina, San Diego, CA USA).

Differential expression analysis

Raw sequence data (BioProject Accession: PRJNA352846) were quality checked using FastQC (Version 0.10.1; Babraham Bioinformatics). TopHat (Verse 2.0.9) was used to align the raw reads against the *M. rotundata* reference genome (BioProject Accession: PRJNA66515). Assembly of mapped reads was conducted using Cufflinks (Version 2.0.2) and differential expression analysis was carried out using Cuffdiff (Version 2.0.2) via the iPlant Collaborative Discovery Environment (Stanzione, 2011). Transcripts were considered significantly differentially expressed when the *q-value* (Benjamini-Hochberg-corrected *p-value*) was below the false discovery rate (FDR) of 0.05. The R package cummeRbund (Trapnell et al., 2012) and the Java-based functional annotation program Blast2GO (Conesa et al. 2005) were used for downstream analysis and the generation of differential expression figures.

Life-stage-specific RNA-seq comparison

To assess whether significant overlap between gene expression profiles exist during low-temperature stress between post-diapause quiescent prepupae (Torson et al., 2015) and

developing pupae Venn diagrams were constructed using the R package VennDiagram (Chen and Boutros, 2011). Statistical significance of the overlap of differentially expressed transcripts between data sets was determined via hypergeometric distribution using the R function phyper. The total number of transcripts for comparison was 11,195, which is the total number of transcripts in the reference transcriptome for *M. rotundata*.

RNA-seq validation

RNA collection

Residual RNA from each RNA-seq sample (2013 field season; described above) was aliquoted and stored at -80°C under 100% ethanol and used for the qPCR validation of the computational methods described above. The 10 most upregulated transcripts in response to FTR were selected for validation.

Sample preparation

RNA samples were diluted to a concentration of 0.333 μg/μl and subsequently treated with Invitrogen DNAase I (Carlsbad, CA, USA) to remove genomic DNA contaminants. A total of 1 μg RNA for each of the three biological replicates per treatment was used as template for first strand cDNA synthesis using Invitrogen Super Script III first strand synthesis system for RT-PCR (Carlsbad, CA, USA).

qPCR

Real-time quantitative PCR reactions were conducted on a Roche LightCycler 480 II (Indianapolis, ID, USA) using Roche FastStart Universal SYBR Green I Master Mix with ROX (Indianapolis, IN, USA). Primers for all targets (10 most upregulated transcripts from RNA-seq experiment, Table B.1) and eight reference gene candidates were designed using Integrated DNA Technologies IDR program (Coralville, IA, USA). Candidate reference genes were selected

using this RNA-seq data set and libraries generated in Torson et al. 2015 (18 libraries total) to identify stably expressed transcripts. Expression of differentially expressed transcripts in qPCR runs were normalized against two most stably expressed reference targets identified using the algorithm geNorm (Vandesompele et al., 2002).

To ensure quality control between and within plates, three controls were used on each plate: a "no template control" consisting of all enzymatic components without cDNA template, a "negative RT control" lacking reverse transcriptase, and a "positive RT control" using a control cDNA template and control primers for each plate. The "positive RT control" also served as calibrator reactions in the event that comparisons spanned multiple plates.

Biogazelle qbase+ (Ghent, Belgium) and Prism Graphpad (La Jolla, CA USA) were used for statistical analysis and graphical representations of the data respectively. Statistical significance for each of the 10 upregulated and 8 non-differentially expressed transcripts was determined by T-test of the calibrated normalized relative quantity (CNRQ) of each transcript from qbase+.

qPCR time series

RNA collection

M. rotundata from the 2014 field season were reared at 29°C until eye pigmentation stage and then split into either the FTR or STR protocol described above. Total RNA for the qPCR time series was collected from whole *M. rotundata* pupae exposed to either the FTR or STR treatment (6 biological replicates; 1 individual per replicate). Before exposure to treatment, pupae were harvested to serve as a baseline for gene expression (T_0; Fig. 3.1). After placement into treatment, individuals were sacrificed during the cold phase (6°C, shortly after the warm pulse in FTR) for each of the seven days of treatment (T_1-T_7; Fig. 3.1). Animals were placed on

ice during dissection from brood cells until they were homogenized for RNA extraction to prevent an additional warm-temperature exposure. The Invitrogen TRIzol protocol (Carlsbad, CA USA) was followed for RNA extraction. Final product was stored as an ethanol precipitate at -80°C until needed. Sample preparation, qPCR runs and similarly to RNA-seq validation (see previous section). Two-way ANOVA with Bonferroni's correction for multiple comparisons determined statistical significance of differential expression.

Results

Assembly

Three biological replicates were harvested from each treatment, totaling six individual transcriptomic libraries (BioProject Accession: PRJNA352846). Each library averaged a total of 47,279,330 reads (Table 3.1). Raw reads were mapped to the *M. rotundata* reference genome (BioProject Accession: PRJNA66515) with an average alignment rate of 69.11%. FastQC (Version 0.10.1; Babraham Bioinformatics) was used to determine baseline quality of raw reads and percentage of reads mapped to the reference genome and mate pair concordance (Table 3.1) were used as a metric of assembly quality.

Table 3.1. Assembly statistics.

Biological Replicate	Total Reads	Mapped Reads	Overall read alignment	Concordant pair alignment
STR_1	42,661,992	29,877,839	70.0%	65.8%
STR_2	43,529,948	28,940,759	66.5%	61.9%
STR_3	36,809,014	25,976,650	70.6%	66.3%
FTR_1	65,929,382	46,612,625	69.2%	64.5%
FTR_2	48,182,900	33,471,363	69.5%	64.7%
FTR_3	46,562,730	32,100,944	68.9%	64.6%
Average:	47,279,330	32,830,030	69.11%	64.5%

Each paired-end (PE) read RNA-seq library was mapped against the *M. rotundata* genome. Each treatment contained three biological replicates

Differential expression analysis and validation

Using the Tuxedo protocol (Trapnell et al., 2012), expression analysis revealed a total of 434 differentially expressed transcripts between STR and FTR treatments after seven days of exposure to treatment (307 downregulated and 127 upregulated in FTR). Real-time quantitative PCR confirmed the expression profiles of 8 transcripts upregulated in FTR, relative to STR and 6 non-differentially expressed transcripts that we used as reference gene candidates (Table B.2). Differential expression was confirmed in all cases with at least one primer pair. We were able to confirm expression of 8 of the top 10 FTR-upregulated transcripts (Table B.2, B.3) and 6 of 8 non-differentially-expressed transcripts (Table B.3).

Gene ontology

A Benjamini and Hochberg corrected Fisher's exact test (one tailed, FDR=0.05) of up- and downregulated transcripts in response to FTR revealed no over or underrepresented functional classes relative to the GO distribution in the reference transcriptome. Due to the lack of enrichment for GO terms, relative to the reference transcriptome, we used direct counts of GO terms of the differentially expressed transcripts in this study for downstream analyses and discussions. Direct counts of the transcripts differentially expressed between FTR and STR treatments on T_7 of treatment revealed classes of transcripts functioning in biological processes including regulation of transcription, oxidation-reduction, signal transduction, and various metabolic processes (Fig. B.1). Also, a direct count of biological process GO terms (Fig. 3.2) revealed the upregulation in FTR of transcripts functioning in signal transduction and trans-membrane transport. Furthermore, GO terms associated with the construction of cellular components, including plasma membrane and various membrane components were upregulated in response to FTR (Fig. 3.2).

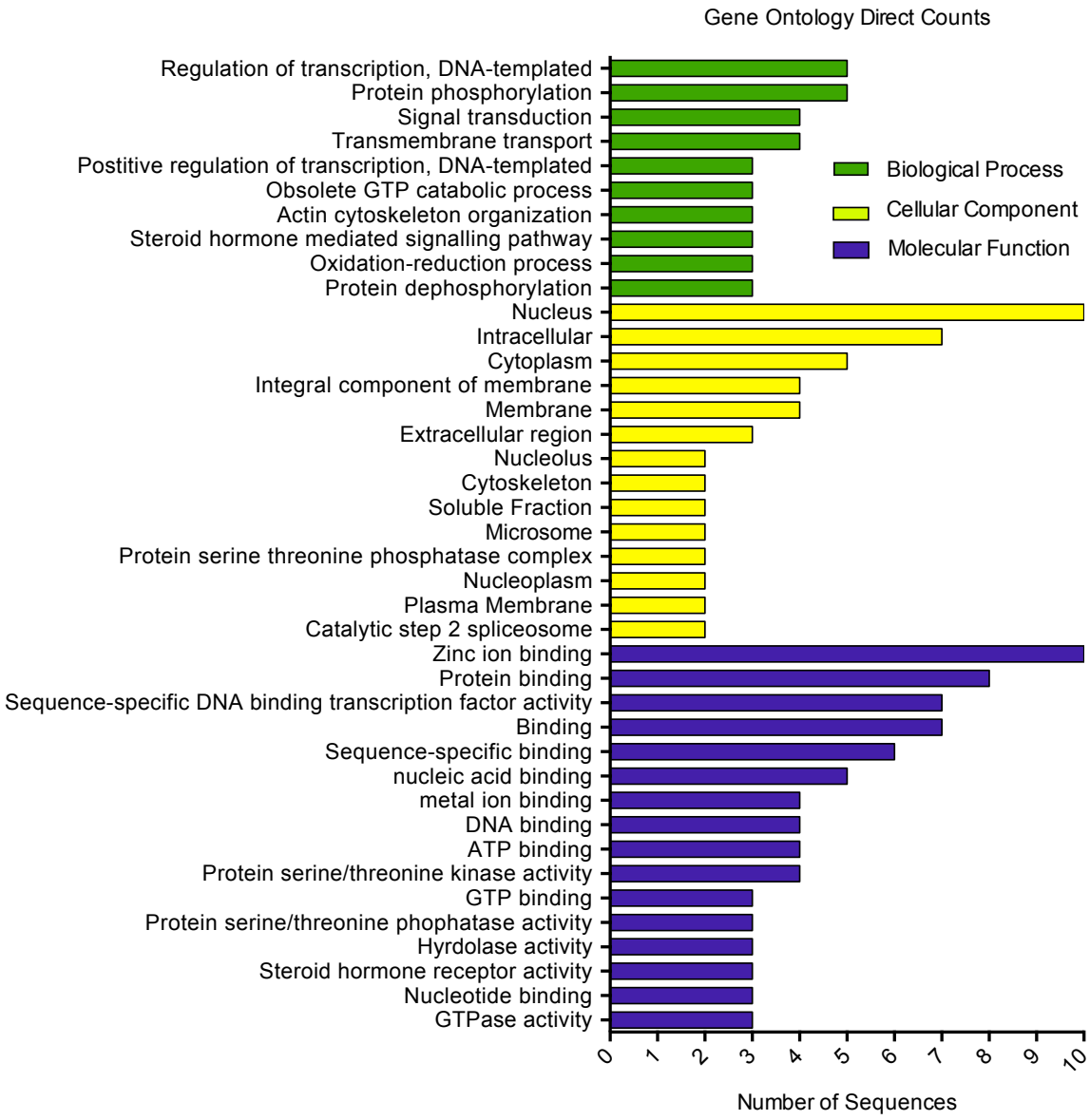

Figure 3.2. Gene ontology of transcripts upregulated in FTR. Most prominent GO terms for biological processes, molecular function, and cellular components of transcripts upregulated in *M. rotundata* exposed to FTR for seven days.

Life-stage-specific comparisons

When the identities of differentially expressed transcripts observed in this study are compared with those presented during the two time points assessed during post-diapause quiescence (Torson et al., 2015), 30 (6.9%) differentially expressed transcripts in the pupal

response (434 transcripts) were also differentially expressed during the early time point (215 transcripts), before mortality diverged in prepupal STR/FTR comparison. This overlap is more than we would expect by chance, based on hypergeometric distribution (p = 1.27E-10). Seventeen of the 434 differentially expressed transcripts (3.9%) overlapped with the late time point (after mortality diverged, 64 transcripts) in prepupae, also more than expected by chance (p = 1.68E-11). Six (1.4%) transcripts differentially expressed in pupae were differentially expressed in both prepupal time points (Fig. 3.3A).

While differentially expressed transcripts overlap more than expected by chance, when direction of regulation (whether the transcript is up- or down-regulated in FTR-exposed individuals) is considered, all overlap among the three differential expression profiles disappears (Fig. 3.3B,C). In fact, the only differential expression patterns conserved between the two life stages are regulated in opposite directions (Fig. 3.3D).

Discussion

Exposure to daily fluctuations in temperature (FTR) during chilling can mitigate the deleterious physiological effects associated chill injury (Colinet et al., 2015). FTR appears to provide a protective benefit across all taxa studied so far, but we lack evidence for a conserved mechanism driving this protective effect. In this study, we exposed developing *M. rotundata* pupae to a static thermal regime (STR), known to lead to chill injury, or to a fluctuating thermal regime (FTR), which diminishes the negative effects of chilling (Bennett et al., 2015). An RNA-seq study after seven days of treatment revealed 434 transcripts that were differentially expressed between the two low temperature treatments. These differentially expressed transcripts provide evidence for cellular damage, oxidative stress, and repair of damage (Table 3.2, B.4). Additionally, we observed a lack of conservation in differential gene expression in response to

FTR exposure across life stages in *M. rotundata*, suggesting a robust physiological response driven by variable transcriptional mechanisms.

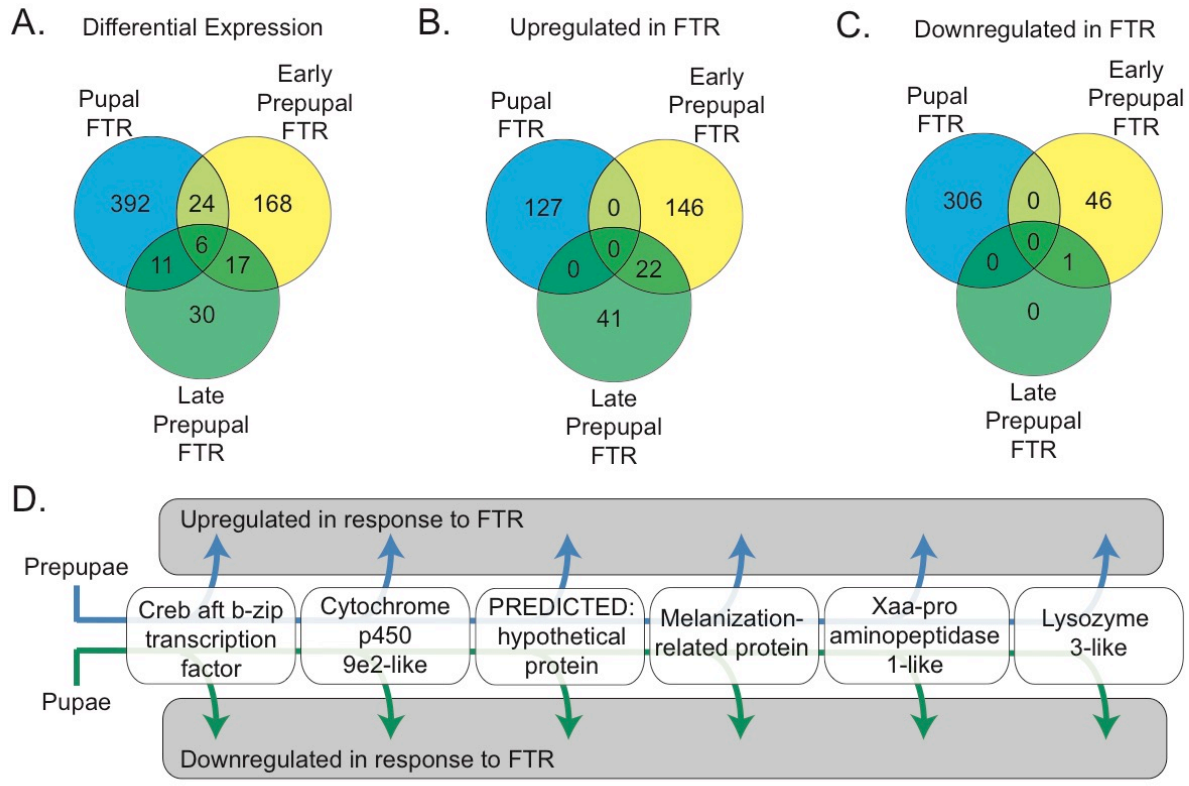

Figure 3.3. Comparison of differential expression profiles of pupa and post-diapause quiescent prepupa exposed to FTR treatment. The differential expression profiles in this study (interrupted development) were compared to two time points sampled during long term FTR treatment during the prepupal life stage (Torson et al., 2015). A) Comparison of differentially expressed transcripts; B) Transcripts upregulated in FTR; C) Transcripts downregulated in FTR; D) Graphical representation of the direction of regulation of the six shared transcripts from panel A.

Table 3.2. Candidate genes involved in cell membrane structure and function.

GO Term	Transcript	Fold Change	Transcript ID
IMC, MT	aquaporin -like	-2.42146	MROT_00006513
IMC, TS	green-sensitive opsin-like	-2.18108	MROT_00001868
IMC	dusky cg9355-pa	-2.13892	MROT_00006341
IMC, TS	isoform b	-2.13224	MROT_00003036
IMC	heparan sulfate 2-o-sulfotransferase	-1.96306	MROT_00008924
IMC	acyl- delta desaturase	-1.94342	MROT_00010957
IMC, TS	toll-like receptor 1	-1.87515	MROT_00011033
IMC, MT	protein patched	-1.78456	MROT_00004594
IMC, MT	sodium potassium calcium exchanger 6	-1.68664	MROT_00007828
IH	transferrin	-1.6421	MROT_00006050
CM, MT	sodium dicarboxylate	-1.55519	MROT_00005087
IMC	tetraspanin isoform b	-1.54449	MROT_00000476
IMC	elongation of very long chain fatty acids protein aael008004	-1.52267	MROT_00002329
IMC, MT	facilitated trehalose transporter tret1-like	-1.49314	MROT_00002610
IMC, MT	solute carrier family 17 member 9-like	-1.43109	MROT_00009520
IMC	slit homolog 1	-1.42611	MROT_00009446
CM, MT	inorganic phosphate cotransporter-like	-1.4077	MROT_00005368
CM, TS	fibrillin-2 isoform x3	-1.38474	MROT_00010592
IMC, TS	protein jagged-1	-1.22979	MROT_00010666
IMC, MT	facilitated trehalose transporter tret1-like	-1.21413	MROT_00010168
IMC	n-acetylgalactosaminyltransferase 7	-1.20159	MROT_00008614
IMC	acyl- delta desaturase-like	-1.17683	MROT_00008401
IMC, MT	aquaporin	-1.13869	MROT_00003003
CM	stromal cell-derived factor 2	-1.09454	MROT_00000939
IMC, TS	adenylate cyclase type 2-like	-1.07231	MROT_00008284
IMC, RA	progestin and adipoq receptor family member 3-like	-1.06359	MROT_00005713
IMC	cg34114 cg34114-pb	-1.05511	MROT_00005193
IMC	atp-binding cassette sub-family g member 1-like	-1.04225	MROT_00007187
CM, TS	abc transporter g family member 22-like	-1.00873	MROT_00003345
IMC, RA	translocon-associated protein subunit beta	-0.974473	MROT_00003491
CM, RA	protein croquemort	-0.929458	MROT_00007021
IMC	acyl- delta desaturase	-0.807356	MROT_00008406
IMC, MT	solute carrier family facilitated glucose transporter member 1	0.759531	MROT_00002239
IMC, MT	synaptic vesicle protein	0.83321	MROT_00003829
IMC	trehalase precursor	0.981384	MROT_00001571
IMC, MT	mitochondrial glutamate	1.16316	MROT_00008195
IH	mucolipin-3	1.2947	MROT_00001219
IMC	osiris 14	2.07664	MROT_00008103

Fold change is $\log_2$ transformed and relative to FTR. A negative fold change is an upregulation in STR. The Gene Ontology (GO) terms used for inclusion into this table are Integral membrane component (IMC), Membrane transport (MT), Transmembrane signaling (TS), IH (Ion homeostasis), Cell membrane (CM), and Receptor Activity (RA).

Cellular damage as a driving factor of chill injury

Plants, microbes, and other animals change the physical composition of cell membranes in response to changes in ambient temperature, namely the proportion of unsaturated fatty acids

in the phospholipid bilayer (Cossins, 1983). In insects, direct evidence of shifts in membrane phase transitions have yet to be observed (Hayward et al., 2014; Lee, 2010), but strong correlative evidence has been provided (Boardman et al., 2011; Kostál et al., 2006; Kostál et al., 2007; Macmillan et al., 2012). Chill injury in insects has been associated with the collapse of ion gradients presumably caused by damage at the cellular level. Extracellular increases of potassium and diminished levels of sodium and magnesium have been observed in chill-injured tropical cockroaches (Kostal, 2004; Kostál et al., 2006; Kostál et al., 2007). These changes in hemolymph ion concentrations could explain the loss of neuromuscular coordination observed in chill-injured insects and may be a direct effect of damage to cell membranes. In this study, the upregulation of transcripts such as sodium potassium calcium exchanger 6, a mitochondrial potassium-dependent sodium-calcium antiporter (Table 3.2) in individuals exposed to the STR protocol suggests an attempt to restore proper ion gradients in response to low temperature stress. Additionally, the upregulation of transcripts with GO terms associated with the construction of various cellular components, including cell membranes and integral membrane components (Fig. 3.2; Table 3.2), in response to FTR exposure indicates that one of FTR's mechanisms of action is to repair the membrane damage that has accumulated over the duration of low-temperature stress.

The upregulation of aquaporins, a water-selective transmembrane channel, has been observed in freeze-tolerant insects (Philip et al., 2011), and the blockage of those channels has been shown to decrease freeze tolerance due to an inability of the cells to move water out of the cell; subsequently causing damage due to osmotic pressure (Philip et al., 2008). We observed the upregulation of aquaporins during constant low temperature stress (STR); suggesting that these individuals may be attempting to return to proper osmotic conditions. In addition to changes in

structural components within membranes, such as aquaporins, an increase in unsaturated fatty acids has been observed in response to decreased temperatures across taxa (Cossins, 1983). In our dataset, three different transcripts coding for acyl-delta desaturases, enzymes functioning in the transition between saturated and unsaturated fatty acids, were upregulated in STR-reared individuals relative to FTR (Table 3.2). The upregulation of transcripts coding for desaturase enzymes has been associated with cold hardiness in the onion maggot, *Delia antiqua* (Kayukawa et al., 2007), suggesting this response is conserved. Increased expression of these transcripts suggest that either bees exposed to STR are trying to compensate for a decrease in temperature or that FTR-reared individuals may have less of a strain on their cell membranes due to the temporary increases in temperature.

Antioxidant system activation and repair of damage in the FTR response

Oxidative stress has been implicated in the occurrence of chill injury in many insect species (Joanisse and Storey, 1996; Joanisse and Storey, 1998; Lalouette et al., 2011; Rojas and Leopold, 1996; Torson et al., 2015) and has been hypothesized to be a downstream consequence of membrane phase transitions (Lee, 2010). Increased oxidative stress is a likely downstream effect of low temperature exposure, but it remains unclear whether reactive oxygen species (ROS) are present at high enough levels in STR-exposed individuals to cause significant damage to nucleic acids, proteins, and lipids.

The upregulation of a gene coding for a myb domain-containing protein in STR-reared individuals (Table B.4) suggests that *M. rotundata* is indeed experiencing a stress response. In plant species this class of transcription factors is known to respond to abiotic stressors, including decreased temperature. The functional unit of the translated proteins are highly conserved suggesting a conserved function in insects (Ambawat et al., 2013). Furthermore, increased

expression of oxidase-peroxidase (Table B.4) suggests that STR reared individuals are experiencing increased levels of peroxide, a consequence of malfunction in mitochondrial respiration (Prasad et al., 1994). If antioxidant systems can't combat the accumulation of free radicals, such as peroxide, damage to cellular structures would be likely.

Previous studies have provided support for the hypothesis that FTR exposure can facilitate the repair of damage caused by indirect chilling injury (Colinet et al., 2015; Renault et al., 2004). The upregulation of transcripts in STR-reared bees such as myofilin isoform b, sestrin-like, and DNA damage-binding protein 1 (Table B.4) suggest that STR-exposed individuals experience DNA damage, potentially caused by increased levels of ROS in the bees during chilling. In *Drosophila,* expression levels of sestrins increase in response to target of rapamycin (TOR) under increased levels of ROS (Lee et al., 2010a). The increased expression of this sestrin has also been linked to genotoxic stress including DNA damage (Hay, 2008; Lee et al., 2010b). While sestrin has not been shown to repair DNA damage directly, it may be part of a larger signaling cascade. Additional cellular-level assessments of damage will be required to assess whether oxidative stress may be playing a significant role in the deleterious effects of chill injury or if high levels of antioxidant expression in FTR-exposed individuals is just an artifact of increased metabolic rate due to transient increases in ambient temperature.

In addition to the transcripts that have been explicitly linked to the repair of damage, transcripts typically associated with developmental processes such as neurogenesis (Table B.4) are upregulated in FTR-exposed pupae. Although the expression of these transcripts may be attributed to noise associated with typical pupal development, the upregulation of transcripts with similar functions in FTR-exposed post-diapause quiescent prepupae (Torson et al., 2015), a life stage characterized by a lack of developmental progression, suggest that these transcripts

may be acting in a reparative manner. The conservation of functional classes upregulated in both life stages assessed so far in *M. rotundata* would suggest that similar mechanisms might be driving FTR's beneficial effects.

Conservation of mechanisms across life stages

Much of what is understood about low-temperature stress response in insects is a composite of discrete studies conducted in a broad range of taxa throughout various life stages and ecologies. For example, a significant amount of work has been done on tropical species exposed to relatively low temperatures (Bale and Hayward, 2010; Kostál et al., 2006; Renault et al., 2004). While these studies provide valuable insight into low temperature stress responses, it is important to view these physiological responses with respect to the environmental pressures under which these organisms have evolved. Selective pressures on cold tolerance will vary throughout development and may result in markedly variable strategies, and thus mechanisms, driving low-temperature stress responses.

In addition to the benefits of FTR observed during pupal development in *M. rotundata*, significant increases in survival have been observed when prepupal post-diapause quiescence are exposed to a similar treatment (Rinehart et al., 2013). For the duration of diapause and post-diapause quiescence in both natural and managed populations, *M. rotundata* are exposed to low temperatures and have mechanisms to cope with these temperatures. Conversely, other life stages, such as developing pupae, which we have assessed in this study, may not be adapted for low temperature exposure and may have different mechanisms driving low temperature stress responses, and subsequently the beneficial effects of FTR. Initially, we predicted that the temperature stress during the pupal stage would elicit transcriptional responses similar to prepupal responses observed in Torson *et. al* 2015. This hypothesis was not supported.

Markedly different differential expression profiles (Fig. 3.3) between the two life stages suggest that either 1) the mechanism responsible for a conserved FTR benefit vary throughout development or 2) that the benefit of FTR exposure changes throughout development.

Adult bees observed after STR treatment in both life stages exhibit diminished neuromuscular function, behavior abnormalities, and diminished flight capacity (Bennett et al., 2013; Bennett et al., 2015). These phenotypes suggest chill injury accumulation in both contexts. However, the comparison of differential expression profiles indicate different mechanisms drive the conserved beneficial effects of FTR in each life stage in *M. rotundata*. This result provides the basis for our hypothesis that chill injury repair in response to FTR is a robust physiological response that is driven by life-stage-specific mechanisms.

Benefits of FTR suggest a robust physiological response

The beneficial effects of FTR exposure have been observed across a wide range of insect taxa. FTR seems to act by repairing chill injury caused by prolonged low-temperature exposure, but evidence of a conserved mechanism driving these apparently similar physiological responses has remained elusive. A comparison of gene expression data from this study and that from Torson et al. (2015) suggests very little overlap in differential expression profiles after similar treatments across life stages in *M. rotundata* (Fig. 3.3). Additionally, gene expression profiles of the most differentially expressed transcripts identified in this RNA-seq study (Table B.2) were not maintained in *M. rotundata* from a different field season that were also exposed to an extended overwintering period prior to pupal development (Fig. B.2). The direction of regulation, relative to STR, was maintained, but high variability in expression was observed, even with a doubling of the sample size. This result suggests that gene expression in response to FTR exposure is highly context-dependent (i.e., environmental history and developmental stage)

and provides further evidence that the conservation of FTR's benefit may not be at the level of gene expression but at a higher level of organization.

In biological systems, an animal's ability to maintain a certain characteristic response under perturbations in environmental conditions is known as a robust response (Stelling et al., 2004). With similar physiological responses having been observed across taxa, and, thus far, a lack of evidence of a conserved mechanism at the level of gene expression, we hypothesize that the beneficial effects of chill injury repair during FTR exposure represents a model for a robust physiological response driven by variable gene expression. Redundancy is one basic mechanism of a robust system. Redundancy provides alternative molecular strategies to carry out an action (Hartman et al., 2001). A sophisticated understanding of redundancy in the context of robustness is lacking, but observations in a family of glycosylphosphatidylinositol (GPI)-linked glycoproteins in yeast, termed fungal adhesions, provide a potent example of how redundancy across developmental stages in *M. rotundata* could work. The proteins within this family have functionally distinct roles separated temporally and spatially via differential expression and compartmentalization of the protein family (Guo et al., 2000), but members of this protein family can replace one another when inappropriately expressed in the organism. Other work in yeast has shown little overlap in gene expression profiles in response to variable stressors (Berry et al., 2011), suggesting that variable downstream effectors (i.e. gene expression response) may enact similar physiological responses (i.e. redundancy or genetic buffering).

Between the larval and pupal life stages in *M. rotundata* a complete restructuring of the animal takes place, resulting in entirely different physiologies. Redundancy in transcript function, or "buffering," may explain why variable gene expression patterns are observed in what appear to be similar physiological response to FTR exposure. Alternative to the hypothesis

presented above, we appreciate that both chill injury and FTR's beneficial effects will affect the animal across multiple levels of organization and that changes in gene or protein expression and metabolite accumulation might be secondary effects rather than direct or adaptive responses to chilling and/or FTR exposure. As a result of this notion, an alternative hypothesis is that the variable gene expression profiles between life stages in *M. rotundata* is not evidence of different life-stage-specific mechanisms driving the same response at the physiological level, but actually different responses driven by stimuli at a higher level of organization. Additional functional genomic work to identify potentially redundant genes and physiological assays to verify similar physiological responses to FTR exposure will be necessary to provide support for the hypothesis that redundancy allows for conservation of the FTR response across life stages in *M. rotundata.*

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CHAPTER 4: OXIDATIVE STRESS IN CHILL INJURED *MEGACHILE ROTUNDATA*

Abstract

In insects, prolonged exposure to unseasonably low temperatures can lead to detrimental physiological effects known as chill injury. Changes in membrane phase as a result of low temperature exposure are hypothesized to lead to the collapse of ion gradients, metabolic imbalance and oxidative stress. RNA-seq has provided support for these responses at the level of gene expression in the alfalfa leafcutting bee, *Megachile rotundata,* but they are characterized by variable transcriptional responses in different life stages. In this study, we assess oxidative stress in low-temperature-stressed alfalfa leafcutting bees, *Megachile rotundata* in two different life stages by comparing chill-injury-inducing static thermal regimes (STR) with protective fluctuating thermal regimes (FTR). We assessed the expression of several transcripts with known antioxidant functions during extended prepupal overwintering as well as total antioxidant capacity and lipid peroxidation during both extended overwintering and low temperature stress during pupal development. Real-time PCR showed differential expression of the antioxidant glutathione peroxidase and several transcripts with known antioxidant function including Vitellogenin, Apolipoprotein D, Glutathione S-transferase, and Nuclear protein 1, but mostly transient differences in the expression of other enzymatic antioxidants. Interestingly, *M. rotundata* did not vary in its ability to respond to an induced oxidative stress and measurements of lipid peroxidation showed no differences between treatments. These results indicate either 1) oxidative damage occurs in other structural components besides lipids or 2) oxidative damage is not a factor in FTR responses to chill injury in *Megachile rotundata.* The results presented here

do not support the hypothesis that FTR exposure results in diminished levels of oxidative stress in chill injured insects.

Introduction

Reactive oxygen species (ROS) are normal byproducts of aerobic metabolism, mainly produced through the mitochondrial respiratory chain, but levels can also increase under environmental stress (Monaghan et al., 2009). Oxidative stress occurs when the production of ROS exceeds the organism's ability to eliminate ROS via their antioxidant system. Excess ROS can lead to structural damage of lipids, nucleic acids, and proteins (Monaghan et al., 2009) and to the malfunction of key physiological systems critical to normal function. Oxidative stress is a constant challenge for all animals and increased levels have been associated with decreased longevity and early senescence across taxa (De Block and Stoks, 2008; Haddadi et al., 2014; Monaghan et al., 2009; Reynolds and Phillips, 2013). In insects, oxidative stress has been associated with increased senescence in long-lived worker honeybees (Aurori et al., 2014), compensatory growth in the damsel fly, *Lestes viridis* (De Block and Stoks, 2008), age-related memory impairment in *Drosophila melanogaster,* and low temperature stress responses in several insect species (Joanisse and Storey, 1996; Joanisse and Storey, 1998; Lalouette et al., 2011; Rojas and Leopold, 1996).

Low temperature exposure in insects can result in a suite of detrimental physiological effects collectively known as chill injury. Chill injury is a complex phenotype spanning multiple levels of organization, but evidence of disruptions in ion gradients, metabolic imbalance, and oxidative stress have all been observed (Findsen et al., 2013; Kostál et al., 2006; Kostál et al., 2007; Lalouette et al., 2011; Renault et al., 2004; Rojas and Leopold, 1996). The mechanism by

which chill injury causes these physiological responses is poorly understood, but it is hypothesized that changes in membrane fluidity could underlie these symptoms (Lee, 2010).

In both laboratory and agricultural settings, insects are typically stored under constant temperatures (static thermal regime, STR), conditions known to lead to chill injury. However, exposure to daily, periodic increases in ambient temperatures (fluctuating thermal regime, FTR) allows for the protection against and/or repair of chill injury (Bennett et al., 2015; Colinet et al., 2006; Colinet et al., 2015; Kostál et al., 2007; Lalouette et al., 2011; Renault et al., 2004; Rinehart et al., 2013). Decreased levels of oxidative stress as a result of FTR exposure in chill-injured insects have only been directly observed in adult lesser mealworms, *Alphitobius diaperinus* (Lalouette et al., 2011), but transcriptomic evidence in the alfalfa leafcutting bee *Megachile rotundata* also implicates oxidative stress as a factor in chill injury and the FTR response, indicating that this response may be conserved across taxa (Torson et al., 2015). However, with this lack of sampling across taxa and even across life stages of a single species, it is unclear whether oxidative stress is a critical component in the beneficial effects of FTR and how stressful levels of ROS in chilliinjured insects could lead to observed chill injury phenotypes. For example, damage to structural components within the insect, as a result of either increased ROS or decrease antioxidant function, could provide a mechanistic explanation for damage caused to cell membranes via lipid peroxidation; leading to the observed collapse of ion gradients in other chill-injured insects (Boardman et al., 2011; Kostál et al., 2006; Kostál et al., 2007).

Observational data of downstream consequences of oxidative stress (i.e. damage) will be critical to determine if oxidative stress and recovery/protection against it are important in chill injury and the FTR response, respectively. Transcriptional evidence of oxidative stress in both

post-diapause quiescent prepupae (Torson et al., 2015) and developing pupae (Torson et al., 2017; in review) in *M. rotundata* provide a rationale for further investigation into the role of oxidative stress and damage in response to chill injury and FTR. In this study, we test the hypothesis that oxidative stress plays a role in chill injury and that FTR exposure provides a protective/reparative effect by investigating antioxidant gene expression during prepupal overwintering and total antioxidant capacity and lipid peroxidation in both overwintering prepupal and low-temperature-stressed developing pupae exposed to either an FTR or STR rearing protocol. We predict that *M. rotundata* exposed to FTR will have increased antioxidant capacity relative to STR in both overwintering prepupae and developing pupae. Furthermore, we expect evidence of damage caused by oxidative stress to be higher in STR-reared, chill-injured bees, but damage may be greatest in late-sampled time points in prepupae due to their long duration of exposure and the increased mortality that is associated with the STR protocol in this life stage.

Materials and methods

Insects

All leafcutting bees of Canadian origin (*Megachile rotundata* Fabricius 1787) were purchased from JWM Leafcutter, Inc. (Nampa, ID) as loose brood cells. Bees for both extended overwintering (prepupae) and interrupted development (pupae) were derived from the 2014 field season and arrived in late March of 2015 after overwintering at a constant temperature (4-6°C). Diapausing prepupae arrived as loose cells and were housed in Percival model I-30BLL reach-in incubators at 6°C ± 0.5°C under darkness upon arrival.

Temperature protocols

Extended overwintering

Post-diapause quiescent prepupae were maintained at 6°C until the end of their typical overwintering period (mid-April). *M. rotundata* were then placed into either a Static Thermal Regime (STR) or a Fluctuating Thermal Regime (FTR; Fig 4.1). The STR protocol consisted of continued exposure at 6°C under darkness. Bees reared under the FTR protocol were exposed to a constant 6°C with a daily, one hour pulse of 20°C with a one-hour ramp-up and ramp-down time (0.23°C/min).

Interrupted development

Post-diapause quiescent prepupae were again maintained at 6°C prior to pupal development. After overwintering, prepupae were transferred to 29°C to initiate development. Developing pupae were maintained at 29°C until they reached a "red eye" stage in development characterized by melanization of the eyes. Developmental progress was assessed via guide plates. For the guide plates, bees not destined for experimental treatment were removed from their brood cells and placed in a 24-well plate within a chamber containing a NaCl solution to maintain 75% humidity (Winston and Bates, 1960). Once ~50% of the guide-plate bees had reached the "red eye" stage, experimental bees were placed into their respective low temperature stress treatments (STR, FTR) for seven days (Fig. 4.1). Both STR and FTR protocols for this life stage are consisted with those that were used during extended overwintering.

Gene expression

RNA collection

RNA samples were derived from the 2009 field season, overlapping with samples collected for a previous RNA-seq study (Torson et al., 2015). Post-diapause quiescent prepupae

were reared under either an FTR or STR extended overwintering rearing protocol. Samples were harvested starting at 20 weeks of exposure and were sampled every two weeks until there were no viable STR-reared individuals.

Sample preparation

Total RNA for qPCR was collected from whole prepupae exposed to either the FTR or STR treatment during extended overwintering (3 biological replicates), using the TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH USA). RNA precipitates were stored under ethanol until needed. RNA samples were diluted to equal concentrations and genomic DNA contamination was removed using Invitrogen DNase I (Carlsbad, CA, USA). First strand synthesis, using 1 µg RNA for each biological replicate, was carried out using Invitrogen Super Script III first strand synthesis system for RT-PCR (Carlsbad, CA, USA). A pooled sample of all biological replicates was used to determine optimal dilution of experimental cDNA samples.

Primer design and reference gene selection

Primers for all experimental targets and reference gene candidates (Table C.1) were designed using Integrated DNA Technologies' IDR program (Coralville, IA, USA). Candidate reference genes (13) were selected using RNA-seq data from *M. rotundata* reared under the same conditions (Torson et al., 2015). The most stably expressed candidate reference genes were identified using the algorithm geNorm (Vandesompele et al., 2002). Two transcripts, ubiquitin-fold-modifier-conjugating enzyme 1-like and peptidyl-prolyl cis-trans isomerase-like 2-like (Table C.1), were selected as reference genes for the normalization of all experimental targets.

qPCR

All qPCR runs were conducted on a Roche LightCycler 480 II (Indianapolis, ID, USA) using Roche FastStart Universal SYBR Green I Master Mix with ROX (Indianapolis, IN, USA).

To ensure quality between plates, three calibrator reactions consisting of a pooled template sample and different three verified primer sets were present on each plate, these reactions also served as a positive controls. Additionally negative, no template, controls consisting of all enzymatic components and lacking template were present for each experimental primer set assayed on each plate. Biogazelle qbase+ (Ghent, Belgium) was used for annotation and normalization against reference genes. Calibrated normalized relative quantities (CNRQ) were $\log_{10}$ transformed to allow for parametric statistics and exported for statistical analysis and graphical representation using Prism 6 Graphpad (La Jolla, CA USA). Statistical significance of each target was determined via a two-way ANOVA of CNRQ for each transcript with Sidak's correction for multiple comparisons.

Total antioxidant capacity

Total antioxidant capacity (TAC) was assayed using the Antioxidant Assay Kit (Cayman Chemical; Ann Arbor, MI USA) in accordance with the manufacturer protocol. Samples were harvested from their respective treatments and immediately frozen on dry ice and stored at -80°C until needed. Samples were homogenized in 1 ml PBS (pH 7.4) using a Bullet Blender Blue (Next Advance; Averill Park, NY, USA) with a mixture of 0.5 mm and 1.0 mm Zirconium oxide beads for three minutes with speed of homogenizer set to 6. Interrupted development and extended overwintering samples were diluted 1:30 and 1:40, respectively. Absorbance was read at 405 nm. Total protein was determined using a modified Bradford Assay (Bio-Rad, Berkeley, CA, USA) read spectrophotometrically at 595 nm against a 0.05-0.5 mg/ml bovine serum albumin (BSA) standard. The results are expressed in mM Trolox equivalents per mg of protein. Differences between treatments were determined using two-way ANOVA with Tukey's test for multiple comparisons. Time effect for each treatment was analyzed using linear regression.

Lipid peroxidation

The concentrations of thiobarbituric acid reactive substances (TBARS), a common metric of oxidative stress, for both pupae exposed to the interrupted development protocol and prepupae exposed to extended overwintering conditions was assayed using a TBARS (TCA method) Assay Kit (Cayman Chemical; Ann Arbor, MI USA). Samples were harvested and stored for both life stages in a similar fashion to the TAC assay. Samples were homogenized at 4°C using a Bullet Blender Blue (Next Advance, Averill Park, NY, USA) in 250 µl RIPA buffer (250 mM Tris-HCl, pH 7.6, containing 750 mM NaCl, 5% NP-40, 2.5% sodium deoxycholate, and 0.5% SDS) with 0.5 mm and 1.0 mm zirconium oxide beads. RIPA buffer was treated with Roche cOmplete™ protease inhibitor cocktail tablets (Roche, Indianapolis, IN USA). Samples were homogenized with same settings as TAC assay. Samples were diluted 1:10 to ensure samples fell within the standard curve. Reactions were run in duplicate and sample TBARS concentrations (nmol/ml) were calculated from a standard curve (0-50 nmol/ml) of malondialdehyde (MDA). TBARS measurements were corrected for total protein using a Bradford assay (Bio-Rad, Berkeley, CA USA) and results are represented as µM MDA/mg protein. Data were analyzed using two-way ANOVA with Tukey's correction for multiple comparisons. Time effect was again analyzed using linear regression.

Results

Gene expression

Antioxidant gene expression was assessed in *M. rotundata* prepupae exposed to either an STR or FTR rearing protocol every two weeks from 20 to 30 weeks of extended overwintering (Fig 4.1; 4.2). Candidate genes involved in oxidative stress were chosen because they were either previously identified as being differentially expressed under these conditions (Torson et al.

2015), or they mediate oxidative stress in other contexts (Imlay, 2003). The expression of Glutathione S-transferase, Apolipoprotein-D-like, Short-chain dehydrogenase reductase, Vitellogenin, Eater, and Nuclear protein 1-like, all transcripts with known antioxidant properties, was assayed in two-week intervals throughout the time series (Fig 4.2A).

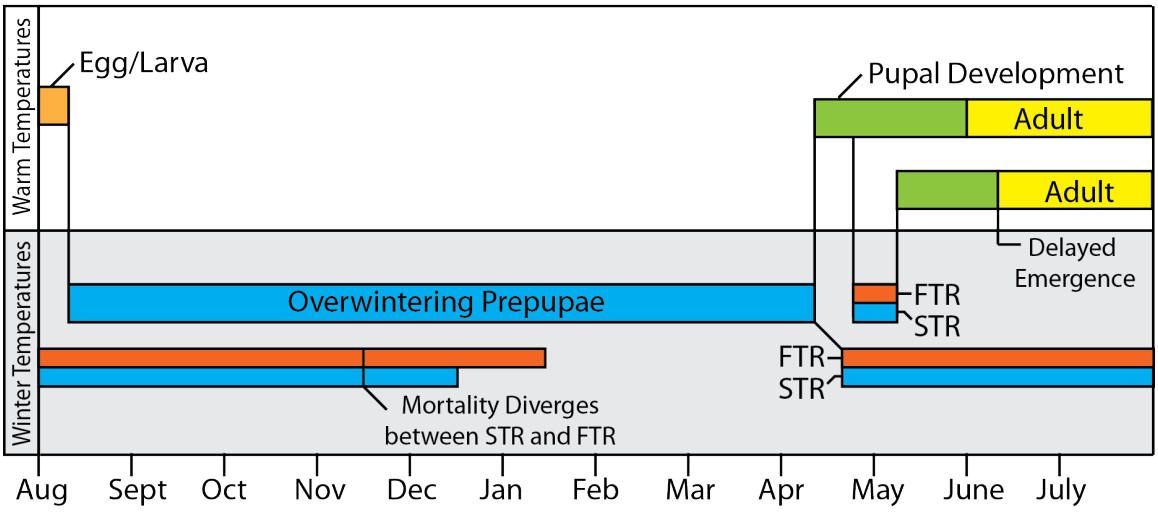

Figure 4.1. Timing of experimental treatments. *M. rotundata* were exposed to either and STR or FTR protocol in either 1) Prepupal extended overwintering starting in April and maintained until STR-reared individuals were no longer viable, or 2) during pupal development to delay emergence to match peak floral bloom.

Expression of Eater was significantly influenced by treatment ($F_{1,4} = 333.3$, $P < 0.0001$), explaining 85.49% of the variation in gene expression. There was no effect of week on Eater expression ($F_{6,24} = 2.09535$, $P = 0.1559$), and significant differences in y-intercepts of the linear regression ($F_{1,39} = 239.003$, $P < 0.0001$) suggests that initial changes in expression happen before the time points sampled. Expression of Apolipoprotein D (ApoD) was also significantly influenced by treatment ($F_{1.4} = 75.31$, $P = 0.0010$) and treatment/time-point interaction ($F_{6,24} = 4.083$, $P = 0.0058$) with all time points except 20 weeks being differentially expressed between treatments with FTR and STR having significant nonzero slopes ($F_{1,19}$, $P = 0.0023$ and 0.0218,

respectively) implying that expression may continue to diverge with increased exposure time. A similar trend was observed in short-chain dehydrogenase reductase expression with treatment explaining 80.41% of the variation in expression ($F_{1,4} = 1000$, $P < 0.0001$). Expression of the egg yolk protein precursor Vitellogenin (Vg) had a significant treatment effect ($F_{1,4} = 63.61$, $P = 0.0013$). Temperature treatment significantly affected expression of Glutathione S-transferase (GST; $F_{1,4} = 16.99$, $P = 0.0292$) with three time points being differentially expressed between treatments. Furthermore, GST expression significantly increased in STR over time ($F_{1,19} = 6.139$, $P = 0.0228$). Nuclear protein 1 (NUPR1) was differentially expressed at 20, 24, and 30 weeks of exposure with treatment explaining 62.71% of variation in expression ($F_{1,4} = 118.0$, $P = 0.0004$). Expression of NUPR1 in either STR or FTR did not change over time ($F_{1, 38} = 0.666368$, $P = 0.4194$), again showing that the initial deviation in gene expression occurred prior to sampling.

With the exception of glutathione peroxidase (GPx), enzymatic antioxidants showed little variation between treatments (Fig 4.2B). Expression of superoxide dismutase (SOD) had a significant time ($F_{6,24} = 3.127$, $P = 0.0349$) and time/treatment effect ($F_{6,24} = 4.035$, $P = 0.0062$), but only one time point (28 weeks) was differentially expressed between STR and FTR treatments. GPx was enriched in STR from 24-32 weeks of exposure (Fig 4.2B). GPx expression also had a significant time effect, with slopes of FTR and STR expression differing significantly ($F_{1,38} = 17.171$, $P = 0.0002$). The expression of Catalase (Cat) did not differ between STR and FTR at any individual time point, but Cat expression in FTR-exposed individuals showed a significant decrease in expression throughout the duration of exposure ($F_{1,19} = 5.233$, $P = 0.0338$). Nitric oxide synthase (NOS) expression was significantly influence by both treatment ($F_{1,4} = 10.45$, $P = 0.0319$), time ($F_{6,24} = 6.902$, $P = 0.0002$) and their interaction ($F_{6,24} = 2.521$, $P = 0.0491$).

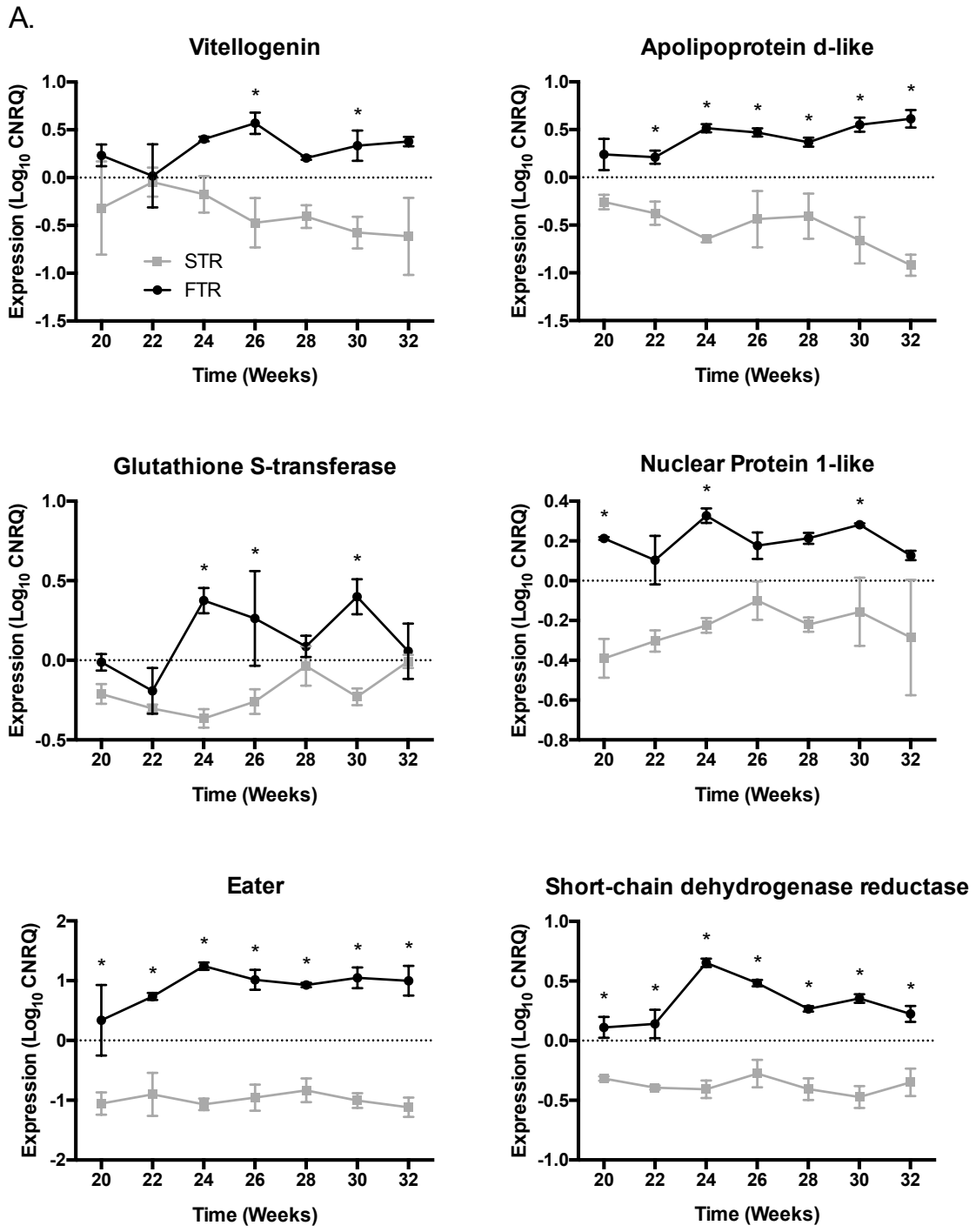

Figure 4.2. Expression profiles of antioxidant-functioning transcripts. Expression was assessed from 20 to 32 weeks of extended prepupal overwintering. Gene expression is represented as Mean ± S.E. of Log_{10} transformed calibrated normalized relative quantities (CNRQ). Asterisks indicate statistical significance between STR (Black) and FTR (Grey). A) Transcripts differentially expressed in Torson et al 2015 with antioxidant function. B) Additional enzymatic antioxidants.

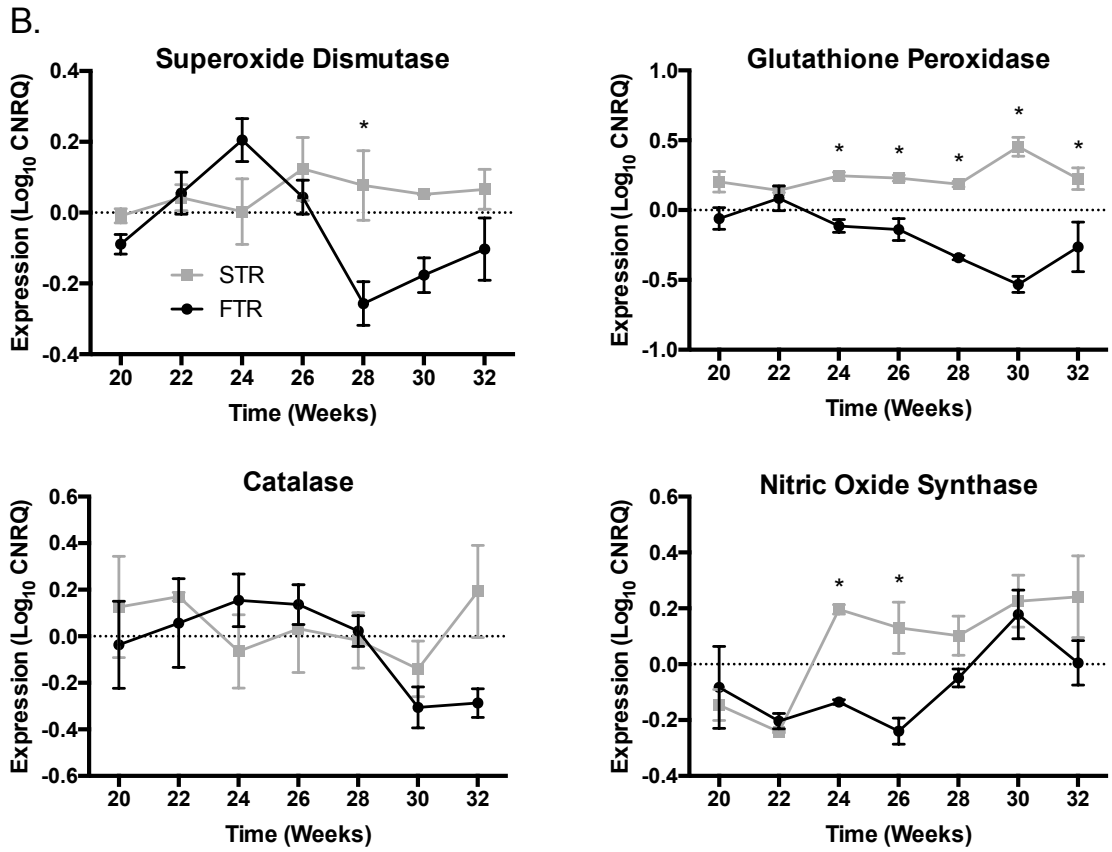

Figure 4.2. Expression profiles of antioxidant-functioning transcripts (continued). Expression was assessed from 20 to 32 weeks of extended prepupal overwintering. Gene expression is represented as Mean ± S.E. of Log_{10} transformed calibrated normalized relative quantities (CNRQ). Asterisks indicate statistical significance between STR (Black) and FTR (Grey). A) Transcripts differentially expressed in Torson et al 2015 with antioxidant function. B) Additional enzymatic antioxidants.

Total antioxidant capacity

The total antioxidant capacity (TAC) during prepupal extended overwintering (leading up to the gene expression time series, 20 weeks) did not vary between treatments (ANOVA, $F_{1,4}$ = 0.6461 P = 0.4665). Time explained 34.76% of the variation in TAC ($F_{9,36}$ = 2.963, P = 0.00097). TAC in weeks 14-20 decreased significantly from week 12 in STR (Fig 4.3A), but no global increase was observed with linear regression ($F_{1,29}$ = 1.019, P = 0.3214). TAC during interrupted pupal development showed similar trends. There was no treatment effect between STR and FTR

($F_{1,4}$ = 0.7891, P = 0.4246) and at no point were TAC measurements significantly different between STR and FTR (Fig 4.3B).

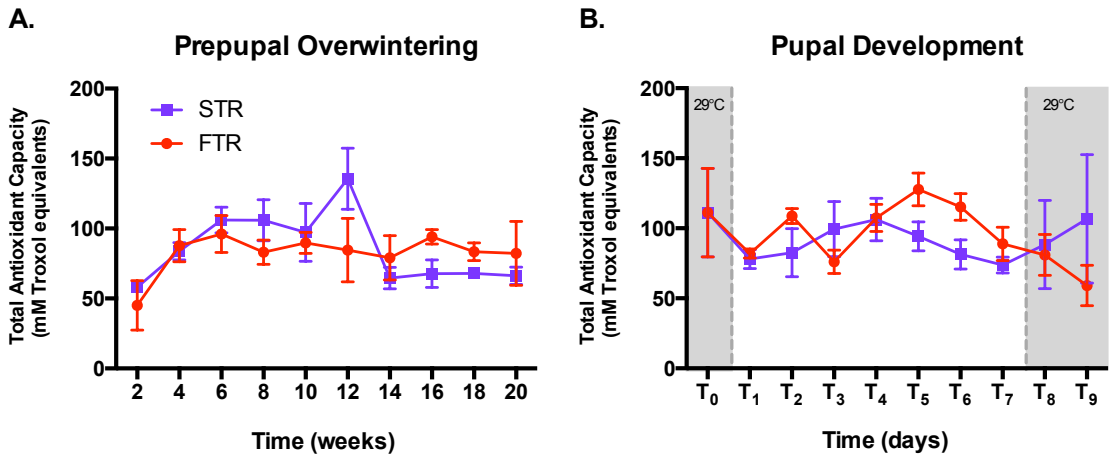

Figure 4.3. Total antioxidant capacity. A) Overwintering prepupae and B) interrupted development pupae. Grey boxes indicate exposure to normal developmental temperatures. Data are expressed as mM Trolox equivalents/mg protein (mean ± S.D.).

Lipid peroxidation

Lipid peroxidation, a common metric of oxidative stress, was measured in *M. rotundata* exposed to either STR or FTR low temperature stress in both life stages. Lipid peroxidation, reported as µM MDA/mg protein, did not vary between treatments before, throughout, and after exposure to low temperature stress in the pupal life stage (Fig. 4.3A). In prepupal extended overwintering, lipid peroxidation between the two treatments was stable for the first 14 weeks of treatment, but increased in both treatments from 16-20 weeks of exposure (Fig 4.4B). There were no statistical differences between treatments (Two-way ANOVA, $F_{1,4}$ = 0.8683, P = 0.4042), but time explained 40.16% of the variation in lipid peroxidation ($F_{5,20}$ = 3.922, P = 0.0122). TBARS measurement was elevated in STR-reared individuals at 20 weeks, but this was not statistically significant.

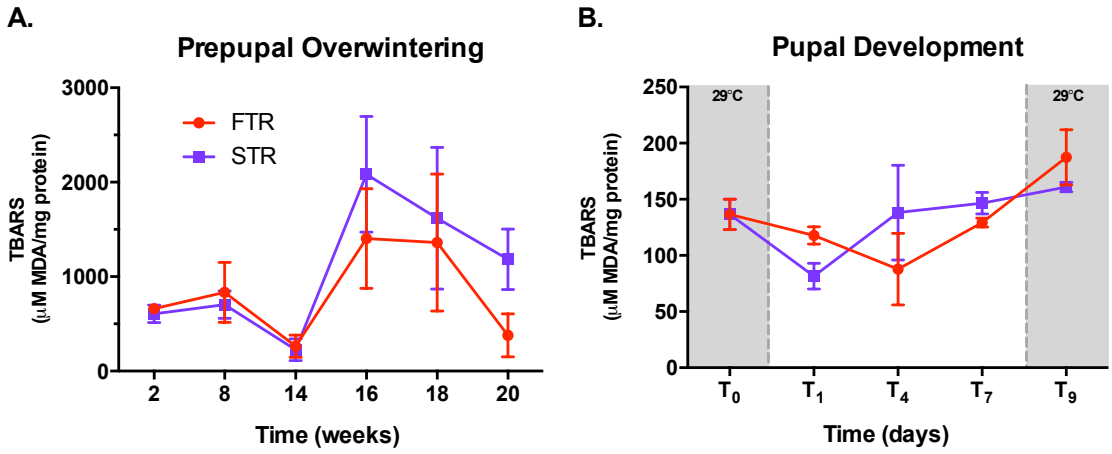

Figure 4.4. Lipid peroxidation. TBARS measurements between STR and FTR in A) extended overwintering prepupae and B) low- temperature-exposed pupae. Grey indicates maintenance at normal temperatures for pupal development (29°C). Measurements are recorded as mean ± S.D.

Discussion

Oxidative stress is hypothesized to be involved in chill injury and, potentially, in the protective and/or reparative effect of FTR (Hayward et al., 2014; Lee, 2010). Prior to this study, few have provided direct evidence of oxidative stress in chill-injured or overwintering insects (Joanisse and Storey, 1996; Joanisse and Storey, 1998; Lalouette et al., 2011; Rojas and Leopold, 1996), and only one of those in insects exposed to FTR (Lalouette et al., 2011). In this study, we assessed gene expression of transcripts with antioxidant function in prepupal extended overwintering as well as total antioxidant capacity and lipid peroxidation in both extended overwintering and during low temperature stress in pupal development.

Antioxidant gene expression

Transcripts functioning in cell membrane structure and function, ion homeostasis, metabolism, neurogenesis and oxidative stress are upregulated in response to FTR exposure (relative to STR) in overwintering, post-diapause quiescent *M. rotundata* prepupae (Torson et al., 2015), prompting further inquiry into oxidative stress. The three main antioxidant groups

Superoxide dismutase (SOD), Catalase (Cat), and Glutathione peroxidase (GPx), were ideal candidate genes to test the hypothesis that an oxidative stress response is involved in the protective/reparative effects of FTR exposure in chill injured insects. SOD acts upon the cell-damaging superoxide ion, converting it to hydrogen peroxide (H_2O_2) and singlet oxygen, both of which are still capable of causing damage to structural components of cells. Resulting hydrogen peroxide is scavenged by both Cat (more localized) and the more widely distributed GPx (Monaghan et al., 2009), while the singlet oxygen is neutralized by non-enzymatic antioxidants such as Vitamin E. SOD was only significantly enriched in STR at 28 weeks, but SOD's increase over time in STR, relative to FTR, suggests that STR-reared individuals are experiencing increased levels of the superoxide ion over time. This result is consistent with relatively constant expression of SOD in overwintering *Epiblema scudderiana* (Joanisse and Storey, 1996) and the variation in slope of the linear regressions between treatments may due to the fact that our samples were taken from individuals in extended overwintering. GPx was enriched in STR-reared individuals from 24-32 weeks of treatment suggesting that *M. rotundata* reared under constant, chilled temperatures are attempting to combat an influx of H_2O_2 produced by SOD. With a lack of differential expression between treatments or time effect in Cat (Fig 4.2B), it is likely that its more localized effect is not contributing differentially to this stress response or that its effect has been masked by our sampling method (whole organism). Increased expression of these transcripts in STR-exposed prepupae indicates they may be coping with an oxidative stress and that this stress may be increasing over time.

The production of endogenous nitric oxide (NO) is critical for proper function of nervous and immune system signaling (Muller, 1997), but increased levels can also lead to oxidative damage. Nitric oxide, produced by nitric oxide synthase (NOS; Fig. 4.2B), and peroxynitrite,

resulting from the reaction of nitric oxide with the superoxide ion, are capable of oxidizing lipids, DNA and protein (Zhang and Li, 2006). NOS also plays a critical role in non-specific immunity and has been shown to regulate anti-inflammatory reactions (Livonesi et al., 2009). In locusts, during development nitric oxide is also a functional component of axonal outgrowth and synaptogenesis (Bicker, 2001). Based on NOS expression increases in STR-exposed *M. rotundata* prepupae alone, it is unclear what functional roles the resulting enzyme would have.

In addition to these enzymatic antioxidants, we assessed expression of several additional transcripts whose products have known antioxidant functions, including Vitellogenin (Vg), Apolipoprotein D (ApoD), Glutathione S-transferase (GST), Nuclear protein 1 (NUPR1), Eater, and a Short-chain dehydrogenase reductase (SDR). These six transcripts were differentially expressed between STR and FTR in the transcriptomic assessment of these responses in Torson et al. 2015. The expression profiles of these transcripts over the time series assessed in this study might provide insight into the protective/reparative effects of FTR.

Vitellogenin is an egg yolk protein precursor involved in lipid transport that is synthesized and secreted from the fat body in insects, but its homologs are present in nearly all egg-laying animals (Havukainen et al., 2013). Vg also has anti-inflammatory and antioxidant properties (Corona et al., 2007; Salmela et al., 2016; Sun and Zhang, 2015) and increased expression is associated with longevity in honeybees (Aurori et al., 2014; Ihle et al., 2015). Vg appears to have localized effects in honeybees (Münch et al., 2015) and its mechanism of action in response to oxidative stress and inflammation may shed light on its importance in the FTR response. Vg expression remained high in FTR throughout the time series indicating that the effect of FTR exposure causes increases in Vg during early exposure to treatment. In honeybees, Vg has a high affinity in binding to damaged cell membranes and can also bind to healthy cells,

increasing oxidative stress tolerance (Havukainen et al., 2013), identifying a potential regulatory mechanism for increased longevity in honeybees. This association with damaged cells could indicate a reparative mechanism, at least in part, for the dramatic increases in survival associated with FTR response in *M. rotundata* prepupae.

We also observed the upregulation of Apolipoprotein D (ApoD) in FTR-reared *M. rotundata.* Similarly to Vitellogenin, ApoD, a lipid transport molecule, is involved in oxidative stress response and longevity in *Drosophila melanogaster* (Walker et al., 2006) and oxidative stress and bacterial challenges in *Bombyx mori* (Chen et al., 2016). The absence of the ApoD homolog (Glial Lazarillo; GLaz) in *D. melanogaster* is associated with reduced longevity and accelerated neurodegradation (Sanchez et al., 2006), indicating a protective effect. Chill-injured pupal *M. rotundata* exposed to the STR protocol show increased neuromuscular dysfunction relative to those exposed to FTR (Bennett et al., 2015). A knockdown of ApoD in *M. rotundata* exposed to the protective FTR protocol could elucidate ApoD's importance in FTR's protective/reparative effect.

In insects, glutathione S-transferase (GST) enzymes are involved in the detoxification of xenobiotic compounds and peroxidative-induced oxidative damage (Liu et al., 2016; Perić-Mataruga et al., 2015; Yamamoto et al., 2011; Zhang et al., 2013). This transcript was up-regulated in FTR-reared prepupae, but an increase in expression over time in STR-reared individuals indicates that this response might be lagging behind those reared under FTR. If these stress responses are indeed slowed down or absent in STR-exposed insects, resulting oxidative damage could play a crucial role in the dramatic survival differences between STR and FTR, especially if Vg and ApoD are providing reparative mechanisms for oxidative damage incurrent in FTR individuals.

The upregulation of Nuclear Protein 1-like (NUPR1), also known as p8 (Goruppi and Iovanna, 2010), in FTR individuals also suggests that fluctuating temperature exposure is allowing for a response to stress. NUPR1 is not as well studied as the transcripts that I have discussed previously, at least in insects, but serves as a transcriptional response regulator to cellular stress pathways in mammalian systems and knockdowns have been shown to lead to increased sensitivity to oxidative stress (Jin et al., 2009). NUPR1 appears to also have a regulatory function associated with DNA repair mechanisms (Gironella et al., 2009). Since oxidative stress is known to cause damage to nucleic acids, the upregulation of NUPR1 could provide repair of this damage caused by low temperature exposure in *M. rotundata*. Although, these observations have not been made in more closely related taxa, these sequences are highly conserved, meaning their stress response roles may also be conserved.

Consistently high levels of Eater in FTR-reared individuals over time, most highly elevated in this study, suggests Eater's role may be important in this response and evidence from other insects indicates that its function may not be directly associated with oxidative stress, but in the identification of damaged cells. Eater is a transmembrane protein located within haemocyte membranes and is known to function in the recognition and binding of pathogens in the initial steps of phagocytosis during an immune response (Kocks et al., 2005).There are several immune response gene upregulated in FTR-reared *M. rotundata* prepupae (Torson et al., 2015), supporting an immune-response-centric function of Eater. However, extracellular domains of Eater show similarity to proteins functioning in the removal of apoptotic cells during tissue remodeling during metamorphosis in the flesh fly, *S. crassipalpis* (Hori et al., 2000) and *C. elegans* (Zhou et al., 2001). During flesh fly metamorphosis, Eater's homolog is present in haemocyte membranes that function to clear apoptotic cells during tissue remodeling. Given

Eater's function during an immune response, the protein may be binding to dead or dying cells, but this apoptotic-function needs additional study (Kocks et al., 2005). If FTR does provide a reparative effect, removal of dead and damaged tissue would be an important component to the response.

Short-chain dehydrogenase reductase family 16c member 6 (SDR) was identified as a candidate gene for an oxidative stress response. Most of the members in the large superfamily are NAD- or NADP-dependent oxidoreductases (Persson and Kallberg, 2013). The majority of these proteins are membrane-bound, but structure and resulting function of these genes are diverse across taxa limiting our ability to make inferences on its function during the FTR response at this point.

Antioxidant capacity and damage

We predicted that *M. rotundata* exposed to FTR would have increased antioxidant capacity when compared to STR-reared individuals in both life stages that we assessed. Counter to our predictions, neither *M. rotundata* prepupae nor pupae reared under an STR or FTR differed in their ability to cope with an induced oxidative stress (Fig. 4.3). Until this point, all assessments of enzyme activity in response to low temperature stress have been at the single-enzyme level and not global responses to oxidative stress. In the freeze avoidant moth *Epiblema scudderiana,* SOD expression remained relatively constant throughout the duration of overwintering (Joanisse and Storey, 1996), a result that is consistent with *M. rotundata* exposed to extended overwintering (Fig 4.2B). Glutathione peroxidase increased over time in *E. scudderiana.* Since GPx expression is also elevated in STR-reared *M. rotundata* prepupae we might expect to see a similar relationship during typical overwintering durations as they could result in the differences that we see between STR and FTR in this study. An absence of an

adaptation of the antioxidant system for cold-hardiness in both *E. scudderiana* and the gall insect *Eurosta solidaginis* (Joanisse and Storey, 1996; Joanisse and Storey, 1998) is consistent with the lack of variation observed at the level of antioxidant expression and lipid peroxidation in *M. rotundata* for both prepupal extended overwintering and pupal interrupted development.

Lipid peroxidation did not vary between treatments throughout the exposure period. This result was counter to our predictions and suggests that neither STR-reared prepupae nor pupa accumulate measurable levels of damage to lipids caused by the accumulation of free radicals. These results, in combination with only marginal differences in expression in enzymatic antioxidants (Fig 4.2B) do not support our initial hypothesis that STR-exposed prepupae and pupae are succumbing to an accumulation of oxidative stress. However, oxidative stress could still be acting on either DNA or proteins, but based on the results presented in this study, this seems unlikely.

The differential expression of antioxidant-functioning transcripts in FTR-reared prepupae during extended overwintering now appears counter to the antioxidant capacity and lipid peroxidation results observed in this study. It is possible that these transcripts, especially Vg, ApoD, NUPR1, and Eater could be acting in a reparative fashion that is independent of oxidative stress in FTR-reared individuals. Further assessment of the expression of these transcripts during pupal interrupted development could provide support for conserved expression profiles between these two life stages. Vg and ApoD's association with longevity still suggests that these could be critical components of the FTR response. For example, Vg is thought to have antioxidant properties, but studies in honeybees support the hypothesis that longer-lived drones are not actually increasing response to oxidative stress or repairing damage but instead an increased tolerance to induced oxidative stress (Li-Byarlay et al., 2016). An increased stress tolerance

could be assayed in *M. rotundata* by, for example, paraquat injection or knockdowns of candidate genes, such as those presented in Fig. 4.2.

Overall, the results of this study indicate that both *M. rotundata* prepupae and pupae reared under FTR or STR do not differ in their ability to combat an induced oxidative stress. I did not observe evidence of lipid peroxidation, but damage to DNA and protein could still occur and assessments of damage to these additional structural components and measurements of ROS production are still needed. The results of the gene expression measurements provide quality candidates for functional genomic assessments in chill injured insects that could yield more concrete functional explanations for the differential expression of these transcripts in this response and how the influence the chill injury phenotype.

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CHAPTER 5: CONCLUSIONS

In ectotherms, exposure to stressful temperatures throughout development can have detrimental effects on adult performance via damage to various physiological systems. In insects, this damage is known as chill injury. However, when chill-injured insects are exposed to daily, periodic increases in ambient temperature (fluctuating thermal regimes; FTR), the effects of chilling can be significantly diminished (Colinet et al., 2006; Colinet et al., 2007; Kostál et al., 2007; Lalouette et al., 2011; Renault et al., 2004; Rinehart et al., 2011). A wealth of data have been collected in a wide range of insect taxa, but mechanistic level hypotheses have been generated with seemingly little attention to the geographic locations of these organisms and that temperature stress responses vary dramatically throughout development, especially in insects located in temperate regions. Therefore, a model system that experiences diverse temperature demands throughout development, has well-defined rearing protocols, and a wealth of genomic and physiological resources is necessary to assess whether chill injury and FTR's protective effect are conserved across taxa.

The goal of this dissertation is to provide the first mechanistic assessments of chill injury and responses to FTR to yield insight into mechanistic-level responses in the alfalfa leafcutting bee, *Megachile rotundata* and offer support for the new transcriptomic data at the physiological level. This dissertation has three primary objectives. First, to identify transcripts involved in FTR response in overwintering, chill-injured prepupal *M. rotundata.* Second, it was necessary to assess whether the transcriptomic responses observed in Objective 1 are conserved across life stages by assessing transcriptional response to a similar treatment in developing pupa. Finally, my third objective was to integrate gene expression data collected in Objectives 1 and 2 with physiological responses to each treatment in both life stages.

Objective 1

In chapter 2, I exposed *M. rotundata* post-diapause quiescent prepupae to an extended overwintering protocol consisting of either constant, typical (managed) overwintering temperatures, or the same constant temperature with a daily, one-hour warm temperature pulse. I conducted an RNA-seq experiment with time points sampled both before and after mortality between the two treatments diverged. I was able to provide support for several physiological responses observed in other chill-injured insects including changes in ion homeostasis, changes in energy metabolism and oxidative stress. In addition to the support for current hypotheses in the literature, I observed transcripts functioning in immune response and neurological development.

Post-diapause quiescence is typically thought of as a developmentally static life stage, so differential expression of developmentally involved transcripts was unexpected. Because there are no gross morphological changes occurring during this life stage, it is possible that the upregulation of these transcripts is evidence of repair of damage in FTR-reared prepupae. Chill injury is characterized by loss of neuromuscular coordination. Upregulation of Neuroligin, Slit, Roundabout-like protein 1, Division abnormally delayed, and dynein heavy chain axonemal-like, among others, suggest that neurological damage could be repaired as a result of the warm-temperature pulses associated with FTR. Further assessments of damage to the neuromuscular system or knockdowns of these candidate genes in FTR-reared prepupae are logical future directions to support this hypothesis.

Objective 2

Chapter 2 was the first transcriptomic-level assessment of an FTR's effect on chill injured insects and yielded the ability to conduct comparative studies to assess FTR's effect

across life stages and throughout and single life stage. To do this, I exposed developing pupae to a "cold snap" consisting of either typical overwintering conditions or overwintering temperatures with a daily, periodic warm temperature pulse, both similar to the thermal regimes in chapter 2, and harvested RNA for RNA-seq after seven days of exposure to treatment. Expression analysis of each treatment revealed differential expression of transcripts functioning in various aspects of cell membrane composition and function. Transcripts with similar functions were also differentially expressed in prepupae (Chapter 2) suggesting that there may be a shared physiological response. Furthermore, evidence of oxidative stress was observed in both life stages. While a significant number of differentially expressed transcripts were present in both life stages, when the direction of regulation is considered (whether a transcript is up- or downregulated in response to FTR), all overlap between the two life stages disappears.

There appears to be conservation in functional classes of transcripts between life stages, but the specific transcripts being differentially expressed are highly variable. This result suggests that there may be redundancy at the level of gene expression that leads to similar physiological responses. This lack of conservation at the transcriptomic level was counter to our initial predictions, but once we considered that there has been a complete morphological restructuring of the bee between the two life stages sampled, it seemed logical, if not likely, that their responses to low temperatures may vary mechanistically. This is reinforced by the notion that an overwintering post-diapause quiescent prepupae and a late-spring-developing pupae have very different ecologies and that temperatures that would illicit low temperature stress responses may vary dramatically.

Objective 3

The variation in differentially expressed transcripts between STR and FTR across these two life stages in *M. rotundata* suggest that different transcriptional mechanisms could be driving similar physiological responses in both life stages. In chapter 4, I assessed oxidative stress responses throughout STR and FTR treatment in both overwintering post-diapause quiescent prepupae and developing pupa. With evidence of antioxidant differential expression in prepupae and oxidative damage in STR-exposed pupa, I decided to assess antioxidant gene expression in an extended prepupal time series and total antioxidant capacity and oxidative damage in both life stages across the duration of treatment. Total antioxidant capacity did not vary between life stages, suggesting the individuals reared under either treatment have the same capacity to cope with an induced oxidative stress. Surprisingly, I did not observe increased levels of lipid peroxidation in, a common metric for oxidative stress, in STR-reared prepupae in extended overwintering. The result is counter to an oxidative-stress centric hypothesis associated with chill injury and the FTR response. While it is still possible that oxidative stress could be damaging other structural components (i.e. DNA and protein), I have not been able to provide support for oxidatively-induced damage at this point.

Synthesis

The three objectives presented in this dissertation present an integrative approach across multiple levels of organization to explain FTR's protective effect against chill injury in *Megachile rotundata.* Evidence at the transcriptomic level suggest that cell membranes are important in this response and their differential expression in both life stages suggests that this may be a critical factor in both chill injury and FTR's protective effect against it. Additionally, upregulation of antioxidants in FTR-reared overwintering prepupae and transcripts suggesting

DNA damage in STR-reared developing prepupae suggest that oxidative stress may also play a role this response.

A lack of increased levels of lipid peroxidation prior to divergence in mortality in prepupae or during pupal stress do not support the hypothesis that oxidative stress is increased, relative to FTR, in STR-reared individuals. As I've noted in the discussion in chapter 4, the time points sampled for the lipid peroxidation assay lead only up the first RNA-seq time point in Chapter 2, two months before mortality between the two treatments began to diverge significantly. This time point selection was based on the significant differential expression of antioxidants at this early time point in the RNA-seq data. It is possible that we could have seen divergence in lipid peroxidation between treatments if I had sampled further into treatment. This continued sampling could be conducted in future field seasons. However, differential gene expression during pupal stress between STR and FTR indicates that DNA damage is present in STR-reared *M. rotundata* during this life stage. Further assessments of DNA and/or protein damage could provide evidence for an oxidative stress response as factor in FTR's beneficial effects.

The work conducted in this dissertation yields insight into responses to FTR in chill injured *Megachile rotundata* at both the transcriptional and physiological level. In addition, to providing support for current hypotheses in the literature, it is important to note that RNA-seq is a powerful hypothesis-generating tool that would provide further study into other components of this response. For example, the differential expression of two facilitated trehalose transporter tret1-like transcripts and a trehalase precursor during pupal interrupted development suggest trehalose, a well-known cryoprotectant, could be involved in chill injury. Metabolites with cryoprotective properties such as trehalose, glucose, sorbitol, glycerol, ribose, and free fatty

acids have been of great interest in assessments of both chilling and freezing in insects (Bale and Hayward, 2010; Colinet et al., 2007; Colinet et al., 2012; Colinet et al., 2016; Hayward et al., 2014; Teets et al., 2012). A metabolomic assessment of responses to FTR in *M. rotundata* would allow for further integration across multiple levels of organization, potentially providing corollary evidence at transcriptomic, metabolomics, and physiological levels. The benefits of an "-omics" assessment of the FTR response would be not only an understanding of how common antioxidants respond, but could identify new metabolites that are associated with this response and inform further metabolite assays in other organisms.

Broader impacts

Megachile rotundata is the most intensively managed solitary bee in the world (Pitts-Singer and Cane, 2011). With an increasing demand for alternative pollinators in the face of colony collapse disorder in honeybees, better management and understanding of the physiology of alternative pollinators is increasingly important. Currently, the United States imports the majority of its alfalfa leafcutting bees from Canada. Canadian-sourced *M. rotundata* have significantly lower immature bee mortality (Pitts-singer and James, 2005). Better management protocols in the U.S. as a result of a better understanding of *M. rotundata's* physiology could allow significantly decreased reliance on imported bees. In this dissertation, I have increased our understanding of how *M. rotundata* copes with low temperature stress at both the transcriptional and physiological level and how a deviation from standard management practices (static temperatures) can increase survival of juvenile life stages, quality of adults, and, subsequently, efficiency of pollination services. A better understanding of temperature stress in this bee will allow for the development of biomarkers that managers can use to assess brood quality. The development of these measurable markers could aid in the identification of stress in managed

populations and prevention potential collapses in population size or decreases in bee quality. Furthermore, an increased knowledge of how to manage this *Megachile rotundata* will inform the management practices of other alternative pollinators. While honeybees have exceptional rearing protocols, their highly social life history does not align well with other pollinator species that are more solitary and gregarious leaving *M. rotundata* as a more appropriate model.

In addition to the direct benefits on agroecosystems, *M. rotundata* provides a potent model for continued investigation into various aspects of cold physiology. With its sequenced genome, well-defined rearing protocols, and the wealth of transcriptomic and physiological data that have been provided in this dissertation, *M. rotundata* allows for continued assessments of cold tolerance across multiple levels of biological organization.

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APPENDIX A: CHAPTER 2 SUPPLEMENTARY MATERIAL

Table A.1. qPCR validation of RNA-seq results.

Transcript ID	Early STR vs Early FTR	Late STR vs Late FTR	Early STR vs Late STR	Early FTR vs Late FTR
MROT_00000781	Y	N	N	N
MROT_00001457	Y	N	N	N
MROT_00001836	Y	N	N	N
MROT_00001974	Y	N	N	N
MROT_00002328	Y	N	N	N
MROT_00002523	Y	N	N	N
MROT_00002561	Y	N	N	N
MROT_00003724	Y	Y	N	N
MROT_00005566	Y	N	N	N
MROT_00005643	Y	Y	N	N
MROT_00005918	Y	N	N	N
MROT_00006349	Y	N	N	N
MROT_00006656	Y	Y	N	N
MROT_00006912	Y	N	N	N
MROT_00008863	Y	N	N	N
MROT_00001358	N	N	N	N
MROT_00002467	N	N	N	N
MROT_00003926	N	N	N	N
MROT_00007147	N	N	N	N
MROT_00008127	N	N	N	N
MROT_00008374	N	N	N	N
MROT_00008548	N	N	N	N
MROT_00008586	N	N	N	N
MROT_00009058	N	N	N	N

The Y (Yes) and N (No) denotation for each comparison refers to whether the transcript was identified as differentially expressed using RNA-seq. Green denotes transcripts for which the RNA-seq results were confirmed by two different qPCR primer sets. Yellow denotes transcripts for which the RNA-seq results were confirmed by one primer set and not another. White indicates that RNA-seq results were not supported by qPCR. Transcript IDs highlighted in grey were not differentially expressed in any of the RNA-seq comparisons, and this lack of differential expression was also validated by qPCR. Only one primer set was tested for each non-differentially expressed transcript, so yellow represents full validation for those transcripts.

Table A.2. Metabolically active transcripts up-regulated in early-sampled individuals exposed to fluctuating temperatures

Sequence Description	Fold change (log_2)	Transcript ID
beta-hexosaminidase subunit beta-like	4.19896	MROT_00001857
gamma-glutamyl hydrolase	4.1399	MROT_00003315
threonine dehydratase catabolic-like	3.79674	MROT_00004634
ribokinase-like	3.15427	MROT_00000976
cysteine dioxygenase type 1-like	3.07038	MROT_00002818
mitochondrial-like	2.96342	MROT_00002591
short-chain dehydrogenase reductase family 16c member 6	2.91369	MROT_00005918
aldehyde dehydrogenase	2.66668	MROT_00000679
trehalase-like	2.62541	MROT_00006110
purine nucleoside phosphorylase-like	2.43582	MROT_00007699
chitinase precursor	2.37257	MROT_00000347
beta-galactosidase-like	2.20709	MROT_00008888
aael014316- partial	2.02077	MROT_00006707
serine--pyruvate mitochondrial-like	1.87673	MROT_00001421
lysosomal alpha-glucosidase-like	1.66036	MROT_00008296

Table A.3. Transcripts differentially expressed between FTR and STR protocol in both early- and late-sampled individuals.

Sequence Description	Direction	Transcript ID
melanization-related protein	UP	MROT_00000056
sodium hydrogen exchanger 7-like	UP	MROT_00000887
placental protein 11	UP	MROT_00000935
microsomal glutathione s-transferase 1-like	UP	MROT_00001619
apolipophorin-iii-like protein precursor	UP	MROT_00001660
gram-negative bacteria-binding protein 1-2	UP	MROT_00001750
cytochrome p450	UP	MROT_00002167
cytochrome p450 9e2-like	UP	MROT_00002409
vasodilator-stimulated phosphoprotein	UP	MROT_00002561
serine threonine-protein kinase d3	UP	MROT_00002562
variable lymphocyte receptor	UP	MROT_00002940
nuclear protein 1-like	UP	MROT_00003724
serine proteinase stubble	UP	MROT_00004427
synaptic vesicle glycoprotein 2b-like	UP	MROT_00004806
myosin light chain 2	UP	MROT_00005203
muscle lim protein mlp84b-like	UP	MROT_00005271
c1q-like venom protein precursor	UP	MROT_00006106
creb atf bzip transcription	UP	MROT_00006656
venom protease-like	UP	MROT_00006932
lysozyme 3-like	UP	MROT_00007603
PREDICTED: uncharacterized protein LOC100877842	UP	MROT_00008787
phosphoenolpyruvate carboxykinase	UP	MROT_00009772
xaa-pro aminopeptidase 1-like	DOWN	MROT_00008138

APPENDIX B: CHAPTER 3 SUPPLEMENTARY MATERIAL

Table B.1. qPCR validation primers.

Transcript ID	Forward	Reverse
MROT_00009775 Set1	CGAAACGCTCGCTTACAAATAG	CATTGAGGTGGCTCGTATCTC
MROT_00009775 Set2	CCTCAGAGACATGAAGGAACAC	GTGGGAAGAACTGGGTGATATG
MROT_0000590 Set1	AATGAGCCTACGGAAAGAAGAG	GCTTTGGTGCTAGAGGATGT
MROT_0000590 Set2	TCGTGCCCTTACAAAGACTG	CGAACTTCTTCTCTCCGGTATG
MROT_00008799 Set1	GACCTGAACCGACGTCTTTAAT	CAGTTGACTCACAGAGGAGAAC
MROT_00008799 Set2	CTCCTGGGAATCAGATGGTTAG	GGCTTGAACTCCGCTGATAA
MROT_00010646 Set1	GCCGATACGATTGTGAGAGAG	GTTCCATGTTGGTTCCGTTTC
MROT_00010646 Set2	ACGCGAGAGGAACAACATAG	GTCGTTGTCCTTCACCAGAA
MROT_00008869 Set1	TGCCAACCAACACAAGGA	AGCTACCGGTTTGCCATATT
MROT_00008869 Set2	CAAAGCCACAGCACACATTC	ATCGCGATGATCTCTGGTTG
MROT_00005226 Set1	GGCAAAGGGAATGACACTTG	GAGTACACATTCACAATCCATGAG
MROT_00005226 Set2	CCAATGGATGAATGCCAGAAAT	GAGAACAAGTGTCATTCCCTTTG
MROT_00008103 Set1	GGTCGTGCCAAGATCAAGAA	CTTAGGCTTCCGAGGATGATTG
MROT_00008103 Set2	CAATCATCCTCGGAAGCCTAAG	GCCGAACAACCTCTGGAAA
MROT_00002094 Set1	TGTGAGATTGGATGCGAAGAA	CAACGAGCCTAGCAGAGATTTA
MROT_00002094 Set2	GCATCGGGCAACAAATCTTC	CACGACTCGTACGGCTTAAA
MROT_00008639 Set1	GTGTGGTTAGCAGTTCAGTAGAG	GAATTTGGCCTTCCTTGTGATG
MROT_00008639 Set2	GTTACGTCCGAGCCTTGAAA	GAGGAATGCAGCTCAGGATAAT
MROT_00004919 Set1	ACAACCCACAACCAGAAGAG	TTCCACTGTGCACATCGTATTA
MROT_00004919 Set2	GAGGAGAATGGCGATCACTAAA	CTTCCCTTCGTAGCGTTTCT
MROT_00001385 Set1	CCAGTTGGTGTTCGAGGAATAG	TGCTCCAAGTCCAGTGTTATG
MROT_00002467 Set1	TACCTCCTCCTCCGACTTTAC	CTGGCCGTCCGCATTATTA
MROT_00003376 Set1	CTGGACCAAAGCAACAAACG	CTGGGCCTGTATCTTCTTCTTC
MROT_00003926 Set1	GGAGGCGATCCTACAAATACTG	GTCTACCTCGACTTTCTCAATCG
MROT_0000714 Set1	CAGTACATGCTTTGGCGTTATG	CCAGAATCTCCTGTTTCTCCTG
MROT_0000812 Set1	AGCAGAGAAGAAGACGAGAAAC	GTGCAGCAGTACGACCAATA
MROT_00008586 Set1	TGAAAGCTTCTGCGGCTATTA	CCAACTTCTGTCTGGTCTCTTC
MROT_00009058 Set1	GATGGCAAACAAGCTAGAAGAAC	GGCTGATAGAGCAACGAACA

Primer sets highlighted in grey were reference gene candidates.

Table B.2. Ten most up- and down-regulated transcripts after treatment.

Transcript ID	Sequence Description	Fold Change (log_2)
MROT_00006789	cuticle protein 6	-8.56374
MROT_00008105	osiris 16	-8.42492
MROT_00010933	PREDICTED: uncharacterized protein LOC100879602	-7.38722
MROT_00010985	leucine-rich repeat-containing g-protein coupled receptor 4-like	-6.34828
MROT_00007545	zinc carboxypeptidase a 1-like	-5.2878
MROT_00002812	carboxypeptidase b	-4.91085
MROT_00002813	trypsin-1	-4.91085
MROT_00001862	heavy metal-translocating p-type atpase	-4.42962
MROT_00002788	PREDICTED: hypothetical protein LOC100741758	-4.41335
MROT_00008052	cuticle collagen 3a3	-4.35375
MROT_00009775	tbc1 domain family member 4-like	1.71071
MROT_00005906	cabut	1.72365
MROT_00008799	zinc finger containing protein	1.78574
MROT_00010646	ccaat enhancer-binding protein	1.87841
MROT_00008869	actin-binding rho-activating isoform 1	2.04768
MROT_00005226	seminal fluid protein hacp027	2.07091
MROT_00008103	osiris 14	2.07664
MROT_00002094	zinc finger protein noc-like	2.26969
MROT_00008639	myofilin isoform b	2.33654
MROT_00004919	protein argonaute-2	3.67798

Log_2 fold change relative to FTR. A negative fold change is an upregulation in STR.

Table B.3. qPCR validation.

Transcript ID	Differentially Expressed in RNA-seq	qPCR Validated
MROT_00009775	Yes	Yes
MROT_00005906	Yes	Yes
MROT_00008799	Yes	Yes
MROT_00010646	Yes	No
MROT_00008869	Yes	Yes
MROT_00005226	Yes	Yes
MROT_00008103	Yes	No
MROT_00002094	Yes	Yes
MROT_00008639	Yes	Yes
MROT_00004919	Yes	Yes
MROT_00001385	No	Yes
MROT_00002467	No	Yes
MROT_00003376	No	Yes
MROT_00003926	No	Yes
MROT_00007147	No	No
MROT_00008127	No	Yes
MROT_00008586	No	Yes
MROT_00009058	No	No

Yellow indicates transcripts that were validated with one primer set and green indicates those validated with two. Genes identified as non-differentially expressed were those used to determine stable reference genes.

Table B.4. Gene ontology of non-cell-membrane transcripts.

Function	Transcript	Fold Change
Apoptotic Process	death associated protein	-1.04078
Apoptotic Process	intraflagellar transport protein 57 homolog	-1.06966
Developmental Process	homeobox protein	1.25495
Developmental Process	udp-glucose 6-dehydrogenase	1.17127
Developmental Process	ras association domain-containing protein 8-like	0.808743
Developmental Process	e3 ubiquitin-protein ligase su -like	0.799208
Developmental Process	paired box protein pax-2-b	-0.916377
Developmental Process	ecdysone-induced protein 78c	-1.30957
DNA repair	myofilin isoform b	2.33654
DNA repair	dna damage-binding protein 1	1.02919
DNA repair	p53 regulated pa26 nuclear protein	0.797203
DNA repair	meiotic recombination 11	-1.27712
Oxidation-reduction	acyl- delta desaturase-like	-1.17683
Oxidation-reduction	acyl- delta desaturase	-1.94342
Neurogenesis	isoform b	0.860505
Neurogenesis	storkhead-box protein 1	-0.726152
Neurogenesis	kn motif and ankyrin repeat domain-containing protein 1	-0.802562
Neurogenesis	peptidylglycine alpha-hydroxylating monooxygenase	0.996843
Oxidation/reduction	cytochrome b5-related protein	-0.85795
Oxidation/reduction	fatty acyl- reductase cg5065-like	-0.954789
Oxidation/reduction	probable cytochrome p450 305a1	-1.11015
Oxidation/reduction	1-cys peroxiredoxin	-1.35647
Oxidation/reduction	d-3-phosphoglycerate dehydrogenase	-1.57624
Oxidation/reduction	homogentisate -dioxygenase	-1.57882
Oxidation/reduction	pro-phenol oxidase subunit 2	-1.712
Oxidation/reduction	cytochrome p450 mitochondrial	-1.73091
Oxidation/reduction	oxidase peroxidase	-2.22979
Oxidation/reduction	10-formyltetrahydrofolate dehydrogenase	-2.33033
Oxidation/reduction	glyoxylate reductase hydroxypyruvate reductase	-2.36061
Oxidation/reduction	short-chain dehydrogenase	-2.41981
Oxidation/reduction	cytochrome p450 6b1-like	-2.44822
Oxidation/reduction	retinol dehydrogenase 14	-2.57532
Oxidation/reduction	osiris 2 cg1148-pb	-3.3388
Oxidation/reduction	fatty acid synthase	-4.00072
Oxidation/reduction	protein kintoun-like	1.41663
Oxidation/reduction	cytochrome p450 4g15	-2.2377
Stress response	myb domain-containing protein	-2.08356

Log_2 fold change relative to FTR. A negative fold change is an upregulation in STR.

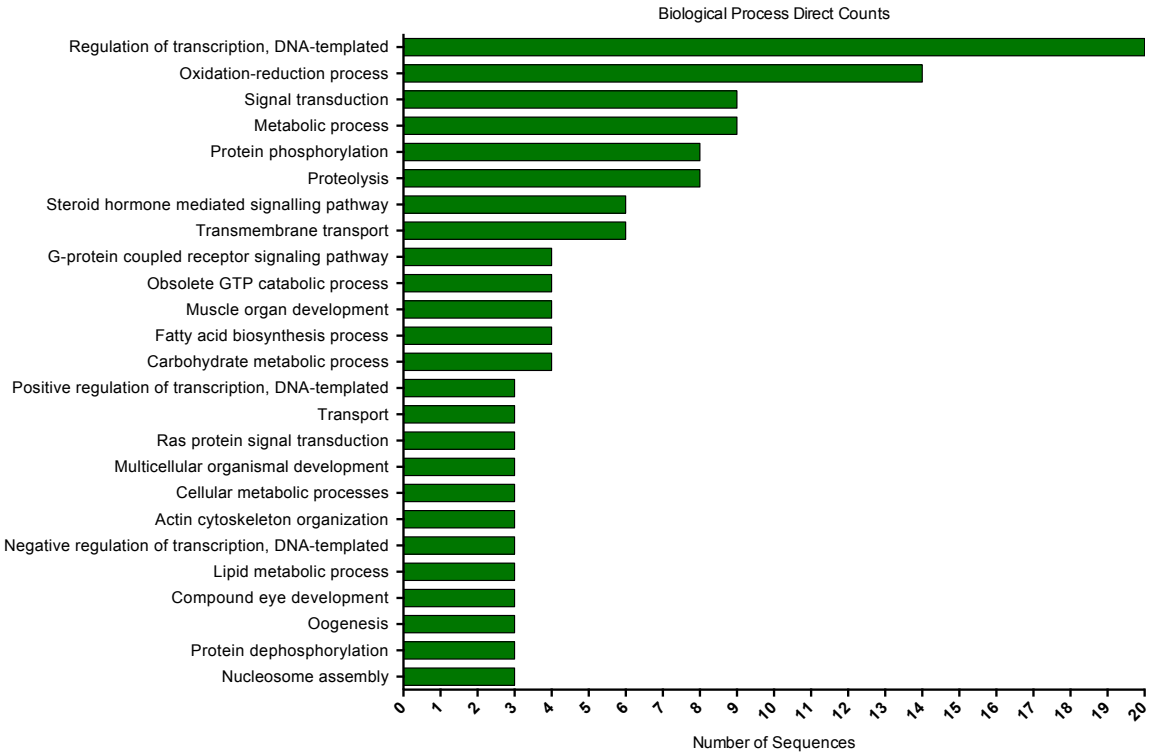

Figure B.1. Biological function gene ontology of differentially expressed transcripts after seven days of low temperature stress.

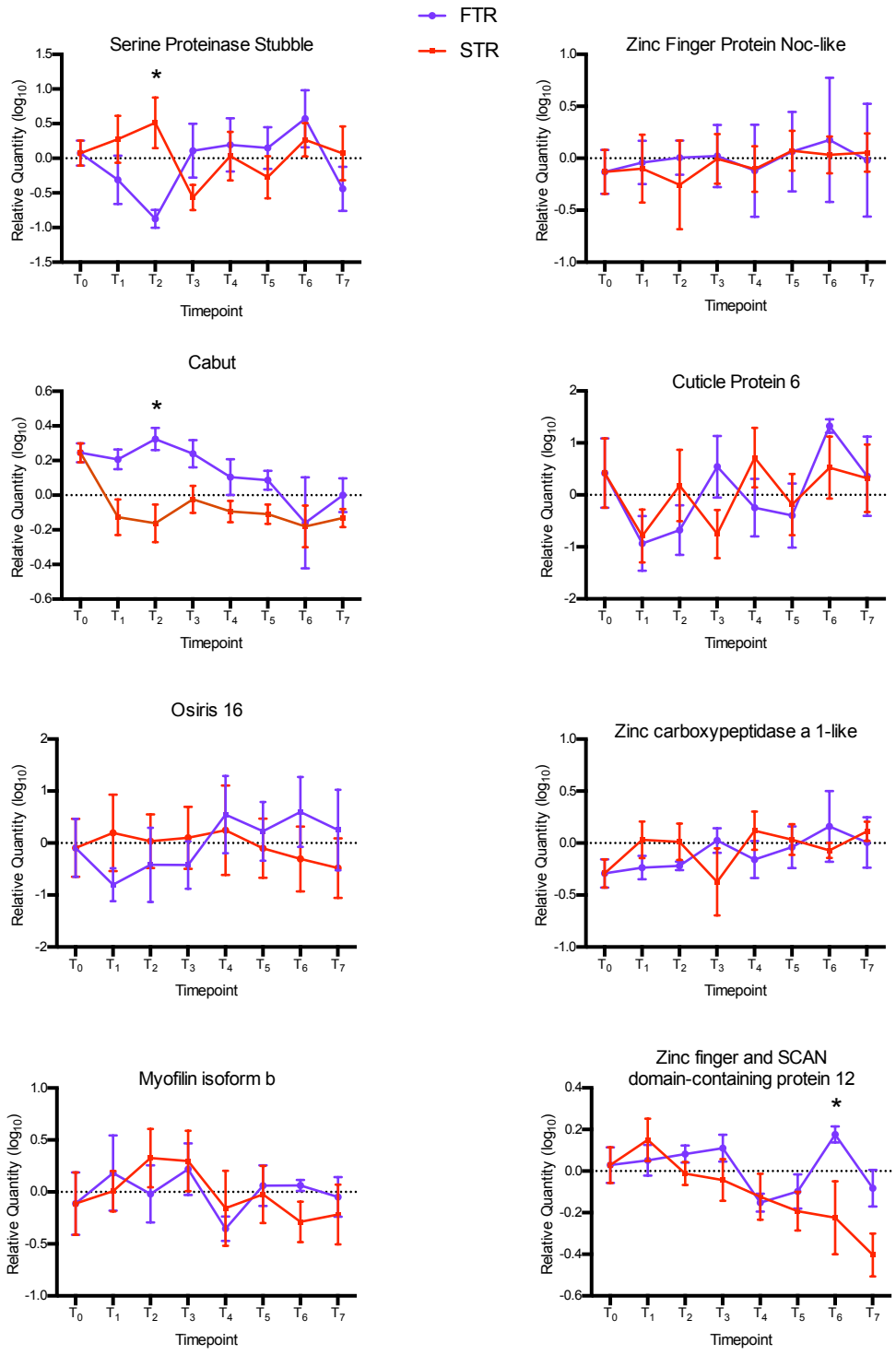

Figure B.2. Expression profiles of candidate genes during temperature stress. The expression of candidate genes identified in the RNA-seq analysis was assessed throughout the entire duration of low temperature stress after extended overwintering (n=6). The data are presented as mean ± s.e.m. Asterisks indicate a significant difference in expression between STR and FTR determined by ANOVA.

APPENDIX C: CHAPTER 4 SUPPLEMENTARY MATERIAL

Table C.1. Time series qPCR primers

Name	Forward Primers	Reverse Primers
Glutathione S-transferase	GCGCCGAAGTACGAAGAATA	GTGCACCATTTAGCTACTCATTTC
	TGCTAAAGCGCCGAAAGA	ACTCAAATCAGCAAGGGTCATA
Apolipoprotein d-like	CGAGATTCTGTTCGCTGATAGT	TTGGGACACTTGGTCTTGTC
	CAACACGTACGTCCTAGATTCA	GACTTGGCTCTCGACTCATAAT
Short-chain dehydrogenase reductase	GGTAGCCGAGGTAATGAGAAAG	AGGTGTGTGATCCAGCAAAT
	TGGTCTACCTAATCTCGTTCCT	GCCTTGTCCTTGTTGGATACT
Vitellogenin	GAAGGGAGATGGACAGCATATT	GTCCAGTGATACCGAAGTGATAC
	CAGCGTCGTGCCATATACTT	CTGCCCTTCAGATCCTTGTT
Eater	ACTGTTCAGGCAGGACTTTG	CGCTGGTAGAAGGAAGAGTATTG
	GTACATGCGTAGGTCCTAATCG	TTCTGCACTGGAACGACATAG
Nuclear protein 1-like	CTCTTACGCACACCAATCATTTC	GCTTTCCCATTGTGTCGTTTAT
	CACGGAAGCAATGTCTGATTAC	CCCGTTTACTTCGTTGTTTACC
Catalase	GCCGAGGAGAGAAAGAGATTAG	TCCACCTGCGTGAAGTTT
	ACGTTGGCGTGAAGATCAA	CGCATAGTGTACGGATGTGTAG
Glutathione peroxidase	CGGCCTTACAGCAACAAATTAC	CCCGGTTCTTGTCCGTTAAA
	CCCAACGTGGATCCCAATAA	TGCAGATACGGAAGATAAGTCTATG
Superoxide dismutase	GTGCGTCATGTTGGAGATTTAG	TGGGACCTTGAAGCTGAATAA
	GTAGCGATTGTCGTTCCGAATA	CCTTTACAGTAGGGCTGTTCTC
Nitric oxide synthase	CTCTTGGAACTGGACGACATAG	CCAAGATTGCTTCCACCAATTC
	CCCAGATGTACCCATCGAATTAC	ACGAGTTGGGCAAATACCTATC
Ubiquitin-fold-modifier-conjugating enzyme 1	AGAAGGTACAAGGTGGTTTGG	CTGGTGCAGTTGTAGGATACG
Peptidyl-prolyl cis-trans isomerase-like 2	GGAGGCGATCCTACAAATACTG	GTCTACCTCGACTTTCTCAATCG