ECOPHYSIOLOGICAL IMPLICATIONS OF SPRING CONDITIONS ON THE ALFALFA LEAFCUTTING BEE, *MEGACHILE ROTUNDATA*

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ABSTRACT

Spring conditions stimulate development of many plants and animals after a period of winter dormancy. Climate change is predicted to cause earlier spring thaws, increasing temperature variability, and more frequent cold snaps. These conditions cause two problems for organisms. First, environmental cues may mislead organisms developing under these scenarios if temperature and photoperiod cues give conflicting information. Second, organisms outside of their overwintering stages can be less tolerant of cold exposure and may be at risk of injury or death. Little is known about the consequences of these conditions on bee species. Therefore, I examined these scenarios in a solitary bee species, *Megachile rotundata.* I hypothesized they would be sensitive to temperature changes to regulate spring emergence because of their cavity nesting life history where photoperiod cues likely buffered. I found light is buffered by the brood cell by approximately 80% and emergence can be synchronized by photoperiod. Furthermore, I demonstrated that *M. rotundata* may be more sensitive to temperature cues compared to photoperiod cues in regulating emergence. To understand how spring cold snaps during development affect adult bees, I comprehensively assayed *M. rotundata* cold tolerance. I discovered that cold exposure during development resulted in numerous sub-lethal effects in adult bees such as a decrease in flight performance and longevity. Furthermore, developmental cold stress affected adult thermal performance, such as chill coma recovery. Cold tolerance varies across development and the post-diapause quiescent stage was more tolerant to cold than pupal or emergence ready stages. Temperature fluctuations of spring may affect the timing of emergence but also the health of adult bees if they experienced a cold snap during development.

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DEDICATION

I would like to dedicate my dissertation to my parents, for their unwavering support and encouragement throughout my college career.

My Mom was an exponential source of support for me. She was there for me every day and every step of the way. I cannot express my gratitude for the sacrifices she has made for me. She is the strongest, kindest and most compassionate person I know. I am proud to be her daughter because her strength is within me. She spent hours on the phone with me when times were tough. My Mom is my rock, and I am forever thankful for her wonderful love and patience.

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I love you both!

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LIST OF ABBREVIATIONS

ANOVA .....Analysis of variation

CTmin ..... Critical thermal minimum

ECO.....Ecological thermal regime

FTR .....Fluctuating thermal regime

SCP .....Supercooling point

STR .....Static thermal regime

nm .....nanometers

T_a ..... Ambient temperature

CHAPTER 1: INTRODUCTION

Overview

Spring conditions stimulate development of many plants and animals after a period of winter dormancy. In temperate regions of the Northern hemisphere, spring conditions are characterized by increasing day length and temperatures. Climate change is predicted to cause earlier spring thaws, increasing temperature variability, and more frequent cold snaps (Inouye 2008; Rigby & Porporato 2008; Augspurger 2013), posing two main problems for developing organisms. First, environmental cues may mislead organisms developing under these scenarios if temperature and photoperiod cues give conflicting information. Second, organisms outside of their overwintering stages are less tolerant of cold exposure. Thus, those in active development may be at risk of injury or death.

Organisms rely on environmental cues to synchronize their biological rhythms to daily and seasonal fluctuations in the environment. Circadian rhythms are important adaptations that synchronize daily patterns of physiological and behavioral processes with fluctuations of the environment. External cues (Zeitgebers) synchronize organisms with the environment by resetting endogenous circadian oscillators, referred to as clocks. Relevant cues vary across taxa, because organisms have evolved to occupy diverse habitats. Understanding the impact of spring conditions on the timing of life history and developing organisms is crucial to making accurate predictions about organismal responses to climate change.

Spring conditions and circadian rhythms

In the spring, most organisms rely on environmental cues for timing of life history transitions. For example, increased average daily temperatures and day lengths in the spring promote budding after dormancy for many tree species and regulate the timing of flowering (reviewed in Singh et al. 2017; Fjellheim et al. 2014; Shi et al. 2017). For many animals, spring

conditions initiate increased activity levels and gonadal maturation (Dawson et al. 2001; Wassmer & Refinetti 2016; Pagon et al. 2013; McAllan et al. 2008). Spring photoperiod is a cue for migration and feeding activity for most non-tropical bird species (reviewed by Dawson et al. 2001; Das & Gupta 2016; Agarwal et al. 2015). Because of its major role in mediating circadian rhythms across taxa, from cyanobacteria to primates, photoperiod has been referred to as the "universal Zeitgeber," (reviewed by Aschoff 1965; Wehr 2001; Saunders 2012). However, the relevance of photoperiod as an environmental cue may vary depending on an organism's evolutionary history.

For organisms that have evolved to live in light-restricted habitats, daily temperature fluctuations may be a stronger, more reliable seasonal cue than changes in daylength, and those organisms may have evolved to respond to thermoperiod rather than photoperiod. Far less is known about the role of thermoperiod compared to the role of photoperiod in regulating the timing of life history events. Many animals that undergo dormancy in the winter do so in light-restricted habitats and rely on temperature cues to inform them of the season. For example, the overall increasing temperatures of spring regulate circannual emergence in many turtle species that hibernate under the sediment (Costanzo et al. 2008; Crawford 1991; Feaga & Haas 2015). Furthermore, thermoperiod regulates circadian regulation of the "spring awakening," or valve opening under the sediment in oysters, *Crassostrea virginica* (Comeau 2014). Ground squirrel, *Spermophilus columbianus*, emergence from winter hibernation dens correlates with the increasing temperatures of spring (Murie & Harris 1982). Since climate change is affecting temperature variability, it is important to understand the role of thermal cues in mediating life history transitions.

One such major life history event in insects is the timing of adult emergence. A well-studied phenomenon, the timing of adult emergence is regulated by photoperiod in many insect species (Zdarek & Denlinger, 1995; Kumar et al., 2007; Umadevi et al., 2009; Guo & Qin, 2010; Thöming & Saucke, 2011; Wu et al., 2014; Yadav et al., 2015). However, insects have evolved to live in many habitats, including many that are light-restricted, and therefore, the relevance of environmental cues may vary across species. For instance, many insects pupate in brood cells or nests where light cues may be buffered. Although temperature regulation of emergence from light-restricted habitats has been studied in other taxa, little is known about the cues trigger emergence in insects living in those environments.

In addition to being in a light-restricted habitat, many insects also develop within a brood cell. Brood cells can be constructed out of many materials, including leaves, specific soils, and glandular secretions from the mother or developing larva (Klostermeyer and Gerber 1969; Gupta et al. 2004). Brood cells may buffer ambient conditions, including incoming light or temperature fluctuations. Studies examining the physical properties of silkmoth, *Bombix mori,* cocoons reported that the cocoon acts both as a buffer of ambient temperature changes and as a humidity trap, due to its waterproof inner barrier (Zhang et al. 2013; Horrocks et al. 2013). I hypothesize that sensitivity to thermoperiods is more important than photoperiod for synchronizing with the environment for insects emerging from these types of habitats. Some evidence supports this hypothesis in dipterans, in which thermoperiod has been shown to influence the circadian control of emergence from beneath the soil (Miyazaki et al., 2011; Watari and Tanaka, 2014; Short et al. 2016). Thermoperiod has also been shown to synchronize emergence in *Megachile rotundata*, supporting the hypothesis that this may be an important cue for timing of adult emergence (Yocum et al. 2015). I hypothesize that light is buffered by the brood cell and that insects

emerging from such habitats respond similarly to the soil-emerging dipterans when exposed to a thermoperiod. Investigating the relevance of environmental cues for insects developing in these scenarios is critical to understanding how circadian systems are affected by changes in spring.

Consequences of spring cold snaps during development

Even though spring temperatures generally increase, spring cold snaps are predicted to occur more frequently with climate change (Inouye 2008; Rigby & Porporato 2008; Augspurger 2013). Changes in temperature away from thermal optima or below the developmental threshold can affect the rate of development and therefore the timing of life history events in plants and animals. For example, cold snaps delay bloom time in cherry trees, *Prunus yedoensis* (Shi et al. 2017) and egg laying is delayed in great tits, *Parus major* (Visser et al. 2009). Spring cold snaps have been recorded to cause injury or death in many reptile species (reviewed in Costanzo et al. 2008; Costanzo & Lee 1996). Furthermore, developing plants exposed to low temperatures may sustain injuries that result in decreased fruit production (Patten et al. 1991; Ercoli et al. 2004). Insects are also susceptible to chill-injury, which can negatively affect physiology, morphology, and behavior (Hutchinson & Bale 1994; Yocum et al. 1994; Kelty et al. 1996; Marshall and Sinclair 2011; Rinehart et al. 2011). Clearly, organisms outside their overwintering stages are less tolerant to cold and may be vulnerable to injury if spring cold snaps occur.

The optimal temperature range for development varies across species, and time spent outside this range may result in interupted development, injury, or even death (reviewed in Day and Rowe 2002). Furthermore, cold tolerance may vary throughout development (Kim & Song 2010; Radchuck et al. 2013; Scheffield 2008). Thus, the timing of cold exposure could make the difference whether the insect lives, dies, or is merely injured. Many insects survive low temperatures during winter because of protections afforded to them in diapause, an adaptive state

in which insects have increased tolerance to environmental stressors, such as low temperature, and are prevented from developing to the next stage (reviewed in Hahn & Denlinger 2011). In diapause, metabolic rate is suppressed and transcripts that function in cold tolerance such as heat shock proteins are upregulated (reviewed in Hand et al. 2016). Spring temperatures initiate active development for many insects and are less tolerant to cold outside their overwintering stages. Thus, if exposed to a spring cold snap during this time, insects may be at risk of injury, interrupted development, or death. If we have a better understanding of an insect species' cold tolerance across development, we can make better predictions about population responses to spring cold snaps.

As mentioned, not all low-temperature exposures are lethal. Instead, low temperature exposure may result in chill-injury, which is hypothesized to affect ion channel function thereby disrupting proper muscle and nervous system function (MacMillan &Sinclair 2011). In addition, the effects of chill injury may be long-term rather than acute, often appearing in the adult stage after the cold exposure has long passed (Hutchinson & Bale 1994; Yocum et al. 1994; Bennett et al. 2015). For example, low-temperature exposure during metamorphosis can impact adult morphology, (Ismail et al. 2013; Bennett et al. 2015), fecundity (Hutchenson & Bale 1994), and timing of emergence (Yocum et al. 1994; Ismail et al. 2013). Sub-lethal effects have the potential to impact fitness, because the effects of chill injury on muscle and nerve function commonly affect locomotion, which is needed for access to food and mates. Understanding the implications of spring cold snaps on developing insects is vital, because these conditions could have long lasting effects on population dynamics. Additionally, because of the widespread consequences of cold exposure, understanding how spring conditions may affect organisms is critical for scientists to be able to make predictions about responses to climate change.

Life history of *Megachile rotundata*

The alfalfa leafcutting bee, *Megachile rotundata* is a solitary bee species native to Eurasia. However, feral populations can be found throughout North America because of their introduction in the 1940's for pollination services (reviewed in Pitts-Singer and Cane 2011). Georeferenced records of *M. rotundata* show sightings as far north as Finland and Saskatchewan, Canada (https://www.gbif.org/species/1335648). Although population sizes cannot be determined, records have identified them across North America, ranging from northern Canada down to New Mexico, as far east as Delaware, and as far west as California (https://www.gbif.org /species/1335648). Understanding the thermal physiology and responses to stress in *M. rotundata* can shed insights on how climate change may affect the natural range of *M. rotundata.* In addition, knowing the thermal physiology will help us to know where this bee can be successfully used for pollination services.

After mating in the summer, female *M. rotundata* build nests in natural or artificial sites such as grooves in tree bark or straws in nesting blocks (reviewed in Pitts-Singer and Cane 2011). Females build brood cells out of cut leaves and provision them with nectar and pollen, lay an egg inside, and then cap the brood cell with more leaf discs. Female *M. rotundata* spend 5-6 hours a day foraging for leaf pieces and provisions (Klostermeyer & Gerber 1969). Depending on the availability and distance of floral resources, the number of brood cells and nests a single female can build can vary (reviewed in Pitts-Singer & Cane 2011). Within each brood cell, a developing larva will consume its entire provision and enter the prepupal stage. Populations at northern latitudes are univoltine, while those at more southern latitudes are bivoltine. In the northern populations and the in the second generation of southern populations, prepupae overwinter, and resume metamorphosis when temperatures increase in the spring. *M. rotundata*

that undergo metamorphosis in the spring are vulnerable to spring cold snaps because they are outside their overwintering stage. Furthermore, because climate change is increasing temperature variability, it is unknown what complications this could cause for emerging *M. rotundata.* Little is known about the cues that regulate adult emergence in the spring or how low temperature exposure may affect development and adult performance of *M. rotundata*.

Emergence of adult *M. rotundata* occurs in the spring and summer months when floral resources are available. Synchronization of adult emergence could be critical for overlapping with nectar production, mating opportunities, and ideal weather conditions. Because they are cavity nesting bees, it is unknown if photoperiod plays a role in synchronizing emergence. One study found that *M. rotundata* did not synchronize emergence to a pulse of light (Tweedy & Stephen 1970). Interestingly, Yocum et al. 2015 showed that emerging *M. rotundata* synchronize to a small thermoperiod, although it was unclear if the thermoperiod activated circadian clocks. Possibly, temperature variability of climate change could be a less reliable cue for timing emergence. Light would be a more reliable cue for synchronizing development, because it is unaffected by climate change, but it is unknown if developing *M. rotundata* are photoreceptive. If the brood cell buffers light, I hypothesize that developing insects rely on changes in temperature to synchronize emergence.

Because populations of *M. rotundata* at northern latitudes are susceptible to spring cold snaps during development, they are at risk of death or chill injury. The cold tolerance of *M. rotundata* has not been well characterized, and the repercussions of low-temperature exposure during development on adult bees are not clear. Because low temperatures are often used for agricultural management of *M. rotundata*, some experiments have examined the effects of low-temperature storage during the overwintering and spring metamorphosing stages (Torson et al.

2015; Bennett et al. 2013; Rinehart et al. 2011; Yocum et al. 2010, 2006). For example, low-temperature exposure during development affected survival of adult bees, and fluctuating low-temperatures were less detrimental than static low temperatures (Rinehart et al. 2011; Yocum et al. 2010, 2006). It is hypothesized that fluctuating low-temperatures grant insects the opportunity to repair chill injury (reviewed in Sinclair et al. 2015). In support of this hypothesis, transcripts functioning in ion homeostasis, metabolic pathways, and oxidative stress responses were up-regulated during fluctuating low-temperatures (Torson et al. 2015). Even if bees survive a low-temperature exposure, sub-lethal effects could severely affect fitness. Therefore, I hypothesize that low-temperature stress during metamorphosis negatively affects adult performance. Furthermore, because cold tolerance varies with developmental stage, I hypothesize cold tolerance depends on developmental stage in post-wintering *M. rotundata.*

Broader impacts: Phenology and pollination services

The majority of wild bee species are solitary like *M. rotundata*, and little is known about what cues mediate the synchrony of emergence for these bees. Emergence patterns have been studied in solitary bees over weeks and months (Rust, 1906; Danforsth, 1999; Vinchesi et al., 2013; White et al., 2009), but not periodic daily rhythms. The mechanisms that synchronize daily rhythms could contribute to changes in the timing of phenological events such as peak bee emergence and flower bloom. Climate change is clearly impacting the phenology of both plants and animals, in turn affecting plant-pollinator interactions (reviewed in Parmesan & Yohe 2003; Visser & Both 2003; Jamieson et al. 2012). For instance, simulations assessing the effects of climate change on pollinator phenology demonstrate potential disruption of temporal overlap between pollinator availability and floral resources and predict local pollinator extinction (Memmott et al. 2007). Understanding which cues are important for developing bees and how

spring conditions may affect their development is crucial to predicting the responses to climate change.

Solitary bees fill an important role as alternative pollinators because of their affinity to feed from certain flowering plants. *Megachile rotundata* is the most intensively managed solitary bee in agriculture, because of their effective pollination of flowering alfalfa crops, *Medicago sativa* (Pitts-Singer & Cane 2011). For agricultural management of *M. rotundata*, low-temperature storage is used at multiple developmental stages. For example, bees are artificially stored under low temperatures during winter in the prepupal stage (Yocum et al. 2006; Rinehart et al. 2011; Yocum et al. 2012) and in the spring to slow down metamorphosis to better synchronize emergence with peak crop bloom (Undurraga & Stephen 1980, Richards 1984, Yocum et al. 2010). Understanding the cues that mediate emergence and how low temperatures affect adult performance is important to improve bee health under commercial management. Furthermore, the more knowledge that exists about the thermal biology of *M. rotundata*, improved temperature regimes can be designed to improve pollinator performance.

Objectives

Objective 1: Examine the cues mediating emergence of M. rotundata

I aimed to test the hypothesis that thermoperiods are dominant cues for insects emerging from light-restricted environments. I determined how much light reaches the developing bee and the relevance of photoperiods for synchronizing emergence. I measured fine-scale patterns in emergence of hundreds of bees using a custom-built recording device (Yocum et al. 2015). Using this method, I examined the roles of thermoperiod and photoperiod cues in mediating emergence. I also examined how sensitive developing and emergence-ready bees are to thermoperiod cues. I predicted that because of their cavity nesting life history, they would not respond to photoperiod

cues. Furthermore, *M. rotundata* have been shown to respond to thermoperiod (Yocum et al., 2016), but whether or not thermoperiod or photoperiod interact with circadian feedback loops is unknown. I predicted emergence rhythms would free-run if thermoperiods are removed because I hypothesized that they have temperature-mediated circadian feedback loops. Additionally, when photoperiods are removed, I predicted no free-running periods or phase-shifting to occur due the presence of temperature-mediated clocks.

Objective 2: Examine the extent of sub-lethal effects induced by low-temperatures in adult M. rotundata

Injury sustained from cold exposure during development can manifest as sub-lethal effects in adult insects. Therefore, I took multiple measures of adult physiology, morphology, and behavior to determine the effects of low-temperature exposure on adult *M. rotundata* (Bennett et al. 2015). I hypothesized that static temperatures would result in more sub-lethal effects than fluctuating temperatures. I assessed flight performance and feeding activity to determine if low-temperature treatments caused physiological and behavioral defects. Furthermore, because temperatures can affect wing development, I examined if low-temperatures affected wing morphology. Taking multiple measures more clearly reflects the consequences of low temperature during development on adult insects.

Objective 3: Characterize the cold tolerance of metamorphosing and adult M. rotundata

Even though work has examined the effects of low-temperature storage on *M. rotundata* (Yocum et al. 2006 & 2012; Rinehart et al. 2011; Bennett et al. 2015), their cold tolerance across metamorphosis has not been well characterized. Management practices could be improved if cold tolerance measures were known. Furthermore, understanding the thermal physiology and responses to stress in *M. rotundata* can aid in making predictions about how climate change may

affect the natural range of *M. rotundata.* I aimed to better characterize cold tolerance of *M. rotundata* across metamorphosis through multiple measures of physiology along with determining their critical thermal minimum and chill coma recovery time in adults. I hypothesized low-temperatures exposure during metamorphosis affects adult thermal minimum and ability to recover from chill coma. Furthermore, I hypothesized the timing and synchrony of adult emergence may also be affected by low-temperature exposure during development. Together these data establish thermal thresholds during development and responses to low-temperature exposure of *M. rotundata.*

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CHAPTER 2: INVESTIGATING RELEVANT ZEITGEBERS FOR EMERGING MEGACHILE ROTUNDATA

Abstract

Photoperiod is considered the universal Zeitgeber, regulating physiological processes in numerous animals. However, for animals in light-restricted habitats (e.g. burrows or cavities), thermoperiod may be a more important cue. Our study tested this hypothesis in the alfalfa leafcutting bee, *Megachile rotundata*, which nests in cavities and undergoes development within a brood cell. We assessed the role of environmental cues (thermoperiod and photoperiod) on the process of adult emergence by examining: 1) if those cues direct circadian rhythms, 2) which cue is more dominant, and 3) how sensitive developing bees and emergence-ready adults are to cues. Although we found that 20% of light penetrates the brood cell, and bees respond to photoperiod by synchronizing emergence, thermoperiod is the dominant cue. When presented with a conflicting Zeitgeber, bees entrained to the thermophase instead of the photophase. When temperature cues were removed, we observed free-running of emergence, indicating that underlying circadian mechanisms can be synchronized by daily fluctuations in temperature. We also found that emerging bees were highly sensitive to even small increases in temperature, entraining to a ramp speed of 0.33°C/hour. The response and sensitivity to temperature cues suggest that *M. rotundata* evolved a temperature-mediated clock to mediate emergence from light-restricted cavities. This may be a general response in other cavity-nesting insects.

Introduction

Circadian rhythms are ubiquitous among organisms and serve to synchronize their biological processes to daily fluctuations in the environment. Circadian systems require an input (stimulus) to regulate the timing of an output (behavior, physiology). For example, circadian

rhythms are mediated by a cue or Zeitgeber (which literally translates as *time giver*) that resets molecular feedback loops, referred to as clocks. Biological rhythms are considered to be under circadian control, if they: 1) are entrainable by a Zeitgeber, 2) have a free-running period of approximately 24-hrs in constant conditions, and 3) are temperature compensated. Photoperiod has been referred to as the "universal Zeitgeber" because of the role it plays in mediating circadian rhythms across many taxa (reviewed by Aschoff 1965; Wehr 2001; Saunders 2012). However, organisms that develop or reside in light-restricted habitats may need to rely on other cues to synchronize their daily rhythms with the environment. For example, thermoperiods mediate "spring awakening," or valve opening under the sediment in oysters, *Crassostrea virginica* (Comeau, 2014). The overall increasing temperatures of spring regulate circannual emergence in many turtle species that hibernate under the sediment (Costanzo et al., 2008; Crawford, 1991; Feaga and Haas, 2015). Still, far less is known about the role of temperature compared to the role of light in regulating the timing of life history events.

A well-known phenomenon controlled by circadian rhythms is the timing of insect eclosion in many insect species, which is the emergence from the pupal stage (Miyazaki et al. 2016; Short et al. 2016; Kikukawa et al. 2013; Dolezel et al. 2008). Many studies have identified photoperiod as the critical cue in synchronizing eclosion (Kumar et al., 2007; Umadevi et al., 2009; Guo & Qin, 2010; Thöming & Saucke, 2011; Wu et al., 2014; Yadav et al., 2015). However, because insects pupate in diverse habitats, the sensitivity to a particular Zeitgeber is expected to vary depending on where an insect pupates. For example, photoperiod may not be the best cue for synchronizing with the environment if an insect develops in a light-restricted environment. Some data support this hypothesis in insects that pupate below ground, where thermoperiod cues were shown to regulate emergence (Short et al. 2016; Miyazaki et al. 2011;

Watari and Tanaka, 2014b; Greenberg et al., 2006). Insects that pupate in other types of light-restricted habitats such as nests, natural or artificial cavities, and brood cells may also rely on thermoperiod cues. Many hymenopterans pupate in brood cells, structures that can be made out of many materials, including leaves, specific soils, and glandular secretions from the mother or developing larva (Klostermeyer and Gerber 1969; Gupta et al. 2004). Thus, insects emerging from these environments may be more sensitive to cues other than photoperiod. In short, consideration of insect life history is important to understanding how sensitivity to different Zeitgebers evolved.

Solitary bees nest in light-restricted habitats, in below- or above-ground cavities, where thermoperiod may be an important cue due to lack of light. One thing that differs between Hymenoptera and other insect taxa is that adult emergence often occurs several days after eclosion (Danforth et al. 1999, Kemp & Bosch, 2000; Yocum et al., 2016; Reznik et al., 2008; Bertossa et al., 2010). Little is known about circadian regulation of adult emergence in comparison to studies on eclosion. The distinction between emergence and eclosion is important, because these events can be differentially regulated by environmental cues. For instance, eclosion in parasitic wasps, *Trichogramma embrophagum,* is not rhythmic, but emergence from the host is regulated by a circadian rhythm (Reznik et al., 2008). We hypothesize that other hymenoptera may behave similarly to *T. embrophagum*.

Emergence patterns of solitary bees have been studied over periods of days and months (Rust, 1906; Danforsth, 1999; Vinchesi et al., 2013; White et al., 2009) but it is unclear what cues mediate more fine-scale hourly and daily rhythms. A previous study showed that small thermoperiods synchronized emergence from brood cells in the alfalfa leafcutting bee, *Megachile rotundata* (Yocum et al. 2015), although it is unknown if the response to thermoperiod was

under circadian control or if it is the dominant Zeitgeber. Furthermore, emerging *M. rotundata* did not respond to a pulse of light (Tweedy and Stephen, 1971), but it is unknown if *M. rotundata* responds to a photoperiod.

In this study, we used the alfalfa leafcutting bee, *M. rotundata* to test the hypothesis that emergence from light-restricted environments is regulated by thermoperiod cues controlled by circadian rhythms. We chose *M. rotundata* to study these questions, because they develop in a brood cell and are readily available due to their management for pollination services (Pitts-Singer and Cane, 2011). We measured fine-scale patterns of spring emergence using a custom-built automated recording device that allowed us to record the emergence of thousands of bees (Yocum et al., 2015). Using this method, we examined the roles of thermoperiod and photoperiod cues in circadian regulation of emergence and determined which is dominant. Furthermore, we examined the sensitivity of emergence-ready adult bees to environmental cues. We predicted that because of their cavity nesting life history, light would be significantly buffered and bees would not respond to photoperiod cues. We predicted that emergence rhythms would free-run if the thermoperiod is removed and that thermoperiod is dominant over photoperiod cues, because they are likely to have temperature-mediated clocks.

Materials and methods

Animals and rearing conditions

Bees were purchased from JWM Leafcutters, Inc. (Nampa, ID) as loose brood cells in 2014 and 2016. Thermoperiod removal experiments were conducted in 2014. Thermoperiod switch experiments and ramp speed experiments were conducted in 2016. In all experiments, prepupae were kept in constant 6°C in darkness for approximately 6 months until development

was initiated by placing bees at 29°C. Temperature regimes were administered in an environmental chamber (Percival models LT-36VL and I30BLL Percival Scientific, Perry, IA).

The Δ4°C thermoperiod had a mean temperature of 29°C and consisted of a cryophase (11 h at 27°C) and thermophase (11 h at 31°C) and two separate 1-h temperature ramping periods. The cryophase ran from 07:00 to 18:00 and thermophase ran from 19:00 until 06:00. The Δ8°C thermoperiod retained a mean temperature of 29°C and consisted of a cryophase (11 h at 25°C) and a thermophase (11 h at 33°C) with 1h temperature ramping periods. The thermophase ran from 07:00 to 18:00, and the cryophase ran from 19:00 until 06:00. Both thermoperiods were administered under complete darkness, except for the dominant Zeitgeber experiment. In all experiments using photoperiod, we measured the increase in temperature due to light and programmed the incubator to compensate to reduce the possibility of temperature fluctuations. Furthermore, we used Percival model LT-36VL which has fluorescent bulbs on the external sides of the incubator, to reduce heat production from lights. Together these measures ensured that any responses we observed were not due to a temperature increase when the lights turned on.

Monitoring emergence

Emergence was monitored using a modified Watari apparatus (Watari and Tanaka, 2010; Yocum et al. 2016), positioned inside an environmental chamber (Percival models PCG-105 and I30BLL Scientific, Perry, IA). A single loose brood cell containing a prepupa was placed in a 0.5 ml microcentrifuge tube (Fisher Scientific Pittsburgh, PA) with the cap cut off. The microcentrifuge tubes were held in place by plastic racks which were designed using SketchUp® (Trimble Inc., Sunnyvale, California) software and 3D printed (Lulzbot, Aleph Objects, CO). On top of the brood cell, a 6-mm plastic ball (Softair, Grapevine, TX) and a 4.5-mm steel ball

(Copperhead, Crosman, NY) were loaded into the tubes. A cover was placed over the loaded tubes, with holes sized to block the possible escape of the bee by the plastic ball. When a bee emerged, it pushed the plastic ball, which in turn pushed the steel ball forward, rolling free from the tube racks down a runway. The steel ball passed through a 5-mm infrared emitter and detector pair (Lite-on Electronics, Inc., Milpitas, CA), recording the date and time of emergence. The apparatus was controlled by an Arduino Nano board (Sparkfun Electronics, Boulder, CO). The temperature (±2°C) and humidity were recorded every 60 seconds using a DHT11 sensor (Adafruit, New York, NY).

Light penetrance of brood cell

To determine if light can penetrate the brood cell we used a MK350 spectrometer (UPRtek, ikan Corporation, Houston TX) to measure light intensity (±5%) outside versus inside the brood cell. Because *M. rotundata* nests inside a cavity (Fig. 2.1), it was difficult to measure light inside the nest, therefore we disarticulated nest cells to measure how much light penetrates a single brood cell. Isolated wavelengths were administered using ultra violet (400 nm), blue (470 nm), green (525 nm) yellow (588 nm) and red (630 nm) LEDs (Super Bright LEDs Inc., St. Louis, Missouri). Light penetrance measurements were taken inside a dark walk-in incubator to eliminate external light. An adapter made from a 6ml syringe, wrapped in black electrical tape was fitted around the aperture of the spectrometer. The back end of the brood cell (not the cap) was cut to fit snugly over the syringe head (adapter). Brood cells were haphazardly chosen from a 24-well plate and used for LED measurements. Any that were damaged while removing them from the adapter were discarded from analysis. The average lux was measured on each brood cell in each wavelength of light, before and after the brood cell was placed on the adapter. The

percent of light intensity was calculated from the mean difference of before and after the brood cell was placed on the adapter.

Figure 2.1. Shown is a cartoon depiction of a male *M. rotundata* emerging from a nest inside. Brood cells are inside a wooden cavity and are made from leaves.

Circadian rhythms and Zeitgebers

Photoperiod removal

To determine if photoperiod affected circadian regulation of emergence, a long day photoperiod 16:8 was applied until approximately 100-200 bees emerged, after which they were exposed to constant 29°C in complete darkness for the remainder of emergence. During the days of emergence with a photoperiod, lights were turned on from 07:00 until 23:00.

Thermoperiod removal

To determine if thermoperiod can regulate emergence via circadian rhythm, bees were exposed to a Δ4°C or Δ8°C thermoperiod until approximately 100-200 bees emerged, then the thermoperiod was removed, and bees were exposed to constant 29°C for the remainder of emergence.

Conflicting (dominant) Zeitgeber

To determine whether photoperiod or thermoperiod was dominant, cues were decoupled (Pittendrigh and Minis, 1964), which is generally referred to as a conflicting Zeitgeber experiment (Short et al., 2016; Watari and Tanaka, 2010). Bees were exposed to a Δ4°C thermoperiod, although the lights turned on during the cryophase and turned off during the

thermophase. The photoperiod was a 12L:12D h cycle with the lights turned on from 7:00 until 19:00.

Sensitivity and Zeitgebers

Emergence-ready: photoperiod response

To determine if emergence-ready bees respond to light, bees were exposed to constant 29°C in complete darkness until approximately 100-200 bees emerged, then a long day photoperiod 16L:8D was applied for the remainder of emergence. During the days of emergence with a photoperiod, lights were turned on from 07:00 until 23:00.

Emergence-ready: thermoperiod response

To determine if emergence-ready bees respond to thermoperiod, bees were exposed to 29°C until approximately 100-200 bees emerged, and then were switched to the Δ4°C thermoperiod for the remainder of the emergence period. The constant 29°C represents the control to compare to the mean time of emergence to the Δ4°C thermoperiod.

Emergence-ready: thermoperiod sensitivity

To determine if emergence-ready bees were sensitive to a change in thermoperiod amplitude, we exposed bees to a Δ4°C thermoperiod until approximately 200 bees emerged, then switched to a Δ8°C thermoperiod for the remainder of emergence.

Sensitivity to ramp speed

The slow ramp speed experiment had a ramp speed of 0.33°C per hour over 12 hours. The ramp to the thermophase started at 07:00 and reached peak temperature (31°C) at 19:00 and immediately ramped down to the cryophase (27°C) at 20:00. The fast ramp speed experiment had a steep ramp speed of 4°C per hour. The ramp to the thermophase started at 07:00 and

reached the peak temperature (31°C) by 08:00. After reaching 31°C, the temperature decreased by 0.33°C per hour until it reached the cryophase temperature of 27°C at 20:00 hours.

Statistical analysis

Circular statistics were used to determine if emergence was synchronous or distributed uniformly around the clock. Emergence times collected on a 24-hour clock (hh:mm:ss), were first converted to angular measurements. To obtain meaningful descriptive statistics for circular data, angular data were transformed to rectangular polar coordinates. This allows calculation of the circular mean which yields better representation of the data. For example, the circular mean of 359 degrees (just before midnight) and 1 degree (just after midnight) is 0 degrees (midnight), rather than 180 degrees (noon) which would be the simple arithmetic mean. Circular ANOVA was used to determine if the mean time of emergence was different before and after Zeitgebers were switched in an experiment.

We tested the hypothesis of uniformly distributed circular data using Rayleigh's test for uniformity. This test is based on the mean resultant vector, rbar, which ranges from 0 to 1. When data are uniformly distributed, the mean resultant vector is expected to be close to zero, and when the data are strongly unimodal, rbar will be close to 1. Rbar was converted to Rayleigh's z ($z = n \times rbar^2$), which follows a X^2 distribution and yields p-values for the test of uniformity. Because rbar has a standard range and is more interpretable by itself, we have provided rbar as the test statistic for Rayleigh's test with the p-value coming from Rayleigh's z (Fisher 1993). For several of the experiments where we were interested in testing the null hypothesis of common directional means, we used the high-concentration F test (Mardia and Jupp, 2000). We used the circSASv1 SAS macros to calculate all circular statistics (http://statweb.calpoly.edu/ulund).

In addition to the Rayleigh test for uniformity, the parameter R was calculated to measure the degree of rhythmicity in emergence (Winfree, 1970; Watari and Tanaka, 2010; Short et al., 2016). The parameter R is a scalar statistic that identifies if emergence is rhythmic or arrhythmic by calculating the highest number of emerging adults in an 8-hour gate then dividing this number by the number of adults emerging outside the 8 h gate, multiplied by 100. All individuals that emerged were pooled to calculate the number of emerging adults for each hour of the day. The theoretical range of parameter R is from 0, if all emergence occurs within the gate, to 200, if emergence is distributed uniformly throughout the day (Winfree, 1970). R values < 60 are considered rhythmic emergence, 60 < R < 90 are weakly rhythmic, and R > 90 are arrhythmic. R values >150 indicate uniform distribution of emergence (Winfree, 1970).

Results

How much light penetrates the brood cell?

Green wavelength penetrance was significantly different from all other wavelengths (Fig. 2.2, ANOVA $F_{4,66}$= 4.433, p <.05). Just over 40% of green light passed through the brood cell, while only 26% of light from other wavelengths passed through (Figure 2.2, Table 2.1).

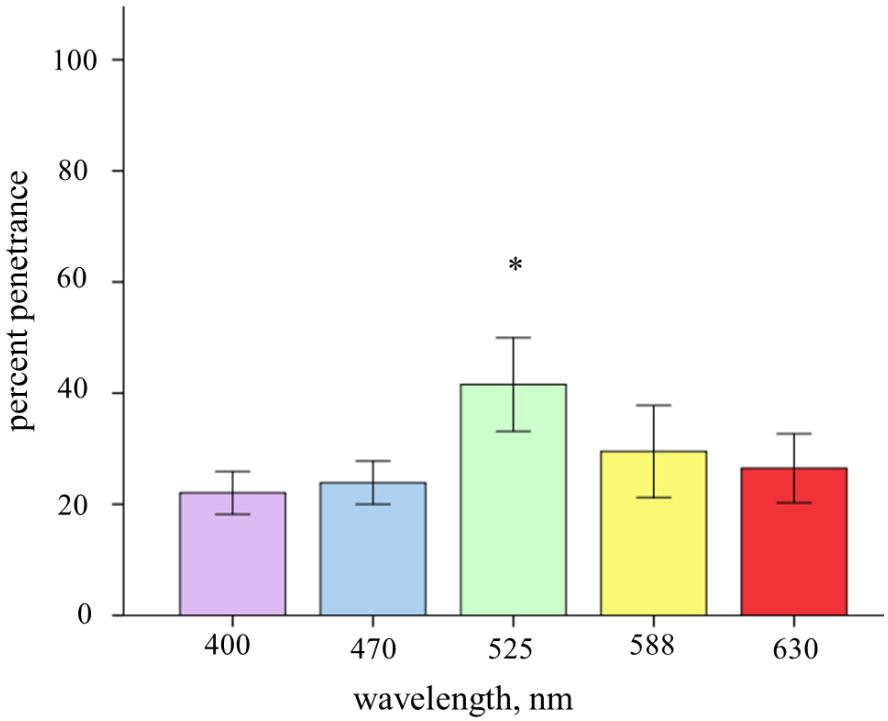

Figure 2.2. Percent penetrance of light wavelengths through the brood cell with cocoon intact. Wavelength in nanometers (nm) is displayed by colored bar, ultra violet (400nm), blue (470nm), green (525nm), yellow (588nm) and red (630 nm).

Table 2.1. Wavelength, ambient lux, brood cell lux and percent penetrance.

wavelength, nm	n	mean ambient lux± se	mean brood cell lux± se	% penetrance
400	8	20.5± 0.83	4.5± 0.428	25.52%
470	16	261.86± 4.14	61.6± 4.73	23.52%
525	10	41.9±1.15	17.2± 1.5	41.05%
588	14	110.8± 1.34	32.33± 4.3	29.18%
630	16	120.81± 1.73	32.125± 3.8	26.59%

Circadian rhythms and Zeitgebers

Does photoperiod interact with the circadian system?

Bees were exposed to a long day photoperiod 16L:8D at constant 29°C for the first 4 days of emergence (n=105), then the photoperiod was removed and bees were in constant darkness for the remainder of emergence (n= 302). Emergence was rhythmic (R= 39.18, rbar= 0.6114, $p < 0.0001$) during the photoperiod, and when removed, emergence remained rhythmic (Fig. 2.3, R= 53.57, rbar = 0.5086, $p < 0.0001$). The mean time of emergence during the photoperiod was not statistically different from the mean time of emergence when the photoperiod was removed (Table 2.2, Circular ANOVA, $F_{1,406} = 0.0454$, $p = 0.80855$).

Table 2.2. Circular test statistics for the circadian and sensitivity experiments.

temperature treatment	circular mean	SD	median	R value	Rayleigh test (rbar), p-value
16L:8D, 29°C constant	11:22:39	03:47:20	10:41:59	39.18	0.6114, p < 0.0001
29°C constant	11:12:38	04:26:40	10:54:31	53.57	0.5086, p < 0.0001
4°C thermoperiod	08:56:28	01:09:51	08:50:33	0.82	0.95463, p < 0.0001
29°C constant	06:47:35	02:29:52	06:51:30	13.97	0.80752, p < 0.0001
8°C thermoperiod	08:27:44	01:08:49	08:12:38	0.47	0.95592, p < 0.0001
29°C constant	05:41:03	02:29:12	05:48:12	11.78	0.80905, p < 0.0001
conflicting Zeitgeber	20:43:59	01:59:18	20:31:04	6.73	0.87330, p < 0.0001
4°C thermoperiod	08:09:14	01:13:20	08:02:14	2.00	0.95009, p < 0.0001
8°C thermoperiod	08:29:11	01:13:23	08:07:44	1.35	0.95003, p < 0.0001
29°C constant	21:09:02	08:50:08	13:18:47	153.48	0.1753, p >0.05
16L:8D, 29°C constant	06:28:09	03:13:27	07:06:29	46.75	0.7003, p <0.0001
29°C constant	02:21:25	08:57:29	13:51:05	131.81	0.06393, p > 0.05
4°C thermoperiod	07:14:28	02:29:41	07:41:15	30.46	0.80794, p < 0.0001
slow ramp speed	06:27:51	03:44:03	08:02:41	35.30	0.62012, p < 0.0001
fast ramp speed	09:00:33	01:49:53	08:53:43	6.04	0.89143, p < 0.0001

Mean, standard deviation (SD), median time for emergence. Rhythmicity index (R value) <60 = rhythmic, > 90 = arrhythmic. Rayleigh test for synchronicity (rbar) p-value < 0.05 = synchronous, > 0.05 = uniform.

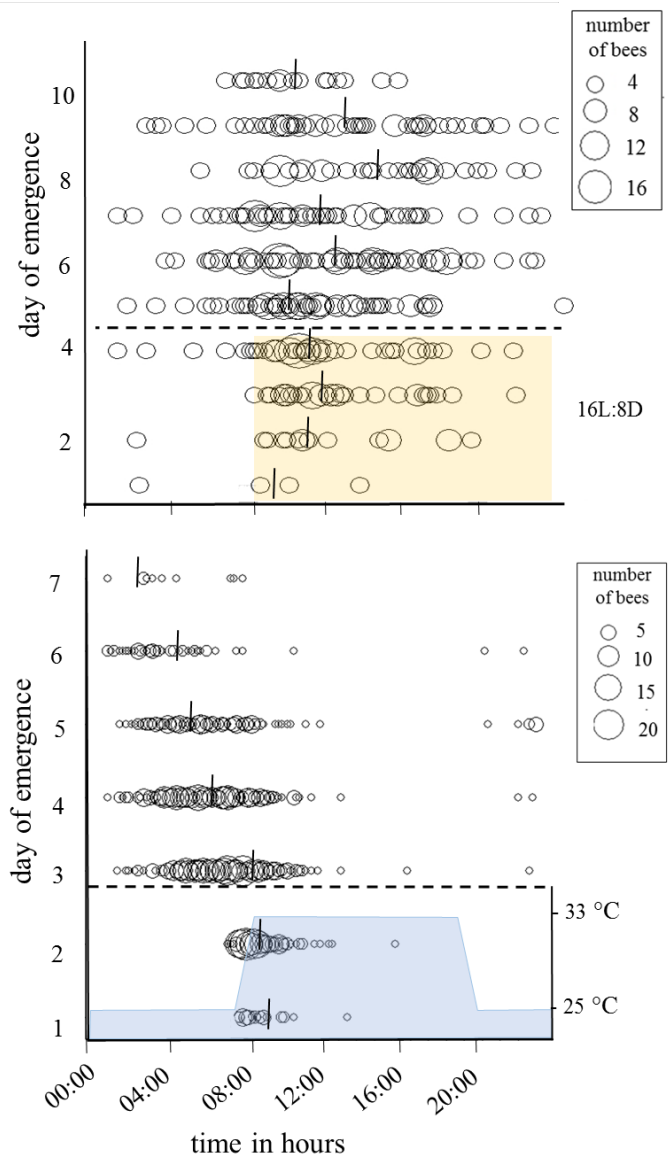

Figure 2.3. The number of emerging bees for the Δ8°C thermoperiod removal experiment (top) and photoperiod removal experiment (bottom). Emergence patterns of *M. rotundata* with a Δ8°C thermoperiod under dashed line and after removal, in constant conditions above dashed line (top). The blue shaded area is Δ8°C thermoperiod, displayed to show temperature marked on the secondary y-axis, which was administered each day but shown here to show timing of temperature ramps (top). For the photoperiod removal experiment (bottom) emergence patterns are displayed at 16:8 photoperiod and constant 29°C (yellow area) under dashed line and after removal, in constant conditions above dashed line. The vertical bars display the circular mean time of emergence for each day. The size of the bubbles indicates the number of bees emerging during 15-minute time intervals.

Does thermoperiod interact with circadian system?

In the first experiment, bees were exposed to a Δ8°C thermoperiod for the first 3 days (n= 243), at which point the thermoperiod was removed and remaining bees were exposed to constant 29°C (n= 791). The Rayleigh test indicated synchronous emergence during Δ8°C thermoperiod (Fig. 2.3, R= 0.8290, rbar= 0.9546, p <0.0001), which remained when the thermoperiod was removed at constant 29°C (R= 13.97, rbar= 0.80752, p <0.0001). Bees exposed to the Δ8°C thermoperiod emerged earlier than when the thermoperiod was removed (Circular ANOVA $F_{1,1033} = 168.207$, p <0.0001). The mean time of emergence significantly differed by day after the switch (Table 2.2, Circular ANOVA $F_{12,1033} = 22.404$, p <0.0001).

In the Δ4°C thermoperiod-removal experiment, emerging bees were exposed to the Δ4°C thermoperiod for the first three days of emergence (n= 210), then the thermoperiod was removed, and remaining bees were exposed to a constant 29°C (n= 854). Similar to the Δ4°C thermoperiod removal experiment, emerging bees maintained synchronicity after the Δ4°C thermoperiod was removed (Fig. 4). The Rayleigh tests indicated directional distribution during the Δ8°C thermoperiod (R= 0.478, rbar= 0.9559, p <0.0001) and when the thermoperiod was removed (R= 11.78, rbar= 0.8091, p <0.0001). The mean time of emergence was statistically different between constant 29°C and Δ8°C thermoperiod temperature regimes (Circular ANOVA $F_{1,1063} = 247.351$ p <0.0001). The mean time of emergence differed significantly by day after the switch (Table 2.2, Circular ANOVA $F_{10,1063} = 46.5802$, p <0.0001).

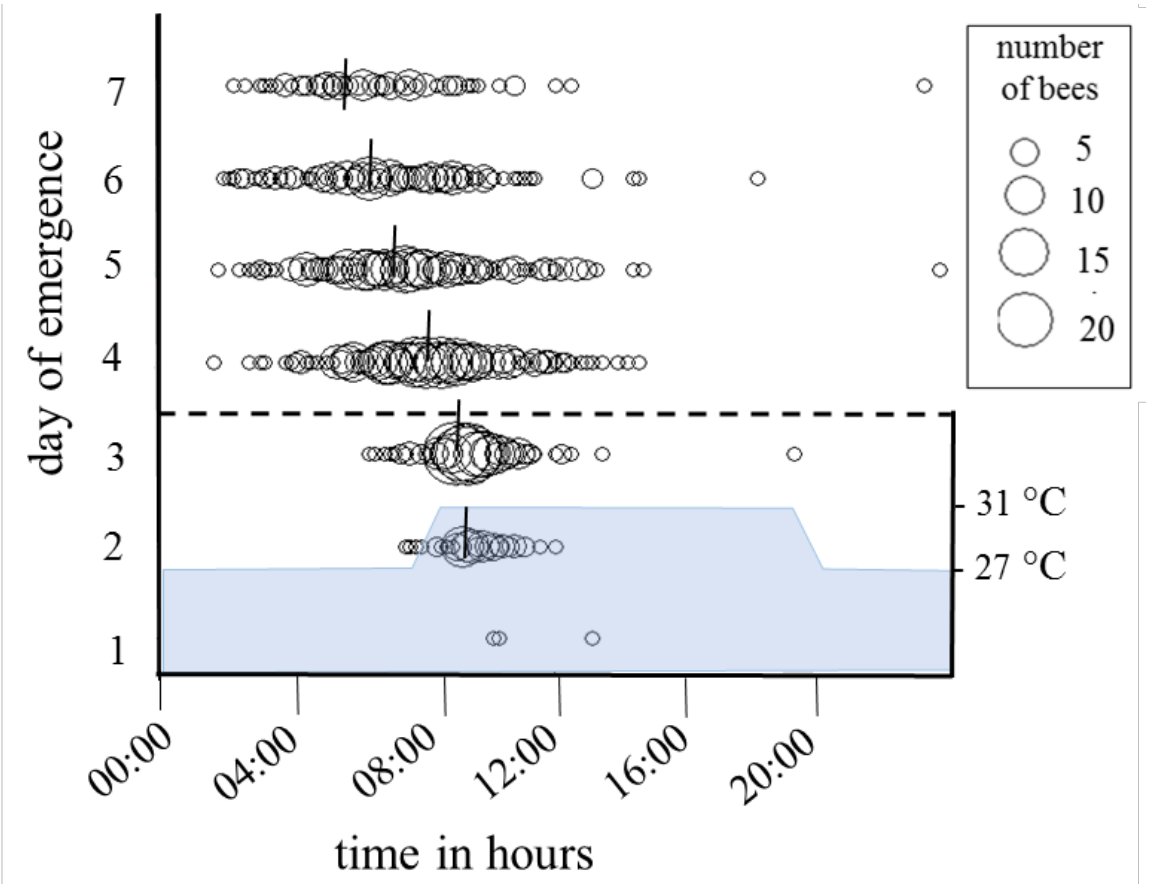

Figure 2.4. The number of emerging bees and mean time of emergence for Δ4°C thermoperiod removal experiment. Emergence patterns of *M. rotundata* with the Δ4°C thermoperiod (below dashed line) and after removal, in constant 29°C conditions (above dashed line). The blue shaded area is Δ4°C thermoperiod, displayed to show temperature marked on the secondary y-axis.

Which cue is the dominant Zeitgeber?

Bees were exposed to a Δ4°C thermoperiod and a long day photoperiod, with the thermophase occurring during the start of the dark phase. Emergence was rhythmic coinciding to approximately the start of the thermophase (Fig. 5, R= 6.73, rbar= 0.87330, $p < 0.0001$).

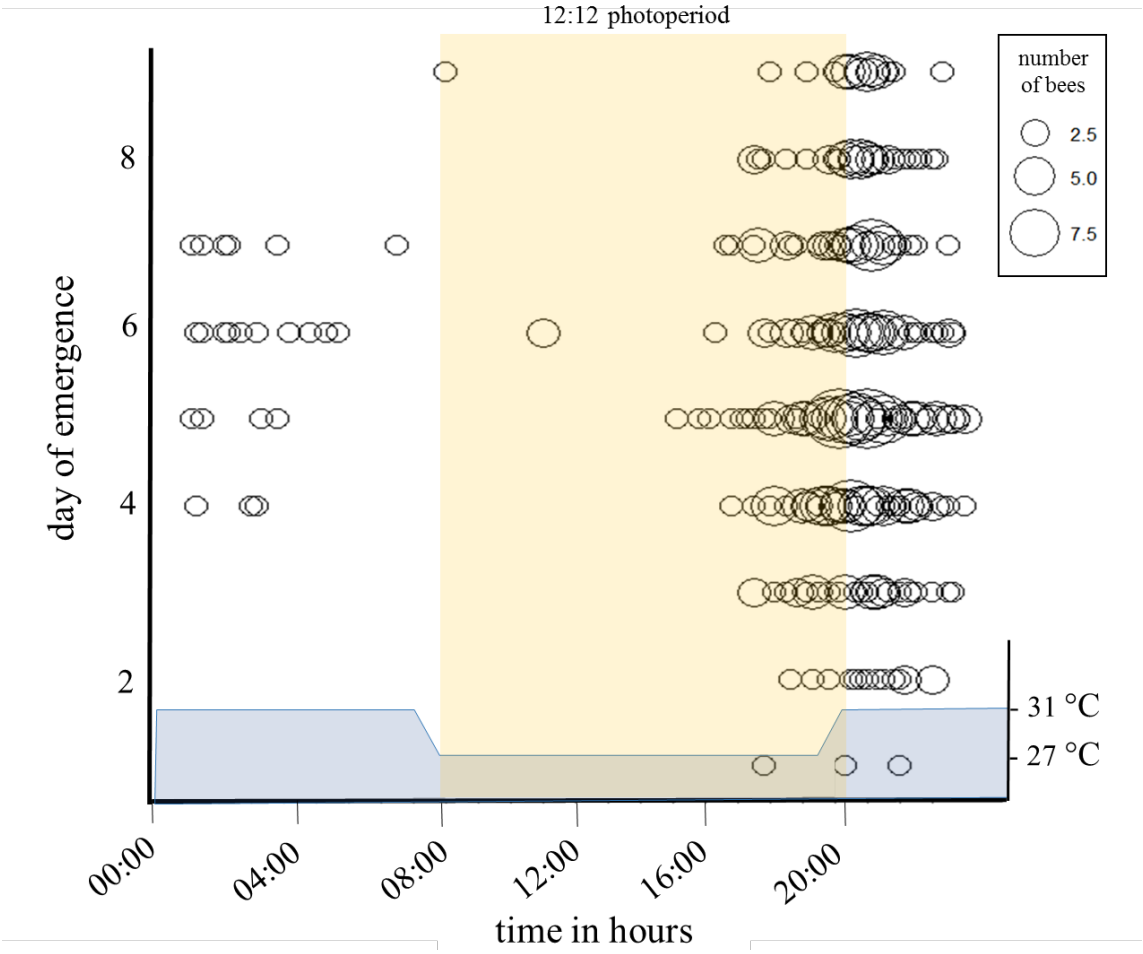

Figure 2.5. Number of emerging bees for the conflicting Zeitgeber experiment. The thermoperiod ramps are shown by blue shaded area, cryophase ramp from 07:00-08:00 and thermophase ramp from 19:00-20:00. The photoperiod was a 12:12-hour cycle where the lights turned on at 7:00 and turned off at 19:00 shown by the yellow area. The size of the bubbles indicates the number of bees emerging during 15-minute time intervals.

Sensitivity to Zeitgebers

Do emergence-ready bees respond to photoperiod?

Bees were exposed to constant 29°C in darkness for the first 4 days (n= 218), at which point a long day 16:8 photoperiod was initiated (n= 443). Emergence was uniform in darkness (Fig. 6a, R=153.48, rbar=0.1753, $p > 0.05$), but after the photoperiod was initiated, emergence was rhythmic (R= 46.75, rbar= 0.7003, $p < 0.0001$). Mean emergence time was significantly different before and after bees were exposed to a photoperiod (Table 2.2, Circular ANOVA, $F_{1, 660} = 67.25$, $p < 0.0001$. Once the photoperiod was initiated, the circular mean time of emergence shifted earlier to 06:28:09±03:13:27 (median 07:06:29).

Do emergence-ready bees respond to thermoperiod?

Emerging bees that initially had no thermoperiod were allowed to emerge for 3 days (constant 29°C; n= 102), and then the remainder of emerging bees were exposed to a Δ4°C thermoperiod (n= 668). Emergence was uniform (R= 131.81, rbar= 0.06393, $p > 0.05$) in constant 29°C. Once the Δ4°C thermoperiod was initiated, emergence was synchronous (Fig. 6b, R= 30.46, rbar= 0.80794, $p < 0.01$). The R value on the day after the thermoperiod was initiated was < 60 (R=58.62), indicating synchronous emergence when first exposed to the Zeitgeber. Bees exposed to the Δ4°C thermoperiod emerged earlier in the day (mean emergence time) than bees exposed to constant 29°C (Table 2.2, Circular ANOVA $F_{1,769} = 25.4330$, $p < 0.0001$).

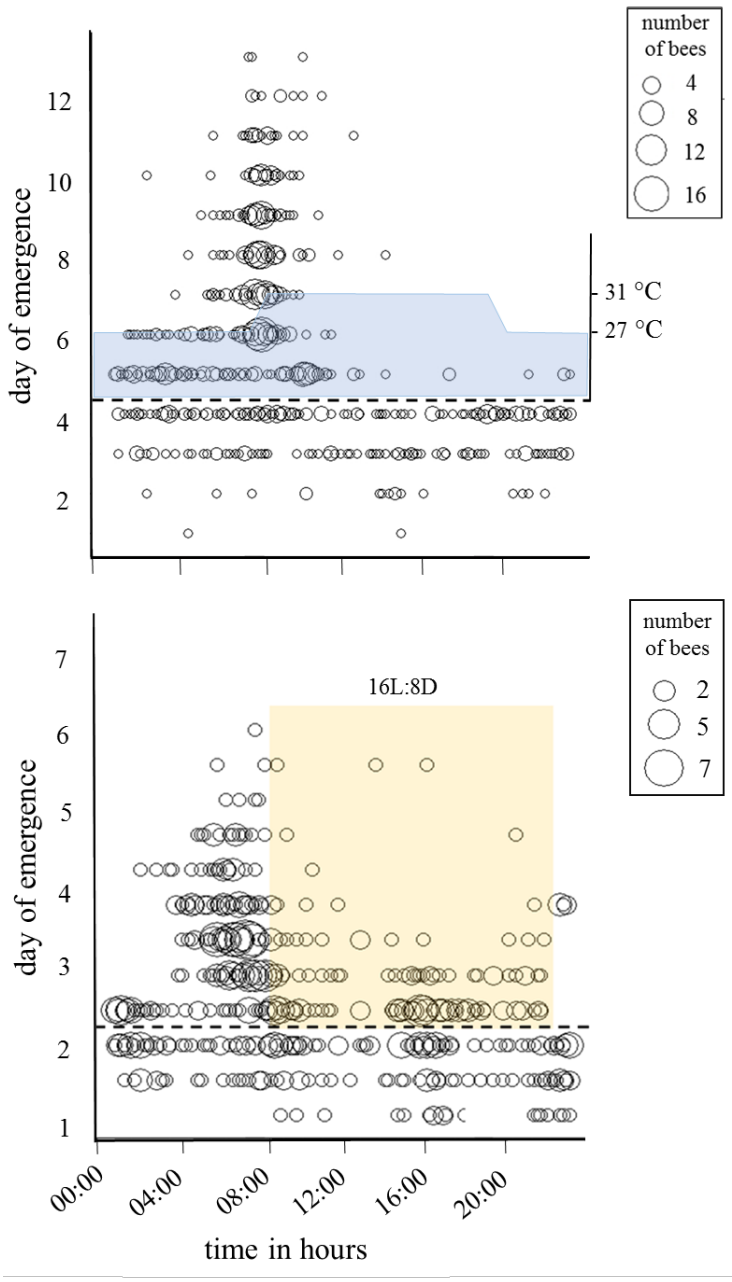

Figure 2.6. The number of emerging bees for the emergence-ready response to Δ4°C thermoperiod response experiment (top) and photoperiod response experiment (bottom). Emergence patterns of *M. rotundata* with constant conditions a under dashed line and after removal, Δ4°C thermoperiod above dashed line (top). The blue shaded area is Δ4°C thermoperiod, displayed to show temperature marked on the secondary y-axis, which was administered each day but shown here to show timing of temperature ramps (top). For the photoperiod response experiment (bottom) emergence patterns are displayed at constant conditions under dashed line and after removal, 16:8 photoperiod and constant 29°C (yellow area).

Are emergence-ready bees sensitive to a thermoperiod?

To determine whether bees distinguish between slight variations in thermoperiod, emerging bees were exposed to a Δ4°C thermoperiod for 3 days (n= 225), then switched to a Δ8°C thermoperiod for the remainder of emergence (n= 559). Emerging bees entrained to both thermoperiods throughout adult emergence. The Rayleigh test supported directional distribution (R=2.00, rbar= 0.9501, p<0.01) during the Δ4°C thermoperiod, indicating synchronous emergence. During the Δ8°C thermoperiod, emergence was synchronous (Fig. 7, R= 1.35, rbar= 0.9500, p< 0.0001). Bees in the Δ8°C thermoperiod emerged later than bees exposed to the Δ4°C thermoperiod (Table 2.2; Circular ANOVA $F_{1,733} = 11.2264$, $p < 0.0001$).

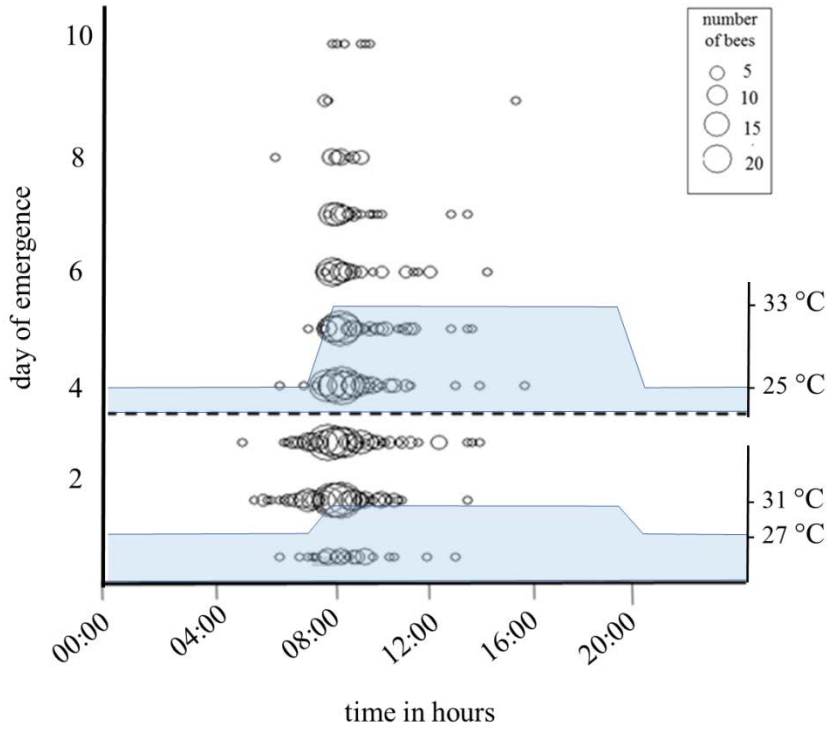

Figure 2.7. The number of emerging bees and mean time of emergence for the 8°C thermoperiod switch experiment. Emergence patterns of *M. rotundata* under Δ4°C thermoperiod (below dashed line) and after removal, in Δ8°C thermoperiod (above dashed line). The blue shaded areas are the temperature treatments which were administered each day but displayed here to show timing of temperature ramps. Temperatures are shown on the secondary y-axis. The size of the bubbles indicates the number of bees emerging during 15-minute time intervals.

How sensitive are developing bees to temperature ramps?

To determine how sensitive emerging bees are to changes in temperature, pupating bees were exposed to slow or fast ramps in the thermophase (0.33°C/h or 4°C/h). In the fast ramp experiments (n= 686), emergence was synchronous (Fig. 8b, R= 6.04, rbar= 0.89143, p<0.0001). Mean time of emergence was 09:00:33 (standard deviation, 01:49:53) which coincides with the end of the ramp period of the thermophase (Table 2.2). During the slow ramp experiment (n= 536), emergence was synchronous (Fig. 8a, R= 35.30, rbar= 0.62012, p<0.0001). Mean time of emergence was 06:27:51 (standard deviation, 03:44:03) coinciding with just before ramp period of the thermophase.

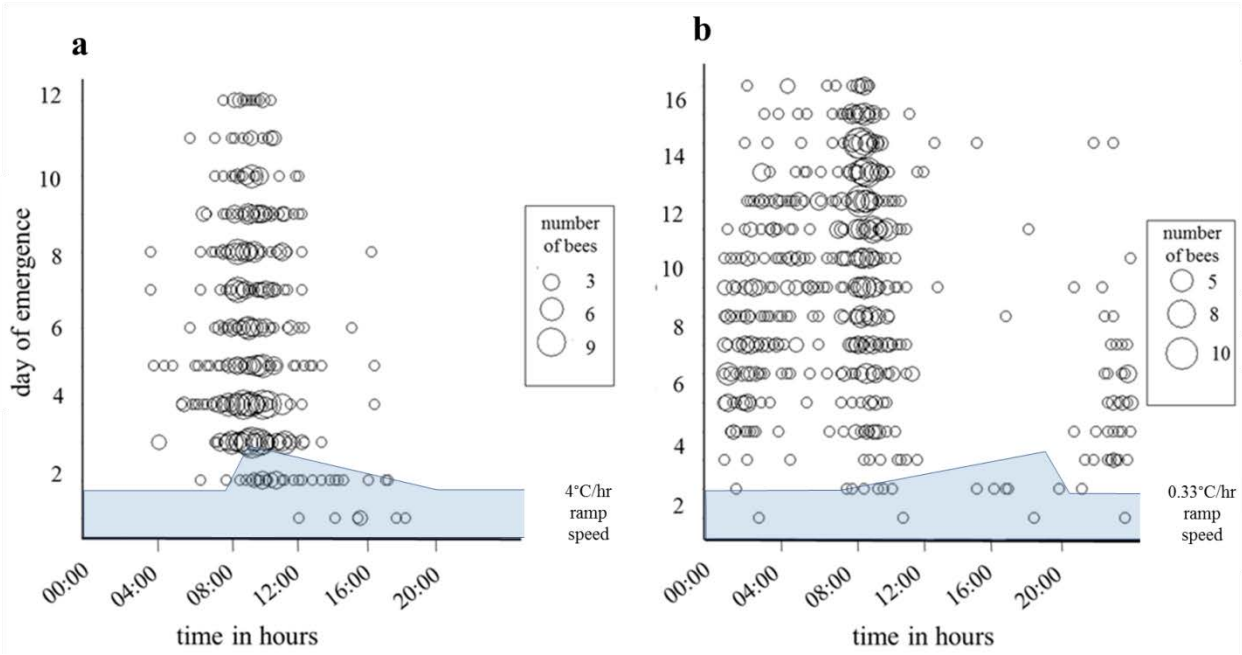

Figure 2.8. The number of emerging bees and mean time of emergence for the ramp speed experiments. a) Emergence patterns from the fast ramp speed (4°C/h) experiment, and b) emergence patterns from slow ramp speed (0.33°C/h) experiment. The blue shaded areas indicate the temperature treatment administered each day but displayed here to show timing of temperature ramps. The size of the bubbles is relative to the number of bees emerging during 15-minute time intervals. The y-axis represents days of emergence, and x-axis is the time of day in hours. secondary y-axis. The size of the bubbles indicates the number of bees emerging during 15-minute time intervals.

Discussion

Our data strongly support the hypothesis that thermoperiod is an important environmental cue for synchronizing emergence of adult *M. rotundata*. Because we observed free-running and phase-shifting of emergence rhythms in constant conditions after exposure to a Zeitgeber, we have shown for the first time that thermoperiod interacts through circadian feedback loops in *M. rotundata*. We also showed for the first time that the brood cell buffered many wave lengths of light by approximately 80 %, suggesting that the brood cell is an important modulator of environmental cues. Even though light is buffered, *M. rotundata* clearly responded to photoperiod cues as emergence-ready adults. Interestingly, they may not be as sensitive to photoperiod cues as they are to thermoperiod cues, because we observed no evidence of phase-shifting after photoperiod removal. Furthermore, when exposed to a conflicting Zeitgeber, emerging bees entrained to the thermophase instead of the photophase, indicating that temperature cues are dominant to light cues. Interestingly, we observed entrainment to the slow ramp speed to the thermophase of 0.33°C/h, alluding to the sensitivity of temperature-mediated clocks in *M. rotundata*. These data support the hypothesis that insects that develop in light-restricted environments may rely on other cues for timing of development and emergence.

Circadian rhythms and Zeitgebers

One way to identify circadian regulation of a process is to expose organisms to a stimulus and then remove it to observe the presence of free-running periods and phase-shifting (Saunders, 2012 & 2013). We observed evidence of phase-shifting (advance) when we removed the thermoperiod, but not the photoperiod. This suggests that photoperiod may be a weaker cue compared to thermoperiod, because phase-shifting is hypothesized to be related to the sensitivity to the Zeitgeber (reviewed in Rensing & Ruoff, 2002; Bradshaw & Holzapfel, 2007 & 2010).

Our finding that thermoperiod entrained emergence is consistent with other studies. For example, free-running eclosion rhythms occur in the flesh fly, *S. crassipalpis* (Miyazaki et al., 2011) and onion fly, *Delia antiqua* (Miyazaki et al., 2016). In the current study, the Δ4°C and Δ8°C thermoperiod removal experiments showed free-running periods and phase-shifting. This is strong evidence that temperature-mediated clocks are involved in regulating emergence of *M. rotundata.*

Two general models have been proposed to describe the mechanisms underlying circadian rhythms for emergence in insects: the single-oscillator model and the two-oscillator model (reviewed in Saunders, 2012). The two-oscillator model (morning and evening oscillators) is proposed for organisms that use both temperature and light cues to mediate emergence, such as in the onion fly, *Delia antiqua,* in which eclosion rhythm is affected by the interacting effect of light and temperature (Watari & Tanaka, 2010). Single-oscillators are reset by one Zeitgeber, irrespective of other cues. It is worth noting that a single-oscillator model includes multiple oscillators, they are just so tightly coupled that they act as a single unit (Wirz-Justice et al., 2003). We found that thermoperiod overrides the photoperiod cue in *M. rotundata*, supporting the single-oscillator model. More experiments need to be conducted to determine the underlying mechanisms mediating the timing of emergence in *M. rotundata*.

To test the relative strength of Zeitgebers on circadian rhythms, one must decouple the phases of the cue (Pittendrigh & Minis, 1964). These types of experiments are called "conflicting Zeitgebers," and they can reveal which Zeitgeber is more dominant (Sharma & Chandrashekaran, 2005; Short et al., 2016; Watari & Tanaka, 2010). Conflicting Zeitgebers occur when cues have different phases, such as the photophase occurring during the cryophase of a thermoperiod. When we exposed *M. rotundata* to these conditions, our hypothesis was

supported because they entrained to the thermophase instead of the photophase. This result is evidence that thermoperiod may be the more dominant cue than photoperiod. Our results are comparable to a study on the flesh fly, *S. crassipalpis* that pupate under the soil, where they entrained to the thermophase of the thermoperiod instead of the photophase of the photoperiod (Short et al., 2016). Complex interactions can exist between the relative timing of photoperiod and thermoperiod phases in mediating insect emergence. For example, the timing and amplitude of a thermoperiod affects whether onion flies, *D. antiqua* entrain to a thermoperiod or photoperiod Zeitgeber (Watari & Tanaka 2010). Thus, we are interested in further investigating the interactions between light and temperature cues for mediating emergence of *M. rotundata*.

Sensitivity to Zeitgebers

Sensitivity to photic stimuli depend on the developmental stage when the signal is received (Joplin et al. 1999; Yadav et al. 2015; Miyazaki et al. 2011; Kumar et al. 2007). For example, *Drosophila* development rate after the third instar has been shown to be affected by wavelengths of green (500 nm), violet (420 nm) and UV (380 nm) (Yadav et al. 1999). Interestingly, honey bees, *Apis mellifera* do not exhibit circadian rhythms in clock gene expression until after adult emergence (reviewed by Moore 2001). Because the hive environment is kept relatively constant by the colony, and newly emerged adults do not leave the hive, there may not be selection to synchronize development with the environment. Previous work showed that adult *M. rotundata* emergence was unresponsive to light cues if exposed to a light pulse during the pupal and emergence-ready adult stages (Tweedy & Stephen, 1970). However, that study only used a single pulse of light, which may have not been a strong enough cue to synchronize emergence. We showed that *M. rotundata* was sensitive to light in emergence-ready adults, when they would most likely be receiving light cues in the field. In a nest, emergence-

ready adults could receive light cues when the sibling in the nearest nest cell emerges, clearing the way for more light to enter the cavity. Sensitivity to environmental cues may change across the lifetime of *M. rotundata* because they undergo development in a cavity and forage during the daytime.

What is intriguing about temperature-mediated clocks is that thermoperiod is presumably a much more variable cue than photoperiod. Thermoperiods can vary by ramp speed, amplitude, and duration of the temperature pulse (Rensing & Ruoff, 2002). Variation in these characteristics can affect sensitivity or responses of insect emergence. For example, thermoperiod amplitude can affect peak eclosion time in some insects (Kikukawa et al., 2013; Miyazaki et al., 2016). We found in the thermoperiod-switch experiment that the mean time of emergence was significantly different when switched from a Δ4°C to a Δ8°C thermoperiod. Furthermore, studies have shown that the thermophase is an important characteristic of a thermoperiod for entrainment of insect emergence (Watari & Tanaka, 2010; Yocum et al., 2016). Similar to these studies, we found that emerging bees entrained to the ramp or the beginning of thermophase versus the cryophase across all experiments in this study. In the slow thermophase ramp experiment, which had a 0.33°C per hour ramp speed, adult emergence was synchronized to the start of the thermophase (ca 07:00). This conflicted with our prediction that bees could not entrain to a slow ramp speed and provides evidence that bees are very sensitive to temperature. These results are comparable to tsetse fly, *Glossina morsitans* for which 0.4°C variations in temperature can synchronize eclosion rhythm (Zdarek & Denlinger, 1995). It would be interesting to determine the smallest temperature increase that could synchronize emergence.

Why would emergence be synchronized? One possibility for synchronous emergence is to increase fitness. We predict that synchronization in the morning could aid in optimizing

availability of locating resources or mating opportunities. Mating success may rely on entrainment to a thermoperiod cue, synchronizing bees in a population to emerge during the same windows of time. Newly emerged adult *M. rotundata* are immediately in search of food and mates, making it important for them to synchronize with the environment. In none of our experiments did we observe synchronization to the cryophase. This makes sense because temperatures increase in the morning and *M. rotundata* are diurnal, foraging during the daylight. Our results suggest emergence-ready adults are more sensitive to temperature than light cues, but this does not mean that photoperiod-mediated clocks do not exist in *M. rotundata.* Such clocks could be more sensitive at other stages of development or for other biological processes.

This study magnified patterns in emergence using automated data collection to better understand the circadian responses and sensitivity to environmental cues. This tool, and our large sample sizes, allowed us to analyze patterns in emergence with more accuracy. Our data support the fact that temperature-mediated clocks play a role in emergence of *M. rotundata*. Testing this hypothesis in other hymenopterans and other insect species will be important to determine if this is a general response of insects that pupate in light-restricted habitats or something specific to this taxa of cavity-nesting bees. Insects relying on temperature cues may be susceptible to temperature variability due to climate change. Because climate change is expected to decouple light and temperature signals to spring insects, understanding what cues drive circadian rhythms and how these may change for animals living in different habitats will be important for predicting how climate change may affect phenologies of not only insects, but the plants they pollinate.

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CHAPTER 3: SUBOPTIMAL TEMPERATURES DURING METAMORPHOSIS REVEALS A CRITICAL DEVELOPMENTAL WINDOW[1]

Abstract

Proper development relies on optimal atmospheric temperatures (T_a), and natural T_a fluctuations, especially in spring or in northern latitudes, could result in interruptions to development. It is unclear how low T_a exposure may affect insects that are actively developing. To understand how suboptimal T_a may affect metamorphosing insects, we used the alfalfa leafcutting bee, *Megachile rotundata*, a solitary, cavity-nesting bee that spends its juvenile and pupal stages within a brood cell. We characterized suites of physiological traits, rather than just using a singular parameter to determine effects of sublethal T_a stress. Metamorphosing *M. rotundata* were exposed to either constant or fluctuating low T_a stress and compared to control bees allowed to develop normally. All bees survived and emerged as adults, but half of the constant low T_a stressed bees were unable to fly. In addition, constant low T_a stressed bees that were able to fly had decreased flight performance (low metabolic rate, shorter flight bouts, decreased wing length), suggesting that the stress altered muscular or neurological development. Constant low T_a stressed bees also had altered activity levels, providing more support for the hypothesis that the low T_a stress caused long-term neurological defects. Exposure to fluctuating

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low T_a also delayed adult emergence, decreased adult lifespan, and resulted in shorter wings. Together these results provide evidence for a critical developmental window during metamorphosis and suggest that there may be severe implications for bees in the wild that are exposed to low T_a stressors.

Introduction

Metamorphosis is a critical developmental window for holometabolous insects. During this time, adult morphology and physiology are established. This process relies on optimal ambient temperatures to proceed without error. While the spectrum of optimal developmental temperatures is relative for all organisms, each species has a developmental temperature threshold (Day and Rowe 2002). When temperatures are too low for normal metabolic processes to continue, development will not be initiated or may be interrupted. Even slight deviations from optimal temperatures can have deleterious effects for insects (Becher et al. 2009; Jones et al. 2005). Natural temperature fluctuations, especially in spring or in northern latitudes, could result in interruptions to development.

Many insects survive low temperatures during winter in a state of diapause, an adaptation that affords protection from low temperatures and prevents insects from beginning the transition to the next stage (Hahn and Denlinger 2011). Insects may still be protected from low temperatures in the state of post-diapause quiescence, a state during which temperatures above threshold can initiate development (Tauber and Tauber 1976). Early resumption of development may occur as our climate changes, because earlier spring thaws are expected (Inouye 2008). This is problematic, because in addition to earlier spring thaws, climate change is predicted to cause increasing temperature variability, including cold snaps (Inouye 2008, Rigby and Porporato 2008, Augspurger 2013). Insects in active pupal or pharate adult development are no longer

afforded protection from low temperatures and may be especially vulnerable to low temperatures during metamorphosis. However, it is unclear how low temperature exposure may affect insects that have resumed development and are no longer afforded protection from low temperatures. Because we rely heavily on insects for pollination, it is critical that we develop accurate, predictive models of population response to temperature stress.

Effects of low ambient temperature (T_a) stress during pupation on adult physiology are contradictory. Insects may have decreased survival, the severity of which depends on the type of temperature stress (fluctuating or constant) (Yocum et al., 2010). Emergence rates and body size may be affected differently with differing types of stress (Ismail et al. 2013). Additionally, adult mortality can be affected not only by the type of stress, but also the timing of the exposure (Rinehart et al 2011). Often, studies examining the effects of suboptimal temperatures during pupal development measure singular parameters such as mortality, emergence rate, fecundity or body size (Ismail et al. 2013; Yocum et al. 2010; Rinehart et al. 2011; Colinet et al. 2006). However, because low temperature stressors may be sublethal (Yocum et al. 1993; Hutchinson and Bale 1994; Kelty et al. 2006; Rinehart et al. 2011), more robust measures may be required to clearly show the effects on whole animals. Furthermore, a single parameter (e.g., adult emergence) may not be a reliable indicator of fitness. While a sublethal stress does not affect survival, it may result in reduced fecundity or longevity (Rinehart et al. 2011; Hutchinson and Bale 1994). Characterizing suites of physiological traits that are affected by sublethal developmental temperatures will help us to understand how insects will respond to temperature variation, such as may occur with climate change.

To understand the implications of suboptimal temperature on metamorphosing insects, we used the alfalfa leafcutting bee, *Megachile rotundata*. *M. rotundata* is a solitary, cavity

nesting bee that spends its juvenile and pupal stages within a brood cell, making them easy to manage in the laboratory. Brood cells are constructed by females and provisioned with nectar and pollen. Eggs are sealed in the brood cell, and juveniles consume the provision before undergoing diapause in the fall and winter as a prepupa. We took advantage of the life history of *M. rotundata* to study effects of suboptimal temperatures during development. Because *M. rotundata* undergo diapause during the prepupal phase, they are not protected from low T_a during metamorphosis when increasing temperatures would initiate pupal development. This allowed us to manipulate temperature during metamorphosis and identify a critical developmental window during metamorphosis.

To test the effect of suboptimal temperatures during metamorphosis, we exposed developing *M. rotundata* to one of two types of low T_a stress: constant or fluctuating. We compared these bees to control bees that had been allowed to develop normally. We assessed many aspects of bee structure and function to determine if one or many parameters would be required to identify effects from a sublethal stress.

Materials and methods

Animals

Loose brood cells containing pre-pupal *M. rotundata* were purchased from JWM Leafcutter, Inc. (Nampa, ID) in the spring of 2013. Prepupae were kept at 6 °C in darkness for approximately 8-9 months during diapause. To simulate natural warming and initiate pupal development, loose cells were placed individually in wells of a 24-well plate (Greiner Bio-one, Monroe, NC) and incubated at 29°C. Humidity was maintained with a beaker of saturated $NaCl/H_2O$ solution in the incubator.

After 14 days at 29°C, following standard industry protocols, actively developing *M. rotundata* were separated into three treatment groups 1) constant low T_a stress, 2) fluctuating low T_a stress, and 3) uninterrupted controls. We interrupted development in constant low T_a stress bees by placing them at a constant 6 °C for 1 week and then replacing them in 29°C to resume and complete adult development. We interrupted pupal development in fluctuating low T_a stress bees by placing them at 6° C with a daily pulse of 20°C for 1 hour with 1 hour ramp times. After one week, fluctuating stressed pupae were returned to 29°C to resume and finish development. The control group was maintained at 29°C with no exposure to temperature stress throughout development. After emergence, all adults were reared as previously described, individually in containers with *ad libitum* access to food (Bennett et al 2013). Bees were fed a sugar solution that was changed every 2-3 days. Bees were maintained at 29°C with a 12:12 light to dark cycle.

Development time and longevity

Pre-pupae from the three treatments were reared as described above (n = 40 bees per group, 20 male and 20 female). Adult emergence was recorded twice daily, in the morning and late afternoon, when bees typically emerged from the leaf pods within well plates. Development time was calculated as the number of days at 29°C. To determine whether temperature treatment during pupal development affected adult lifespan, we reared the bees as adults until they died. Adults were observed in the morning and afternoon, and we recorded whether they were alive or dead. An adult was considered dead if prodding with a dissection probe elicited no movement. Longevity was calculated as the difference between the day of death and the day of emergence from the leaf pod.

Flight performance

Bees were haphazardly selected for flight metabolic rate measurements. Tethered flight metabolic rate was indicated by CO_2 emission rates of bees using flow-through respirometry in a temperature-controlled incubator (29°C). The incubator door was covered with a plastic sheet to maintain constant temperature yet allow access to the respirometry chamber. Dry, CO_2-free air was made with a purge gas generator (Balston, Haverhill, MA). Flow rates were maintained using a mass flow controller (MFC-4; Sable Systems, Inc., Las Vegas, NV) and mass flow meters (Sierra Instruments, Monterey, CA). Voltage outputs from the CO_2 analyzer and mass flow controller were digitized and recorded using Sable Systems hardware (UI2) and software (EXPEDATA version 1.4.16). Bees were weighed with an analytical balance (Mettler Toledo, Columbus, OH) to the nearest 0.01 mg prior to measurement of flight metabolic rate.

A previously described method of sling-tethering (Bennett et al. 2013) was used to collect flight metabolic rates. Briefly, Adults were chilled at 6°C for approximately 5 minutes to inhibit movement during tethering. Bees were lassoed around the cervix with a strand of polyester which was threaded through a blunt-ended 18-guage hypodermic disposable needle. A luer-lock glued to the inside of the chamber held tethering needle in place. After tethering, bees were positioned in the respirometry chamber, and the animal was allowed to acclimate to the chamber for 10 minutes. The chamber (471ml) was flushed with dry, CO_2-free air at 1,250 ml min^{-1}. Before initiating flight, bees rested on a moveable stage, constructed from a barbed, elbow tubing connector and Tygon ^{TM} tubing. Bees were stimulated to fly when we removed the stage was removed from the tarsi. CO_2 emission rates were recorded for three flight bouts per bee. A flight bout was identified by behavioral characteristics of flight: the front legs tucked underneath the body, back legs splayed outward from the body and wings beating rapidly. During flight bout

measurements, bees that did not display these characteristics or exhibited aberrant behaviors, such as kicking, were omitted from the analysis. Flight bouts were indicated in the data file using a foot pedal that sent a voltage to the data acquisition hardware and digitized a marker on the raw CO_2 emission trace. We averaged flight bout duration and CO_2 emission rate from the three flight bouts per individual.

Activity

Activity levels across treatment groups were assessed by visually observing behavior within the adult housing units as previously described (Abdelrahman et al. 2013 in review). Three days after bees emerged, activity was assessed for one minute, five times a day for three days. Behavior was categorized in one of three ways, non-active, active, or feeding. Non-active bees were those not moving. To be classified as feeding, a bee must have had its proboscis touching the muslin cloth. Any other movements were classified as active but not feeding.

Morphological measurements

Animals used for wing measurements were at least three days old, to allow for wing hardening and acclimation to the housing, and less than days old to minimize possible damage to the wings from being contained. Adults were chilled at 6°C for 5 minutes and body mass was measured using an analytical balance (Mettler Toledo, Columbus, OH). While the animals were still cold, wings and head were dissected using Student Vannas spring scissors (Fine Science Tools, Foster City, CA). Wings were positioned under the dissection microscope using a paintbrush and laid flat with a cover slip. Images of wings were taken using a Leica MZ12 dissection scope attached to a DFC 295 camera the left wing was always next to the crescent shape at the top left corner of the picture for reference (Figure 3.1). Wing lengths were measured using ImageJ (version 1.47). Each image included a ruler for calibration of measurements for

wing dimensions. Head width was measured using digital calipers (Fisher Scientific, Hampton, New Hampshire) dorsally (approx. where the antennas are located) to the edge of each eye.

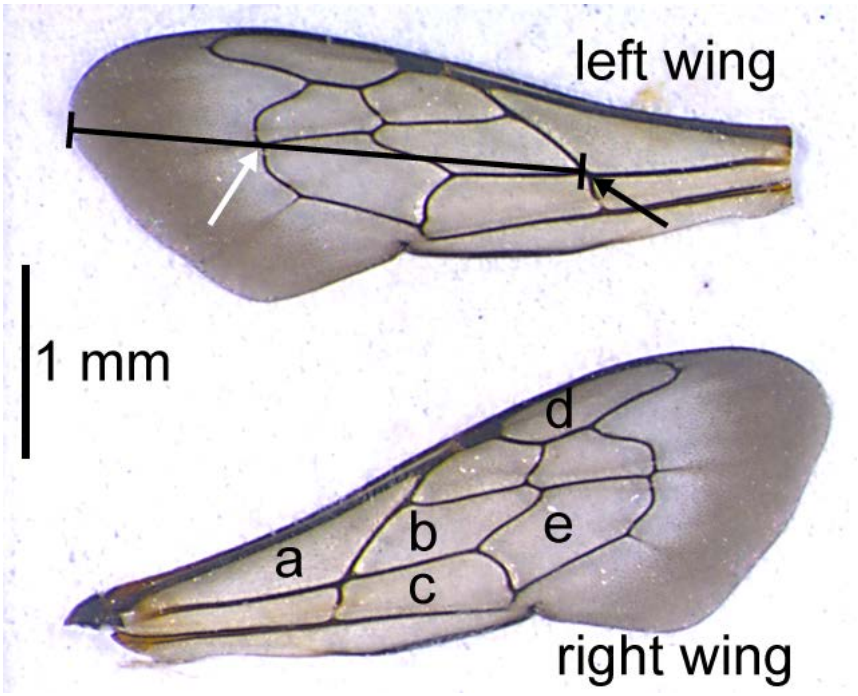

Figure 3.1. Left and right wings of *M. rotundata* mounted for analysis. A ruler was used for calibration in Image J. Our index of wing length was measured from the intersection of the basal vein and 1st cubital cell (black arrow) through the intersection of the 3rd transcubital vein and 2nd recurrent vein (white arrow) then to the tip of the wing, as noted by the black line. Cell areas were measured for the radial cell (a), the 1st medial cell (b), the 2nd cubital cell (c), the marginal cell (d) and the 2nd medial cell (e).

Some wings were dissected too far away from the body, so we used the distance from the intersection of the basal vein and 1st cubital cell through the intersection of the 3rd transcubital vein and 2nd recurrent vein then to the tip of the wing, as our index of wing length (Figure 3.1). We also measured the area of five individual wing cells on the forewing; the radial cell, the 1st medial cell, the 2nd cubital cell, the marginal cell, and the 2nd medial cell (Figure 3.1) (Michener et al. 1994). Wing loading was calculated for each individual as mass divided by total wing area. However, the constant low T_a stress group had a large percentage of animals that had

incalculable wing areas due to wing damage, so wing loading was only calculated for individuals with no damage.

Statistical analysis

Data were analyzed using IBM SPSS Statistics (version 21, Armonk, NY, US). Development time, flight performance parameters, and some morphological data were analyzed using two-way ANOVA with sex and treatment as factors. Longevity was analyzed using Kaplan-Meier with sex and treatment as factors. Activity data were analyzed with a proportional odds model using SAS (version 9.3, Cary, NC, US) and the PROC LOGISTIC command. Because bee activity changed over the three days, data from each day were analyzed separately. Time of day, treatment, sex, and their interaction were used as factors in the model. Wing cell areas were analyzed using repeated measures ANOVA, with cell location and body side as within-subjects repeated factors and temperature treatment and sex as factors. Because the data had unequal variances, we used the Greenhouse-Geisser adjusted degrees of freedom. In all tests, p values less than 0.05 indicated statistically significant differences.

Results

Development time and longevity

Temperature stress of any kind during pupal development affected development time differently depending on the sex of the bee (Figure 3.2; sex x treatment interaction $F_{2,266} = 6.99$, $p < 0.01$). There was no difference in the developmental delay between fluctuating or constant low Ta stress (Bonferroni-corrected post hoc tests, $p = 0.115$). Male and female development times were 35-50% longer under constant and fluctuating low T_a stress compared to normal bees 10-14 days delayed rather than our prediction of a 7 day delay. Females emerged around 8 days

later than males in both temperature stressed groups, whereas in control rearing conditions, females emerged at nearly the same time as males.

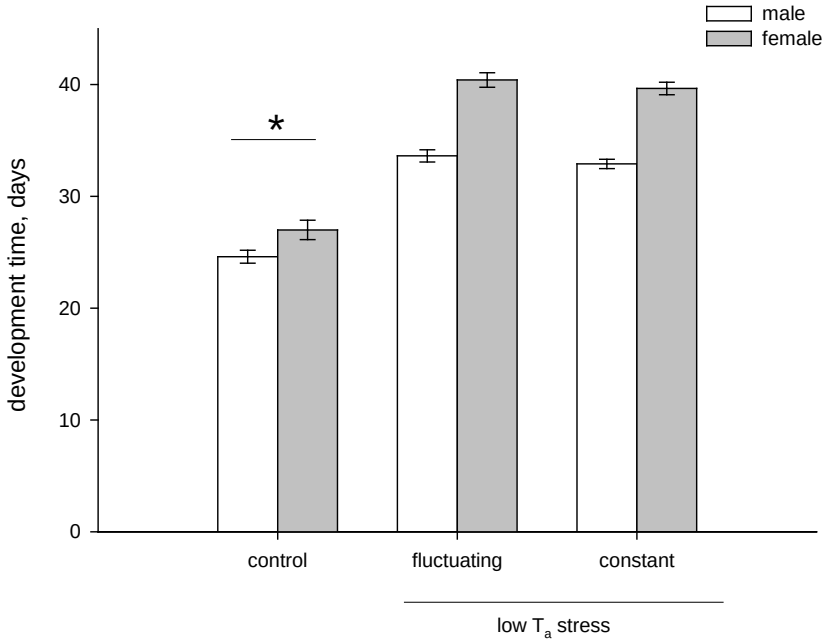

Figure 3.2. Development time at 29°C until adult emergence as a function of T_a treatment. Females (gray bars) and males (open bars) were exposed to constant or fluctuating low T_a stress or control rearing conditions during pupation. Asterisk indicates a significant effect of temperature $p < 0.05$.

Adult lifespan of males was significantly shortened when given any temperature stress during pupal development (Figure 3.3; log rank test, $X^2 = 17.724$, $p = 0.000$). Both constant and fluctuating low T_a stressed males lived half as long as control bees. Females showed a non-significant trend toward shorter lifespans (log rank test, $X^2 = 2.89$, $p = 0.089$). There was no significant difference between male and female lifespans in control rearing conditions (log rank test, $X^2 = 0.71$, $p = 0.40$). Fluctuating and constant low T_a stress affected the lifespan of males with males living approximately half as long as control males (log rank test, FTR: $X^2 = 12.16$, $p < 0.001$; constant stress: $X^2 = 6.22$, $p < 0.01$).

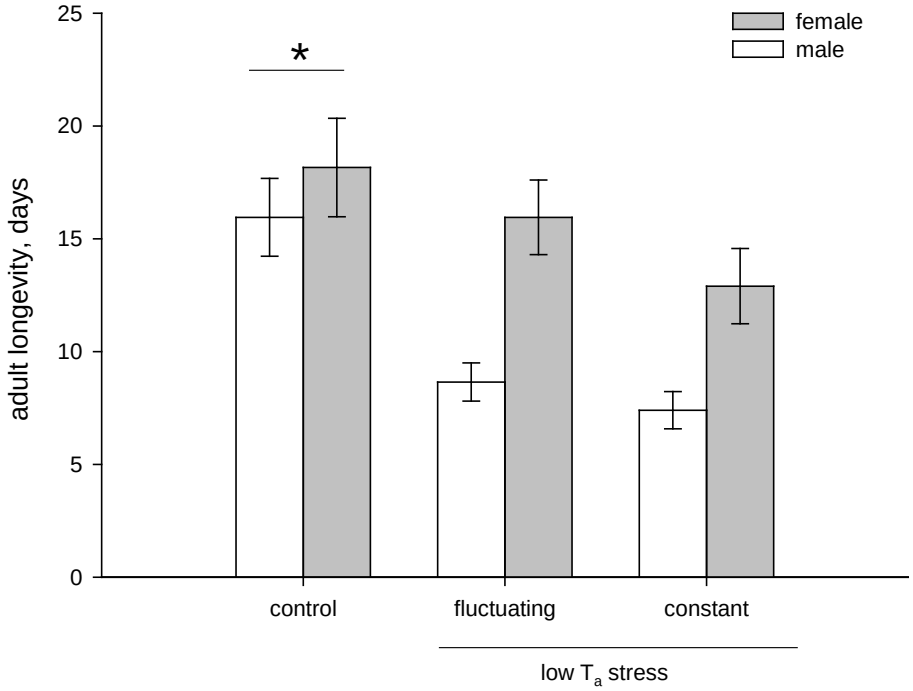

Figure 3.3. Adult longevity in days post emergence as a function of T_a treatment. Females (gray bars) and (males) were exposed to constant or fluctuating low T_a stress or control rearing conditions during pupation. Asterisk indicates $p < 0.05$.

Flight performance

When adults were haphazardly selected for flight metabolic rate measurements, many bees from the constant low T_a stress treatment were unable to fly (see supplemental video 1 for flight defect, see supplemental video 2 for normal flight), nearly half of the males and 10 % of females were unable to fly. Of bees that were able to fly, there were no treatment effects on body mass, although constant stressed males were significantly smaller than females ($F_{2,42} = 36.77$, $p < 0.03$). Absolute metabolic rates (µmol h^{-1}) did not vary with temperature or sex. However, mass significantly affected absolute metabolic rate ($F_{1,41} = 10.50$, $p < 0.01$), and there was a significant interaction between treatment and sex, indicating that males and females responded differently to the temperature treatment ($F_{2,42} = 3.39$, $p < 0.05$). Constant low T_a stressed males had absolute flight metabolic rates 26% lower than control reared or fluctuating T_a stressed males (Fig 3.4).

Mass-specific metabolic rate was not affected by treatment or sex, but there was a non-significant trend mirroring the response of absolute metabolic rate ($F_{2, 42} = 2.56$, $p = 0.09$).

Temperature treatment affected the duration of flight bouts differently depending on sex (sex x treatment $F_{2,42} = 3.38$, $p < 0.05$). Constant low T_a stressed males had 30% shorter flight bouts compared to control or fluctuating low T_a stressed males (Fig 3.5). Thorax temperature immediately after flight did not vary with treatment or sex. Body mass accounted for 17% of the variation in thorax temperature ($y = 0.095x + 27.85$, $F_{1,47} = 9.72$, $p < 0.01$).

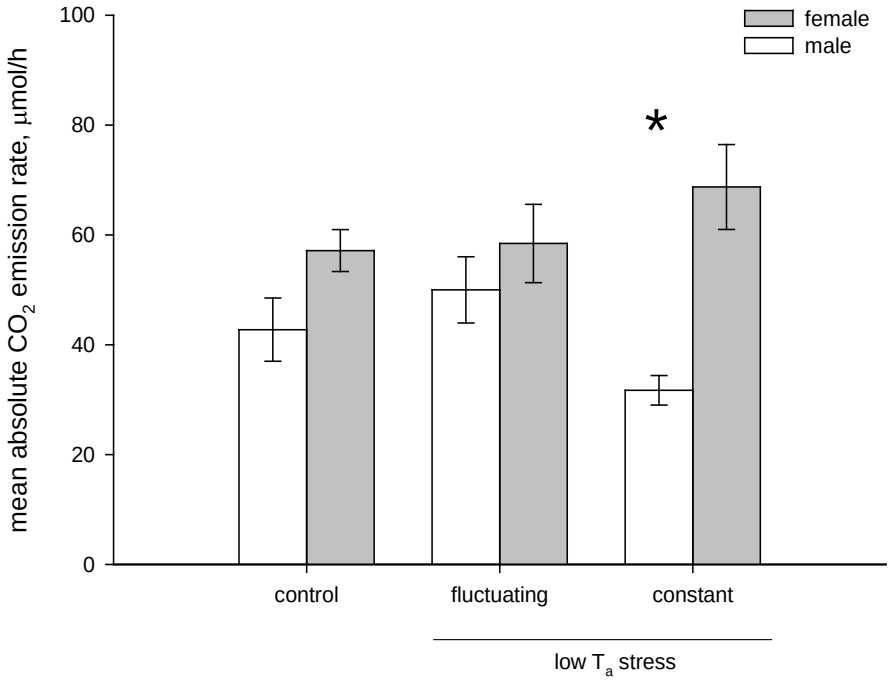

Figure 3.4. Absolute CO_2 emission rate during tethered flight as a function of T_a treatment. Females (gray bars) and (males) were exposed to constant or fluctuating low T_a stress or control rearing conditions during pupation. Asterisk indicates $p < 0.05$.

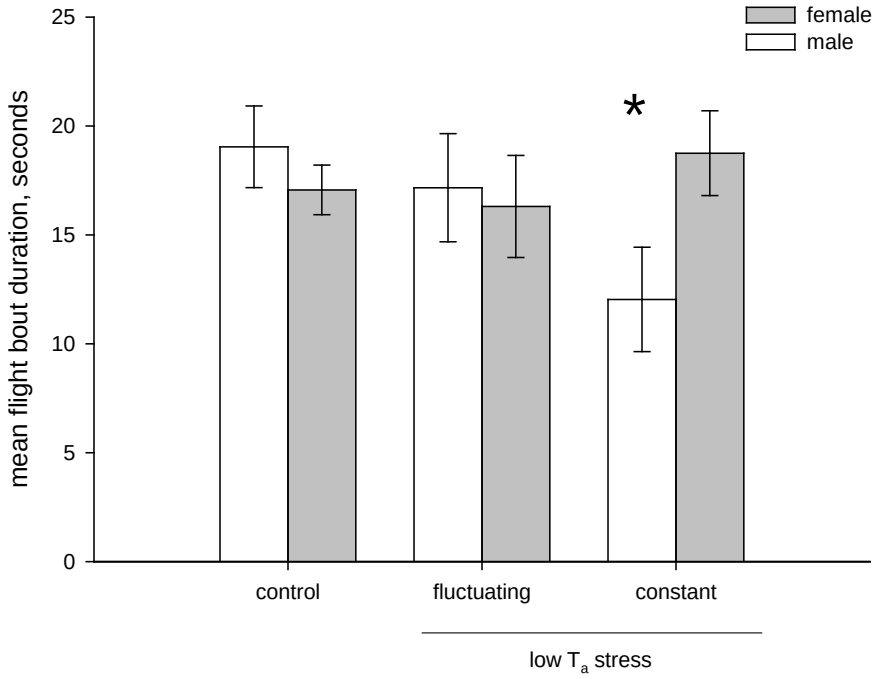

Figure 3.5. Females (gray bars) and (males) were exposed to constant or fluctuating low T_a stress or control rearing conditions during pupation. Asterisk indicates $p < 0.05$.

Activity

Temperature treatment significantly affected adult bee activity regardless of which day activity was observed (Figure 3.6). Across all three days, control bees spent the most time active but not feeding compared to bees that were exposed to constant or fluctuating low T_a stress (Figure 3.6). Constant low T_a stressed bees were most likely to be non-active (Figure 3.6). By day 3, bees exposed to constant low T_a stress were less likely to be feeding (Figure 3.6). On the first and third days of observation, there was a significant effect of sex. In addition, activity was related to time of day. All observations from each day are pooled in Figure 3.6 for simplicity.

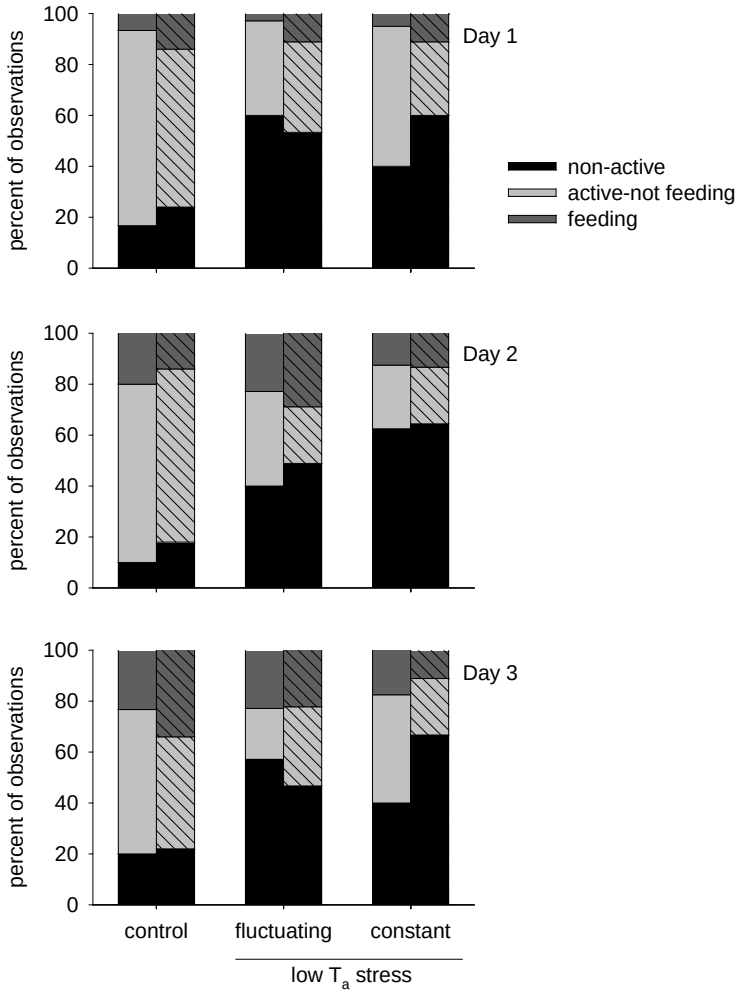

Figure 3.6. Percent of observations for bees that were not active, active but not feeding, or feeding as a function of sex, T_a treatment and day of observation. Percent of time that adults were observed in different behaviors.

Morphological measurements

Body mass and head width did not differ across treatment groups, although males are always smaller than females (female mass =43.91±0.81 mg; male mass = 34.15±0.87 mg; $F_{1,84}$ = 45.82, $p < 0.001$; Figure 3.7). Wing length was significantly shorter in the constant low T_a stressed males and females (effect of temperature: $F_{2,80} = 11.62$, $p < 0.001$; Fig 3.7). Right forewing compared to left forewing length were similar (RM-ANOVA, $p = 0.114$). Fifty-seven% of constant low T_a stress adults had damaged wings, with the tips of this wing appearing tattered.

Fluctuating T_a stress and control bees had less than 10 % of bees with damaged wings (8% and 3% respectively). Wing cell area varied differently depending on the cell location, the temperature treatment and the location (three-way interaction, $F_{5.15,239} = 2.51$, $p < 0.03$, Figure 3.8). In addition, wing cell area varied differently by location depending on the sex of the bee ($F_{2.534,236} = 19.87$, $p < 0.0001$, Figure 3.8). There was no effect of sex ($p = 0.2$) or temperature treatment on wing loading ($p = 0.2$ and 0.6 respectively). Mean wing loading was 13.86 ± 0.26 mg/mm^2.

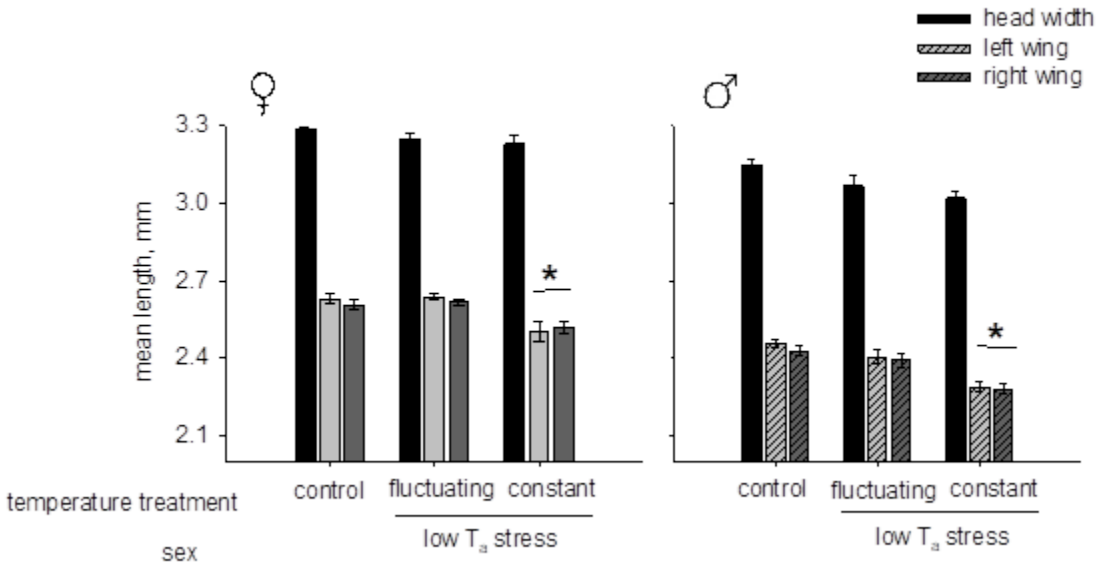

Figure 3.7. Head width (black bars) and wing lengths as a function of temperature treatment. Females are shown on the left panel with open bars and males are shown on the right panel with hatched bars. Left (light gray bars) and right (dark gray bars) wings are also shown. Asterisk denotes $p < 0.05$.

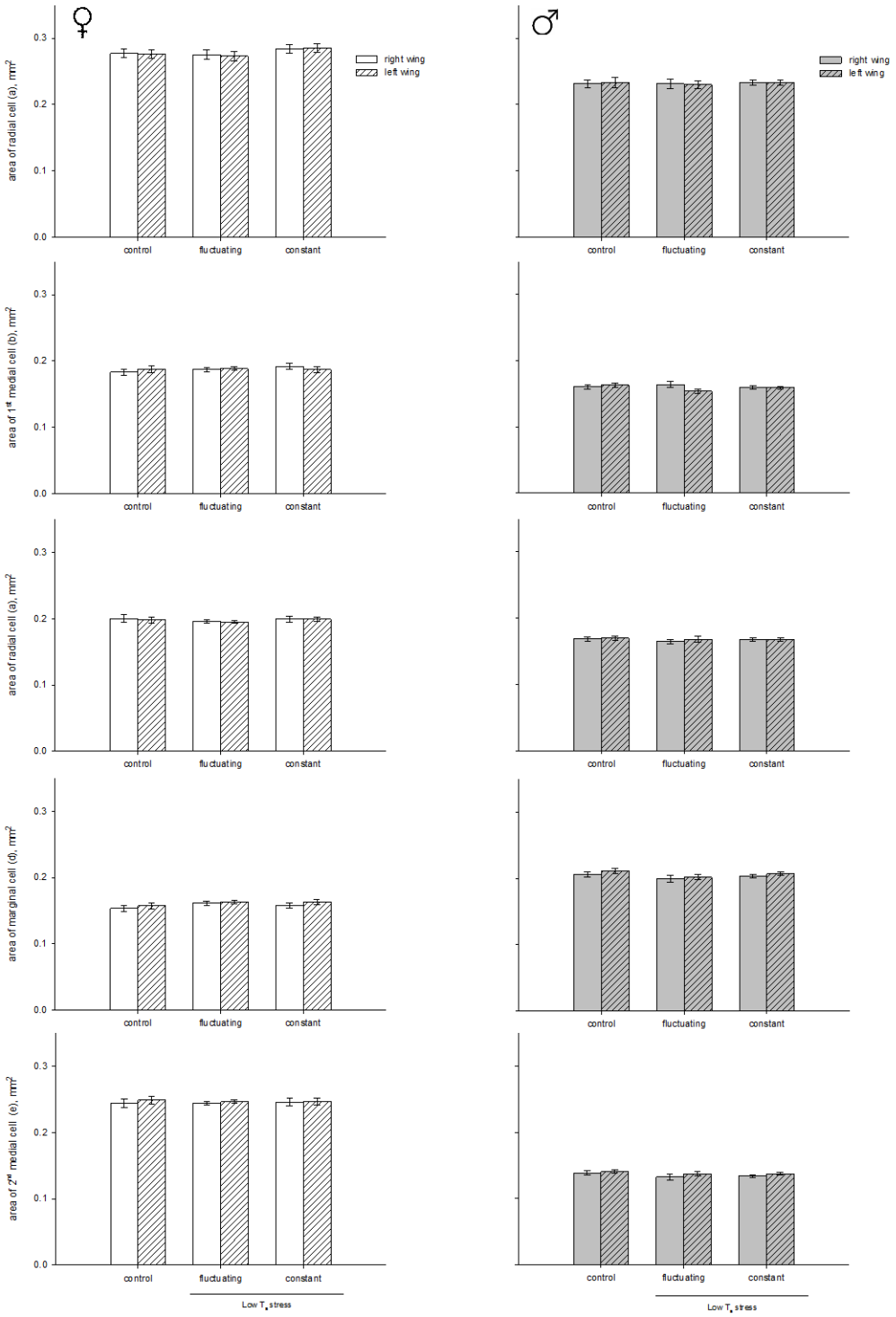

Figure 3.8. Each row shows data from a specific wing cell area. Right (open bars) and left (hashed bars) side wings are shown for each treatment group. Female wing cell areas (white bars) are the left side panels, and male wing cell areas (gray bars) are right panels. The y-axis for each graph indicates which wing cell was measured.

Discussion

Our hypothesis that exposure to a suboptimal temperature below the developmental threshold during pupal development of *M. rotundata* would be a sublethal stressor was supported. Furthermore, this stressor affected many aspects of adult physiology that would have gone unnoticed had we investigated only one parameter, such as emergence or body size. From our findings, we can begin creating a more holistic index of how suboptimal developmental temperatures affect adult organisms. Many aspects of adult physiology measured in this study were affected by a constant low T_a stress. While bees survived and emerged as adults, many of them were unable to fly. In addition, constant low T_a stressed bees that were able to fly had decreased flight performance, hinting at altered muscular or neurological physiology. Constant low T_a stressed bees also had altered activity levels, suggesting neurological defects. Although, it is more likely that developing insects would encounter a fluctuating low T_a stress rather than a constant low T_a stress, the effects of a fluctuating low T_a temperatures delayed adult emergence, shortened adult lifespan and shortened the length of the wings. Additionally, there were noticeable sex-specific differences in physiology after exposure to constant low T_a stress during development, providing evidence for a critical developmental window during metamorphosis. Together these results indicate that there may be severe implications for bees in the wild that are exposed to low T_a stressors.

Research examining the effects of low developmental temperature stress in hymenopterans exists (Jones et al. 2005; Becher et al. 2009; Luczunski 2007), but is lacking for solitary bee species. Research on the honeybee, *Apis mellifera,* shows that even a slight deviation from the optimal pupation T_a changes the division of labor in the colony (Becher et al. 2009) and decreases short-term memory (Jones et. al 2005). However, these T_a were not below the

developmental threshold and were maintained throughout development. In addition, our study detected differences in numerous adult physiological phenotypes when pupae were exposed to a week-long suboptimal T_a stressor. Many studies use body size indices to determine if there is a significant effect. Had we used body size or head width as our readout, we would not have detected the massive effects of low T_a on these bees.

Effects of low temperature on adult emergence and longevity

Emergence rate and longevity are important fitness parameters, especially for solitary bees where their life history strategy involves male and female synchronization of emergence for successful mating. Male and female emergence rates and average lifespans, if not given a T_a stress, were not significantly different from each other in this study. We predicted that emergence would merely be delayed proportionally to the duration of the low T_a stress. However, we found that when given any suboptimal T_a stress, compared to our control, adult emergence was delayed twice as long as the duration of the stress itself (Figure 3.2). Because exposure to any T_a over the developmental threshold is expected to contribute to development, it is unclear why there was a disproportionately long development time.

Furthermore, this was the first study to report data on the adult longevity of *M. rotundata* under these rearing conditions. We found the constant low T_a stress treatment significantly decreased adult male longevity, living only eight days versus the control males who lived an average of sixteen days. The timing of this poses a problem, because if constant low T_a stress males live for only eight days and females emerge eight days later, the opportunity for mating could be missed altogether.

Effects of suboptimal pupal temperature on adult flight performance

In this study, constant low T_a stress had the greatest impact on the flight performance of *M. rotundata*. In our study, we found that half of the constant stress bees were unable to fly. Normal flight behavior is described as legs tucked under the body with the back legs slightly splayed outward and the abdomen pumping rapidly along with the wings (Dudley 2002).While we did not quantify these aspects of the flight behavior exhibited by bees, video taken shows that the constant low T_a stress animals that were unable to fly demonstrated the characteristics described above, but lacked rapid movement of the wings (Supplemental Video 1). Instead, the wings moved slowly and asynchronously away from the body, unable to produce effective wing beats. Additionally, of those who could fly from the constant low Ta stressed group, males had lower flight metabolic rates and shorter flight bouts.

What is the mechanism underlying the differences in flight performance? One possibility is that muscular development was deleteriously interrupted during the constant low T_a exposure. When white fly parasitic wasp, *Encarsia formosa*, pupae were exposed to durations of low T_a exposure adult flight ability and ability to parasitize aphids decreased (Luczunski 2007). Low T_a exposed flesh flies, *Sarcophaga crassipalpis,* exhibited muscular dysfunction, which interfered with adult emergence (Yocum et al. 1993; Kelty et al. 2006). However, in our study we found that thorax temperature immediately after flight was not significantly affected by treatment possibly indicating no difference in how hard the flight muscles are working between treatments. However, the constant low T_a stressed males had shorter flight bouts, perhaps indicating that the muscles fatigue more quickly. An alternative hypothesis is that there could be neurological damage to the nerves controlling the flight muscles (Yocum et al. 1993; Kelty et al. 2006). Our activity data supports this idea, because constant low T_a stress bees had decreased observations

of activity and feeding. Chill-injury in insects exposed to low T_a has been shown to affect ion channel function, which is critical for proper muscle and nervous system function (MacMillan 2011). While chill-injury is a term usually reserved for short-term T_a stress, it is possible that effects observed in this study are long-term effects of chill-injury during metamorphosis.

These data provide evidence that there is a critical window of development for flight performance. Suboptimal T_a stress clearly affected males and females differently. In general, males seemed more sensitive to the stress. For example, a higher ratio of bees male bees were unable to fly compared to females. We hypothesize that the sex-specific differences observed in this study could be due to the difference in development rate between males and females. Because females and males develop at slightly different rates, and all pupae were interrupted at the same time, it is likely that more males were developing flight-related physiological systems at this time. Our study provides support that days 14-21 of pupal development are a critical developmental window for male bees. Perhaps if the timing of the stress were delayed by a few days, we would hit the critical window for females and see more females that were unable to fly.

Effects of low temperature on morphology

Many studies looking for effects of a treatment use body mass or some other indication of size as evidence that the treatment was not affecting insect development. In our study, neither body mass nor head width significantly differed with treatment (Figure 3.7). Interestingly, we found that wing morphology was very different in constant low T_a stress bees. Plasticity in adult wing morphology has been documented in studies where lower developmental temperatures lengthened the wings versus higher temperatures (Ottenheim & Volmer 1999), and fluctuating temperatures during juvenile development caused the wings to narrow compared to constant temperatures (Kjærsgaard et al. 2013). In this study, wings from bees in the constant low Ta

stress group were shorter and appeared tattered (Figure 3.7). There were also difference in certain wing cell areas, and the interaction between body side, location and temperature indicate that the temperature treatment may induce asymmetry in wing areas (Figure 3.8). While we did not detect differences in wing loading, we suspect this is due to being unable to analyze several of the tattered wings from the constant low T_a stress bees. Using wing length, which differed with temperature, as an index of wing area, we would expect to see higher wing loading in the bees with tattered wings, since body mass did not vary. In bumblebees, *Bombus impatiens*, animals of with smaller wing surface areas had higher metabolic rates and thorax temperatures compared to bees of similar mass with larger wing area, suggesting that bees have to work harder to compensate for the increased wing loading (Skandalis & Darveau 2012). This result is in contrast to our study, where constant low T_a stressed males had lower metabolic rates with smaller wing areas and no difference in thorax temperature. This suggests that constant low T_a stressed *M. rotundata* are unable to compensate for the increased wing loading, either because of neurological, muscular or morphological defects.

Implications of low T_a stress and their effects on population dynamics

In addition to the possibility of missing a mate due to differences in development times when given a suboptimal temperature stress, many other implications may result from the altered timing. Females could get a late start constructing brood cells if males are hard to find, because they do not start building the leafy brood cells until after they have mated with a male (Rossi et al. 2010; Pitts-Singer & Cane 2010). Even worse, because of their shortened lifespans, they might die before completing them. If many males die before females emerge, the living males will likely mate multiple times because of their scramble competition mating system (Rossi et al.

2010). This could decrease genetic diversity in a population if fewer males are mating multiple times.

In addition to the problems associated with altered phenology, not being able to fly will have serious effects on an individual's fitness. Adults must fly to locate food, mate, and provision brood cells. For females, this defect would significantly jeopardize fitness because the females fly 5-6 hours a day to build brood cells, carrying loads of pollen, nectar and leaf pieces (Pitts-Singer & Cane 2010). For males, shorter flight bouts and a lower metabolic rate associated with low T_a stress could jeopardize their ability to mate because male leafcutting bees must chase and harass females to mate with them (Rossi et al. 2010). Together, the implications of T_a stress during the critical developmental window will have huge fitness effects.

Conclusions and future directions

Suboptimal constant low T_a stress during the pupal development of *M. rotundata* significantly impacted many adult phenotypes measured in this study. Beyond emergence data, we now have better knowledge of the effects of a low T_a stress on adult physiology during a specific developmental window for *M. rotundata*. From this data, we have a better understanding about how the whole organism is affected under temperatures below the developmental threshold during active periods of development for *M. rotundata*. This study is a starting point for recognizing sublethal effects of temperature stress in insects. Our use of *M. rotundata* is particularly important, as not much is known about the physiology of these solitary bees, and timely, as honeybee populations are in danger.

Temperature strongly affects physiological processes, and predicting how global climate change may affect insects, particularly pollinators, is critical. Our climate is predicted to change by not only increases in the number of warm days and decreases in the number of cold nights

(Alexander et al. 2006) but also increases in temperature variability and increased likelihood of spring cold snaps (Inouye 2008). While many insects are tolerant of cold temperatures in their overwintering stages (i.e., diapause or post-diapause quiescence), insects that resume active development early in the spring due to an increase in the diurnal temperature range may be particularly vulnerable to sudden cold spells. Suboptimal temperature stress during a critical developmental window could cause plasticity in developmental trajectories, which may affect organisms on multiple levels such as species, population, and ecosystems. Inclusion of physiological analyses is necessary for accurately predicting changes in population sizes, range, and phenological events, because phenotypic plasticity is an important response to thermal stress (Helmuth et al. 2005). Because we rely on pollinators for food production, identifying critical developmental windows sensitive to environmental variation is important in understanding how long term implications affect overall adult condition.

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CHAPTER 4: DEVELOPMENTAL EFFECTS OF LOW TEMPERATURE ON ADULT THERMAL PHYSIOLOGY

Abstract

Cold tolerance is difficult to measure because it requires multiple assessments of an insect's physiology. Temperatures experienced during development can affect adult morphology and physiology. Less is known about how exposure to cold during development affects adult thermal performance. Sensitivity to low temperatures can vary across development. We found that cold exposure during development affects recovery from chill coma but not critical thermal minimum. Furthermore, we saw that cold tolerance varies across development in supercooling points. Post diapause quiescent stage were more tolerant to cold than stages in active development. Furthermore, assessing the presence of sub-lethal effects is essential to understanding the consequences of low temperatures during development. We observed some red-eye pupae who were exposed to cold resulted in adults with wing deformities. We comprehensively assessed cold tolerance using multiple assays and responses of adults to developmental cold stress.

Introduction

Insects experience multiple types of environmental stressors during their lifetime. One such stressor that has been well-studied is low temperature exposure. The response and ability to withstand low temperatures is referred to as cold tolerance (reviewed in Teets & Denlinger 2013; Sinclair et al. 2015). Cold tolerance can be evaluated in numerous ways, including survival, physiological thresholds, or thermal performance (Andersen et al. 2015; Hazell et al. 2008; Rinehart et al. 2008 and 2011; Teets et al. 2011; Yocum et al. 1994; Rajamohan & Sinclair 2008). Assessment of an insect's cold tolerance is difficult, because an accurate assessment

cannot be achieved by a single metric. To further complicate this assessment, studies have shown that cold tolerance varies across development (Rinehart et al. 2011; Le Bourg 2011; Tran & Takagi 2007). Therefore, an accurate representation of a species' cold tolerance can only be achieved by incorporating multiple measures across several developmental stages.

Physiological threshold temperatures are commonly used as indicators of cold tolerance, because they are easily identifiable responses to low temperature. Supercooling point (SCP) and critical thermal minimum (CTmin) are two frequently used thresholds. Supercooling point is the temperature at which intracellular freezing occurs. A useful measure, SCP can tell us if an insect is freeze-tolerant or freeze-avoidant. If an insect survives freezing, this indicates they are freeze-tolerant versus freeze-avoidant insects, where SCP is the lowest temperature threshold before death (reviewed in Teets & Denlinger 2013; Overgaard & MacMillan 2017; Sinclair et al. 2015). CTmin is the temperature at which loss of muscle coordination, termed chill coma, while the time it takes an insect to recover is another useful index of cold tolerance (reviewed in Overgaard & MacMillan 2017; Hazell & Bale 2011). A study examining SCP and CTmin across species of *Drosophila* found these two measures were the best predictors of cold distribution limits (Andersen et al. 2015). Therefore, characterization of an insect's cold tolerance should include at minimum these two metrics, because they can aid in making predictions about the types of environments or thermal ranges where an insect species may be found.

The last commonly used measure of cold tolerance is survival. However, measuring survival alone does not accurately represent cold tolerance due to the possibility of sub-lethal effects in those who survived to adulthood. Sub-lethal effects negatively affect physiology, morphology, and behavior (Bennett et al. 2015; Hutchinson &Bale 1994; Yocum et al. 1994; Kelty et al. 1996; Marshall & Sinclair 2011; Rinehart et al. 2011). Sub-lethal effects are

hypothesized to be due to chill-injuries, which are thought to affect ion channel function, disrupting proper muscle and nervous system function (MacMillan & Sinclair 2011). Sub-lethal effects of low temperature stress can be severe, resulting in problems with locomotion (Bennett et al. 2015; Kelty et al. 1996) and fecundity (Hutchinson & Bale 1994) and clearly demonstrate that survival alone is not a good measure of an insect's ability to withstand cold exposure.

Insect responses to low-temperature stress can vary with the duration (acute or chronic) and frequency of exposure. Chronic exposures have been shown to correlate with increased severity of the injury compared to acute exposures (reviewed in Overgaard & MacMillan 2017). Furthermore, the shape of the thermoperiod can also affect the amount of injury sustained. For example, it has been shown that low temperatures which are fluctuating with a brief warming period, are less detrimental than static treatments (Rinehart et al. 2011; Bennett et al. 2015). Thus, the presence of sub-lethal effects from different types of stress should be incorporated into assessments of cold tolerance.

An emerging insect model for studying physiological processes is the alfalfa leafcutting bee, *Megachile rotundata*. In the wild, these solitary bees undergo development within a brood cell and enter diapause in the prepupal stage. The following spring, metamorphosis resumes, and adults emerge. Interest in the cold physiology of *M. rotundata* is driven by two things. First, they are intensively managed for pollination services (Pitts-Singer & Cane 2011), and low-temperatures are frequently used to overwinter bees and to delay emergence in the spring (Undurraga & Stephen 1980, Richards 1984). Second, bees in natural settings may be exposed to low temperature stress during development from spring cold snaps. Survival of *M. rotundata* to low temperatures varies across development, with low-temperature tolerance decreasing as developmental stage advances (Yocum et al. 2010; Rinehart et al. 2011). Furthermore, low-

temperature exposure during the pupal stage results in numerous sub-lethal effects in adult *M. rotundata* (Bennett et al. 2015). Low temperatures during metamorphosis could be harmful, because adult structures are forming. Although effects of some cold treatments have been documented, the overall cold tolerance profile of *M. rotundata* has not been established. To comprehensively assess cold tolerance, we used multiple measures of physiological thresholds and responses to stress across development in post-wintering alfalfa leafcutting bees, *M. rotundata.* We hypothesized that low-temperature exposures during metamorphosis would affect adult thermal thresholds. Furthermore, we hypothesized that cold tolerance varies across the stages of metamorphosis. We predicted that bees more recently finished with overwintering would be more tolerant to cold compared to later stages.

Materials and methods

Animals

Loose brood cells containing pre-pupal *M. rotundata* were purchased from JWM Leafcutter, Inc. (Nampa, ID) in the spring of 2016. Prepupae were kept at 6°C in darkness for approximately 8-9 months during diapause and post diapause quiescence. Individual brood cells were placed individually into 24-well plates (Greiner Bio-one, Monroe, NC) and incubated at 29°C to stimulate metamorphosis.

Survival

To determine how developmental stage affects acute low temperature survival, we exposed developing bees to -5°C or -10°C. Three developmental stages were selected: 1) post-diapause quiescent, 2) red-eye pupal stage, 14 days after initiation of metamorphosis at 29°C (Rinehart et al. 2011)), and 3) emergence-ready (adults that have eclosed but not emerged from the brood cell). Temperature treatments were administered using water baths to maintain

constant temperatures. Only bees still inside their brood cells were used in experiments. Bees were removed from the water baths after 24, 48, 72, 96 hour time points. After being removed from the baths, bees were placed into 24-well plates (Greiner Bio-one, Monroe, NC) and put into an incubator at 29°C to resume development to adults. The well plates were checked daily for emergence, and the sex of emerged all bees and survival of bees that emerged was also recorded.

Supercooling point

To determine how developmental stage affects lower lethal thresholds, we measured supercooling point across metamorphosis. Bees from several developmental stages (post-diapause quiescent, first pupal molt, pink-eye pupa, red-eye pupa and emergence ready) were adhered to iButtons (San Jose, CA, Maxim Integrated) with petroleum jelly. The bees were then placed in a cooling container at 1°C/minute inside a -80°C freezer and removed after 45 minutes. Temperature data were extracted from iButtons using a 1-Wire adapter (San Jose, CA, Maxim Integrated) and analyzed to identify the supercooling point (SCP) defined as the temperature before latent heat release upon freezing.

Emergence

To determine how chill injury during development affects adult bees, we measured the timing of adult emergence after exposure to low-temperature during development. The control group was uninterrupted and maintained at a daily Δ4°C thermoperiod, with an average temperature of 29°C, a cryophase of 27°C, and a thermophase of 31°C in darkness. Treatments were kept at the Δ4°C thermoperiod pre- and post-exposure to low-temperatures (Fig 4.1 temperature treatments). Static thermal regime (STR) treatment was maintained for a week at a static 6°C and then returned to the Δ4°C thermoperiod. We used this treatment to assess the effects of chill injury on emergence because Bennett et al. 2015 showed that this STR treatment

resulted in numerous sub-lethal effects, hypothesized to be a result of chill injury. The fluctuating thermal regime (FTR) group was maintained for at 6°C with one hour at 20°C each day for seven days and then returned to the Δ4°C thermoperiod. The ecological thermal regime (ECO) was designed from climatological data from a spring cold snap occurring in Missoula, Montana (N 46°54′59″ W114°05′26″, NOAA National Climatic Data Center) where *M. rotundata* are used for alfalfa pollination. This spring cold snap occurred in April of 2013, when *M. rotundata* would likely have been actively developing. The high temperature was 6°C and the lowest temperature was -9°C for 4 days.

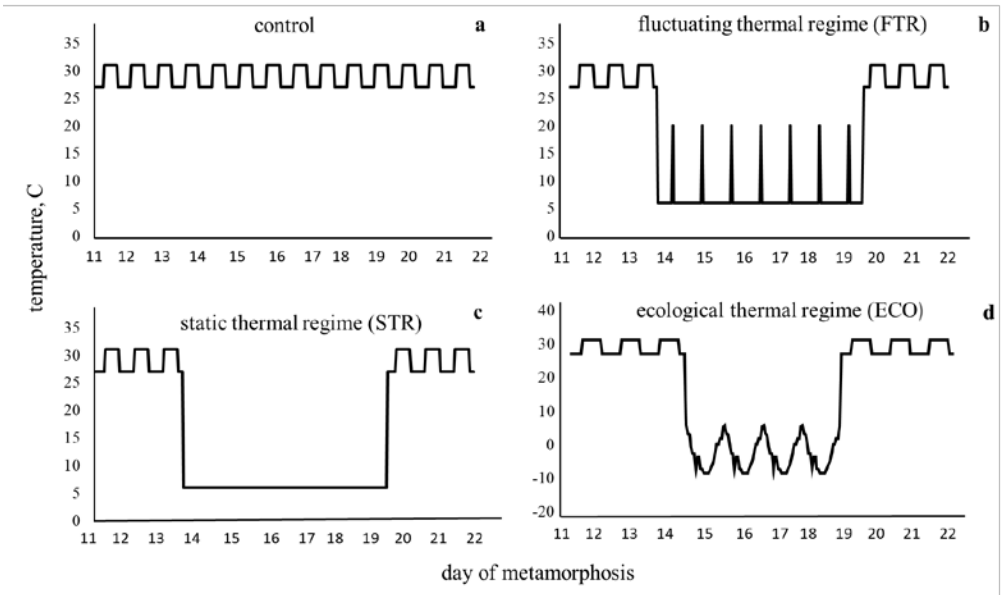

Figure 4.1. (a) The control group was uninterrupted and maintained at a daily 4°C thermoperiod with a mean temperature of 29°C. (b-d) Low temperature treatments for STR, FTR, and ECO. Bees were kept at the daily 4°C thermoperiod pre- and post-exposure to low-temperatures. The x-axis only shows day 11 – 22 to better see the low-temperature exposure treatments starting on day 14.

Developing eye-pigmented pupae were exposed to either the STR, FTR and ECO treatment before adult emergence rhythms were recorded. Inside an environmental chamber (Percival models PCG-105 and I30BLL Scientific, Perry, IA), emergence was monitored using a custom-built, automated, recording device (Yocum et al. 2016). A brood cell containing a

prepupa was placed in a cap-less 0.5 ml microcentrifuge tube (Fisher Scientific Pittsburgh, PA). The microcentrifuge tubes were inserted into plastic racks that we custom 3D printed (Lulzbot, Aleph Objects, CO). On top of each brood cell in the microcentrifuge tube, we placed a 6-mm plastic ball (Softair, Grapevine, TX) and a 4.5-mm steel ball (Crosman, NY). A cover was placed over the loaded tubes with openings big enough to only allow the smaller steel ball to pass, preventing the bee from escaping past the larger plastic ball. When a bee emerges, it pushes the large plastic ball into the steel ball, which rolls free from the tube racks and down a runway. On the runway, the steel ball breaks the signal from a 5-mm infrared emitter and detector pair (Lite-on Electronics, Inc., Milpitas, CA). The signal from the infrared emitter and detector pair was continuously monitored and its break triggered recording of the date and time of bee emergence (hh:mm:ss). The system was controlled by an Arduino Nano board (Sparkfun Electronics, Boulder, CO). Each treatment was designated a different channel. The temperature and humidity were recorded every 60 seconds using a DHT11 sensor (Adafruit, New York, NY).

Adult thermal performance

To determine how chill injury affects adult thermal performance, we exposed developing bees to low temperatures and assessed critical thermal minimum (CTmin) and chill coma recovery after emergence. During the red-eye pupal stage bees were exposed to STR, FTR or control, constant 29°C with no low-temperature exposure. CTmin was measured by recording the temperature at which loss of muscle function occurred. We measured ambient temperature inside of 15 mL conical tubes (Corning, Tweksbury MA) using a custom-built, multichannel, thermocouple microcontroller (Max31855 breakout board, Adafruit, New York, NY). An Arduino Nano (Adafruit, New York, NY) was programmed to read five temperature channels. An adult bee was placed into a 15mL conical tube with a thermocouple, to measure the

temperature, taped to the side. The conical tubes were placed into an incubator that was set to go down to 0°C with a ramp speed 1°C/min. The CTmin was designated as the temperature at which the bees became ataxic and could no longer hold onto a toothpick that was glued to the inside of the conical tube.

To assess how developmental low temperatures affects adult thermal performance, we exposed adult red-eye pupae to STR, FTR, or control conditions and allowed to emerge as adults. Then we exposed adults to a cold shock to induce chill coma, and measured likelihood of recovery from chill coma. The cold shock was administered to emerged adult placed inside conical tube into a water bath at 0°C for 44 hours. Once removed from the bath, they were held at room temperature and checked after 1, 3, and 24 hours for signs of recovery from chill coma. Insects were recovered from chill coma if they exhibited the righting response.

Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics 23 software (Armonk, NYl, IBM Corp.). We used analysis of variance with Tukey's post hoc tests to identify significant difference among supercooling points with development stage as a factor. We used univariate analysis of variance with Tukey's post hoc tests to for analyzing days to emergence, and CTmin, with low-temperature treatment as a factor. Days to emergence was calculated by totaling the number of days at 29°C post diapause. The low-temperature treatment duration was subtracted from the total days. A generalized linear model was used to test for the likelihood of chill coma recovery. To examine how developmental stage affected survival at -5°C, we used a product Kaplan-Meier, limit survival fit with developmental stage as a factor. To examine how time spent at -10°C affected survival, we used a contingency analysis of response by stage. Circular analysis of variance was performed on emergence data to determine if the mean time of

emergence was affected by developmental low-temperature exposure as previously described (Chapter 3). Circular statistical analysis was conducted using circSASv1 SAS macros to calculate all circular statistics (http://statweb.calpoly.edu/ulund). Means ± S.E.M or circular means are presented throughout.

Results

Survival

To determine how stage affects survival of a cold exposure, we exposed bees to -5°C for increasing amounts of time. Survival differed across all three developmental stages, post-diapause quiescence, red-eye pupa and emergence ready (Fig. 4.2, χ^2 (df 2) = 154.2361, p <0.0001). Post-diapause quiescent bees had the highest survival across all timepoints and emergence ready bees had the lowest survival. In addition, some of the surviving red-eye pupa group at -5°C exhibited wing deformities (Table 4.1). Furthermore, survival differed between the -10°C post-diapause quiescent stage and red-eye pupa stage, with none of the red-eye pupa surviving -10°C (χ^2 (df 1) = 451.332, p <0.0001). Because of this and because -5°C resulted in low survival at the emergence ready stage, we did not examine if emergence-ready bee survival of -10°C.

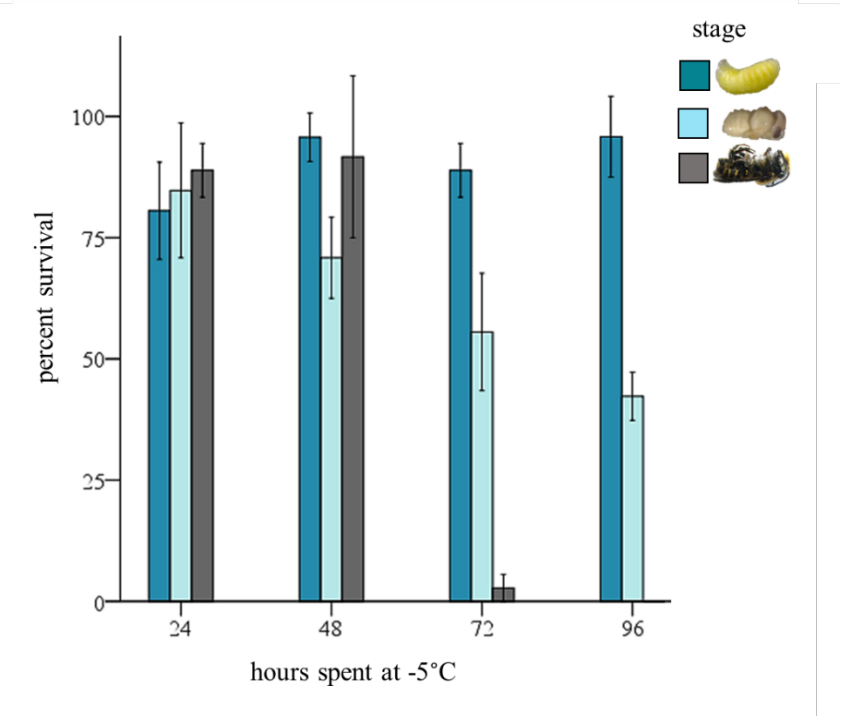

Figure 4.2. Shown is the percent survival versus the amount of time spent at -5°C. The dark blue bar is the post-diapause quiescent prepupa, in light blue is the red-eye pupa and grey is the emergence ready adult.

Table 4.1. Number of bees that survived -5°C at the red-eye pupa stage exhibiting a deformity.

hours	total	survived	deformity
24	72	61	6
48	69	49	7
72	72	40	3
96	72	30	6

Supercooling points

SCP was measured for bees at the first pupal molt (n=11), the pink-eye (n=7), red-eye (n=7), and emergence ready (n=10) stages. The SCP of the post-diapause quiescent stage differed from all other developmental stages (Fig. 4.3; $F_{4,47}$= 17.642, p <0.0001). But the SCP did not significantly differ between pupal stages. The mean SCP of the pupal stages and emergence ready bees was -13.68 ± 0.781°C. The post-diapause quiescent stage had the lowest SCP at -23.66 ± 0.921°C. For all stages where sex could be identified (pupal stages, emergence ready), SCP did not differ between males and females ($F_{1,35}$= 1.796, p=0.227, data not shown). Mass was significantly different for male and female bees ($F_{1,26=}$ 40.026, p <0.001) although, mass did not affect SCP ($F_{1,34}$=1.650, p =0.208, data not shown).

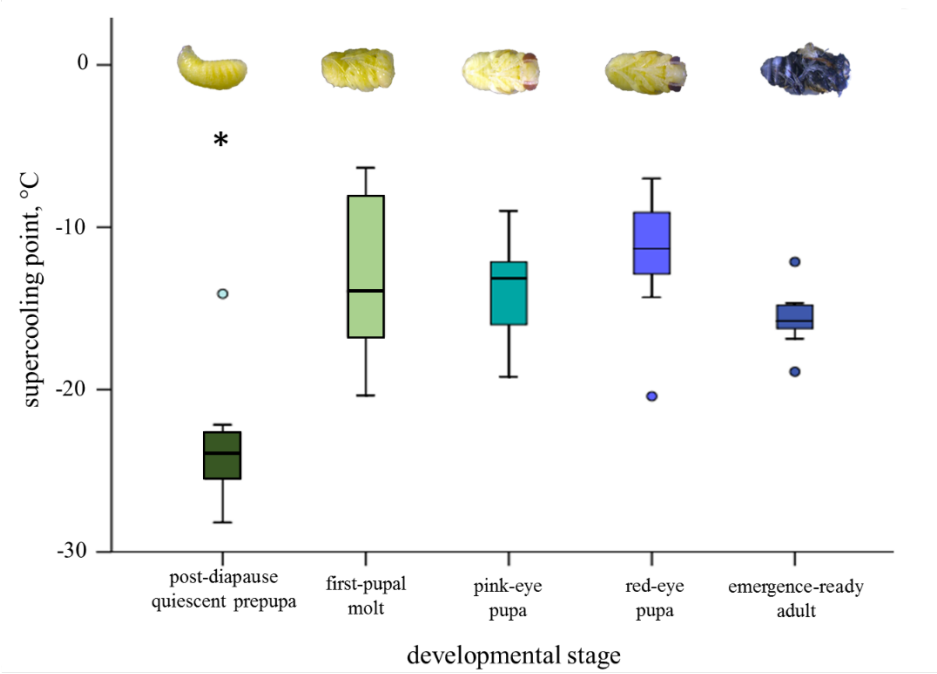

Figure 4.3. The y-axis starts at -30°C, displaying SCP across the stages of metamorphosis. Above each box is an image showing what the developmental stage looks like. Boxes indicate interquartile range, whiskers indicate minimum and maximum values, black bar is median and points show outliers. Asterisk indicates a significant difference of SCP.

Emergence rhythms

Cold stress during metamorphosis did not affect synchronization of adult emergence (Fig. 3.4, $F_{3,557}$= 0.6639, p= 0.536). The number of days to emergence significantly differed between from the control and low-temperature treatments (Fig. 4.5, $F_{3,556}$=124.889, p<0.0001). A Tukey's post hoc test revealed the ecological stress and fluctuating stress did not differ in the number of days to emergence (p=0.960), but all the other cold exposed treatments significantly differed from each other (p <0.0001). The control bees on average emerged the earliest (n= 196, 22.12 ±0.12), while the ecological stress emerged the latest (n=120, 25.97 ±0.16). The fluctuating stress (n= 164, 25.84 ±0.21) treatment took longer to emerge than the static stress treatment (n= 77, 24.39 ±0.21).

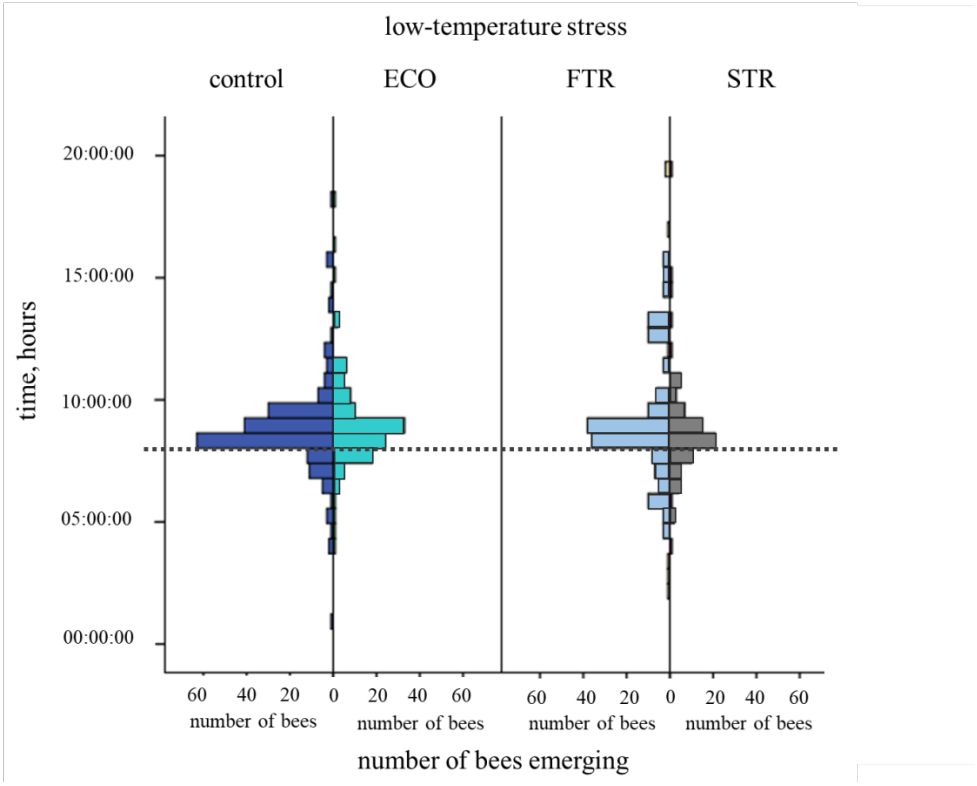

Figure 4.4. Frequency histograms of the number of bees emerging at a given time of day. The dotted line indicates the time of the thermophase ramp (8am).

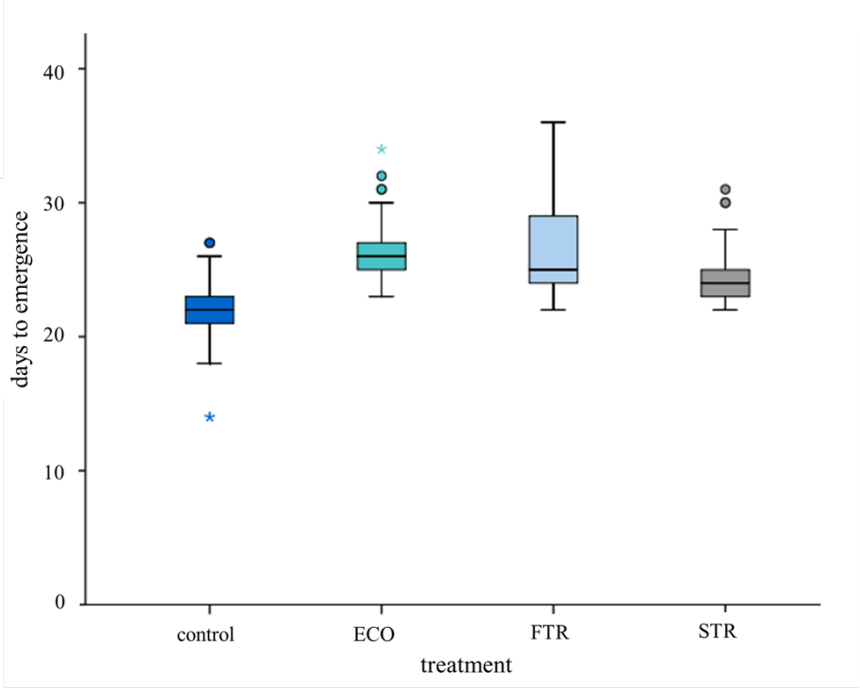

Figure 4.5. Box and whisker plot showing the number of days it took for bees to emerge as adults. Boxes indicate interquartile range, whiskers indicate minimum and maximum values, black bar is median and points show outliers. Asterisk indicates a significant difference between control and low-temperature treatments.

Critical thermal minimum

When we exposed red-eye pupae to STR (n=30), FTR (n=30) and control (n=21) conditions, we found low-temperature exposure during metamorphosis did not affect the critical thermal minimum (CTmin) of adults (Fig. 4.6, $F_{2,75}$=0.068, p =0.936). However, CTmin was significantly different between males and females (Fig. 4.6, $F_{1,18}$=19.637, p =0.045).

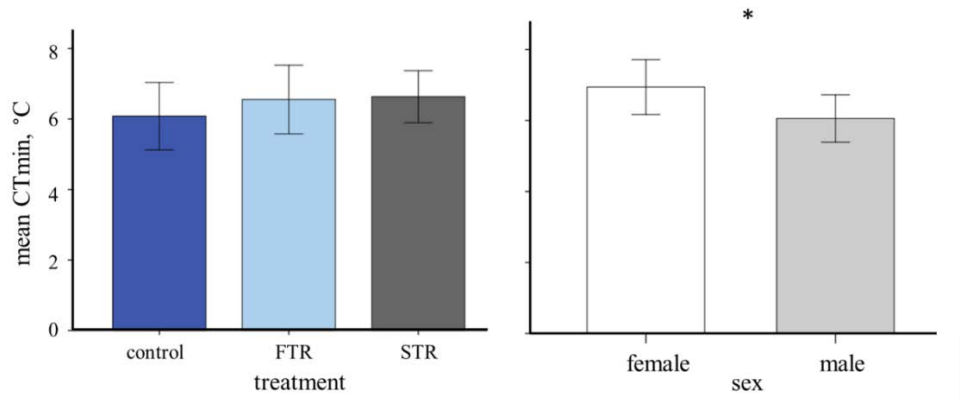

Figure 4.6. The mean CTmin for control bees and low-temperature exposed bees on the left. The control group is the dark blue bar and low-temperature treatments are colored in light blue (FTR) and grey (STR). Male and female CTmin are shown on the right.

Chill coma recovery

Overall, male bees were more likely to die after exposure to chilling (data not shown χ^2(df 2)= 6.019, p <0.049). Furthermore, STR-(n=47) and FTR-treated bees (n=45) were more likely to die post-chilling exposure versus control (n=60) bees (Figure 4.7; χ^2 (df 1)= 5.507, p<0.019).

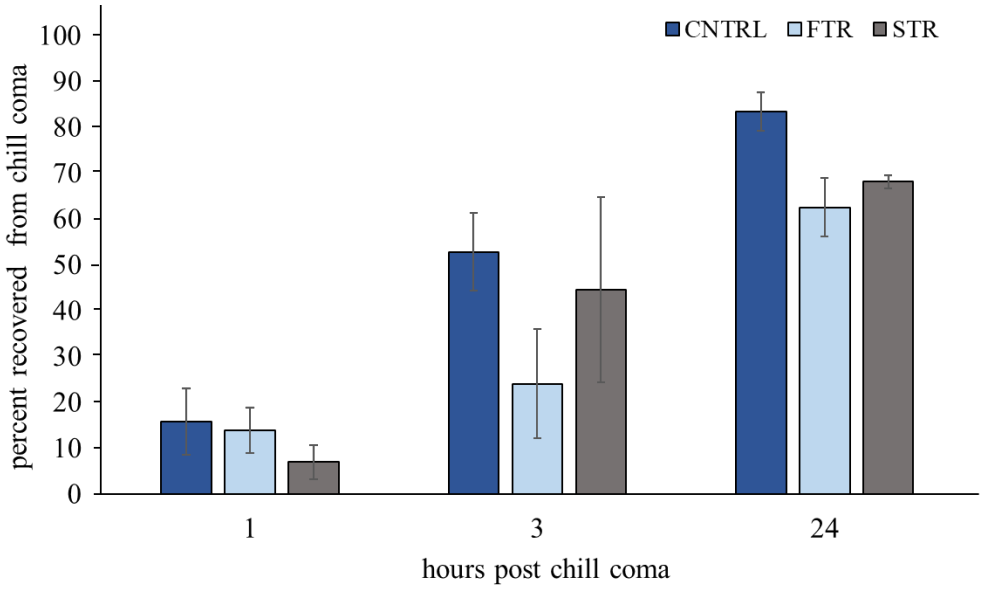

Figure 4.7. Percent of bees that recovered from chill coma versus the number of hours after the exposure to 0°C. Developing red-eye pupae were exposed to FTR (light blue), STR (grey) or control (dark blue). Bees were counted as recovered if they flipped over at 1, 3, or 24 hours.

Discussion

We examined the effects of low-temperature stress on several physiological thresholds and took measures across metamorphosis to comprehensively describe the cold tolerance of *M. rotundata.* Our data indicate that *M. rotundata* is freeze-avoidant, and its cold tolerance varies throughout metamorphosis. Previous studies suggested that cold tolerance declines with age post-wintering (Rinehart et al. 2011; Yocum et al. 2012). Support for this hypothesis was mixed. Our hypothesis that *M. rotundata* recently finished overwintering are more tolerant to cold versus later stages was supported by SCP and survival data. However, when we assessed sub-lethal effects, later stages seemed more cold tolerant. For example, wing defects were observed after exposure to low temperatures in red-eye pupae but not emergence-ready bees. We also showed that low-temperatures during metamorphosis affected adult physiology in some ways but not others. For example, after exposure to chronic low-temperatures during metamorphosis, the likelihood of recovering from a second acute low-temperature exposure as an adult decreased. Collectively, our data indicate that cold tolerance is a complex parameter that varies depending on stage and measurement, further demonstrating the importance of including multiple analyses in examining cold tolerance.

Insects can experience many types of stress during development, which can have differential effects on physiology. Many studies have shown that fluctuating stressors are less detrimental than static stressors (Rinehart et al. 2011; Bennett et al., 2015; Bale et al. 2001; Budejovice et al. 2004; Colinet et al. 2007; Ismail et al. 2013). The number of days to emergence was affected by fluctuating or static temperature exposure in this study. In comparison to acute low temperature exposures, chronic exposures often correlate with the increased severity of the injury (reviewed in Overgaard & MacMillan 2017). Furthermore, exposure to an acute stress can

increase the likelihood for surviving a chronic stress later, a process referred to as thermotolerance (reviewed in Colinet et al. 2015; Overgaard & MacMillan 2017). In our study, we did not see this effect, most likely because the static and fluctuating low temperatures were chronic, week-long stressors. These chronic exposures during development negatively affected recovery from an acute stress as adults. One hypothesis is that both low-temperature exposures caused an accumulation of chill injuries, leading to death. In the field, this result could have serious implications. A severe cold snap during metamorphosis could decrease the likelihood of surviving another bout of cold for wild or even managed populations of *M. rotundata*. Had we not examined the effects of repeated cold exposure on thermal performance and only considered CTmin, the effects of our treatments may not have been fully understood. Examining insect responses to different types of stress and repeated exposures are clearly important to accurately characterize cold tolerance of a species or even a population.

Thermal thresholds are important parameters because they can limit the range of environments an insect can live in. In this study, the ecological treatment was designed using actual climate data from Missoula, Montana during a spring cold snap in April of 2013. We chose this location because *M. rotundata* is used for pollination of alfalfa crops in this region. The temperature during the cold snap simulation dropped to -9°C at the lowest point during the red-pupal stage and significantly affected adult survival. Furthermore, we found that none of red-eye pupae survived exposure to -10°C, possibly because the ecological stress was near their SCP of approximately -13.68±0.781°C. We found that SCP was approximately 10°C lower in post-diapause quiescent prepupae than the pupal and emergence ready stages, which is consistent with findings from another study (Sheffield 2008). When we exposed developing bees to low-temperature exposure above the freezing-point, we found that adult CTmin was unchanged. The

CTmin is useful because if spring and summer temperatures dip below their CTmin, adults cannot fly and pollinate crops. Even though CTmin was unaffected by developmental low-temperature exposure, the likelihood of recovering from adult chill coma was increased. The time it takes to recover from a cold exposure is crucial because when insects are in a chill coma, they cannot move which makes them vulnerable to predation or death. Together, these data suggest that *M. rotundata* has the potential to recover from multiple occurrences of chill-injury.

Our study showed sub-lethal effects of low-temperatures in red-eye pupae exposed to -5°C, with some adults exhibiting wing deformities. These deformities were not seen in post-diapause quiescent or emergence-ready bees. Thus, this result is contrary to our prediction that cold tolerance would be greater during earlier stages. We hypothesize that the deformities may be caused by chill injury during the red-eye stage, because wings are actively developing during metamorphosis. Adult wing deformities can occur when *Drosophila* are exposed to suboptimal temperatures during metamorphosis (Milkman 1962). Previously, we showed that chronic, low-temperature exposure of red-eye pupae resulted in numerous sub-lethal effects in adult bees such as flight defects, reduced adult activity and overall decreased flight performance (Bennett et al. 2015). Emergence ready bees would have already inflated their wings before they were exposed to low-temperatures, explaining why no bees with wing deformities were observed in this group. Furthermore, the post-diapause quiescent group likely did not suffer chill injury, because we predict their wings would most likely have been imaginal disks at the time of the low-temperature treatments. Hence, the timing of low-temperature exposure during development affects the severity or appearance of sub-lethal effects retained in adult bees.

In addition to sub-lethal effects on flight, timing of adult emergence has also been shown to be affected by low-temperature stress during development. For example, cold exposure during

development shifts the timing of emergence to later in the thermophase in flesh flies, *S. crassipalpis* (Yocum et al. 1994). When we exposed metamorphosing pupae to low-temperatures, our treatments affected the days to emergence but not the time of day that they emerged. It is interesting that even after the static treatment, which we know is a severe chronic stress, emergence was still synchronized. This suggests that the circadian system is resilient against low-temperature stress. This is important because it suggests that bees exposed to spring cold snaps may still be able to emerge together, maintaining synchrony in emergence for mating and foraging.

Conclusions

Our study identified critical thermal thresholds and responses to low-temperatures across development of an important pollinator, *M. rotundata*. Characterizing cold tolerance is especially important because of the widespread use across latitudes in pollination of alfalfa (*Medicago sativa* L.) for seed (Pitts-Singer & Cane 2011). Even though some work has examined the effects of low-temperature storage on *M. rotundata*, cold tolerance across metamorphosis has not been well characterized until now. Understanding the thermal physiology of *M. rotundata* is important for designing storage treatments that are less likely to be lethal or sub-lethal to adult bees. Incorporating multiple measures of cold tolerance, including physiological thresholds and various types of low temperature exposures over multiple developmental stages enables a comprehensive assessment of cold tolerance of a species or population. Furthermore, characterizing cold tolerance can aid in making predictions about species ranges as the climate is changing. It would be interesting to know if *M. rotundata* reared at lower latitudes have similar cold tolerance profiles to those used in this study.

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CHAPTER 5: CONCLUSIONS

Environmental cues of spring mediate the timing of life history events for many plants and animals. Precise timing of events through the coordination of physiological and behavioral processes with the environment is critical for maximizing fitness. Because animals occupy diverse habitats, relevant cues for such timing vary across species. Climate change is expected to result in earlier spring warming, increased temperature variability, and more occurrences of cold snaps (Inouye 2008; Rigby & Porporato 2008; Augspurger 2013). These scenarios pose problems for animals that are actively developing. First, they could be receiving misleading signals about the season, which in turn could affect the timing of life history events, and eventually impact the phenological overlap of interacting species. Second, organisms outside of their overwintering stages may be less tolerant to low temperatures, which could cause delays in development or reproduction, injury, or death. Therefore, understanding how these phenomena affect plant and animal species is critical because they rely on environmental cues to coordinate life history events.

In chapter 2, my finding that *M. rotundata* synchronizes and senses light is the first evidence of this response in this species. Light could be a more reliable cue if temperatures are misleading, although I found that thermoperiod is the dominant Zeitgeber and *M.rotundata* is sensitive to small temperature fluctuations. Because of their sensitivity to temperature, increased temperature variability and my data shows that this species is highly susceptible to climate change. This research is an important addition to the body of knowledge of circadian rhythms and cold physiology. This is especially important for bee species, because limited knowledge exists about what cues mediate the timing of adult emergence. Understanding the potential consequences of climate change is crucial, because it is impacting phenologies of both plants and

animals (reviewed in Parmesan and Yohe 2003; Visser & Both 2003; Jamieson et al. 2012). This is an important and complex issue, because mutualistic pollinator-plant interactions could be disrupted. Understanding what cues are relevant for mediating circadian rhythms in insects is crucial to understanding large scale impacts on phenology by climate change.

Animals that develop in light restricted environments may rely on other cues to synchronize their biological clocks during spring. Little is known about what cues mediate the timing of emergence in solitary bees, many of which pupate in light-restricted environments. I discovered *M. rotundata* likely possess a temperature-mediated clock and temperatures are dominant over light cues. These data contribute to the field of chronobiology by testing the hypothesis that organisms in light-restricted environments evolved other means of synchronizing development with their environments. Understanding how spring conditions affect life history of animals is important to making predictions about the effects of climate change. Since an increase in spring temperature variability is predicted, it is crucial to understand how these conditions may affect the timing of emergence. I have shown that *M. rotundata* are sensitive to even slight changes in temperature, which could be problematic if they are relying solely on temperature cues to time emergence. Future research should further examine the underlying mechanisms of clocks to better understand the effect of environment on circadian system.

Metamorphosis is a critical time during development when adult physiology and morphology are established. Insects are far less tolerant to cold outside their overwintering stages. Thus, insects that undergo metamorphosis in the spring are vulnerable to spring cold snaps. Even if insects survive exposure to a low temperature during metamorphosis, the presence of sub-lethal effects from injury can occur. In chapter 3, I examined the presence of sub-lethal effects on adult *M. rotundata* after experiencing low-temperatures during metamorphosis. I

broadly assessed adult performance by assessing sub-lethal effects on physiology, morphology, and behavior. I observed numerous sub-lethal effects on flight performance such as shorter bouts, smaller wings, flight defects and lower metabolic rates (Bennett et al. 2015). Flight is critical to the success of adult *M. rotundata*, because they must fly to access food, mates, and nest materials. Furthermore, I found decreased longevity and activity levels in adult bees. It is clear low temperatures can affect bee fitness, which could impact populations and ecosystems. Measuring multiple aspects of adult physiology, morphology, and behavior is necessary for understanding how thermal stress affects bees.

Knowledge about the consequences of developmental low-temperature exposure in *M. rotundata* is also relevant for their use in agriculture. If weather events delay flower bloom, low-temperature storage is used by farmers to interrupt metamorphosis to better synchronize emergence with peak crop bloom (Undurraga & Stephen 1980, Richards 1984, Yocum et al. 2010). I examined the same temperature regimes used as a standard management practice, and identified numerous sub-lethal effects on adult flight performance. Pollination of crops requires flight and is not possible if flight is compromised. Because I used the same management practices, this knowledge can be easily translated so farmers understand the consequences of interrupted storage at these temperatures.

Understanding the consequences of developmental low temperature is also important for making predictions about the effects of climate change on wild bee species. Most wild bee species are solitary, and many share a similar life history to *M. rotundata*, making them susceptible to the same types of stress. This knowledge can be used to make predictions about responses of other solitary bee species to spring cold snaps. There is little knowledge about the ecological consequences of spring cold snaps on bees, and the climate envelops are lacking for

the majority of bees (Brown & Paxton 2009). Climate envelopes are derived from modeling existing species distributions and environmental variables to define a species' tolerance to make predictions about how a species range has or will change. This is crucial for understanding the impacts of climate change on bee species. Thus, there is a need for more research examining the responses of solitary bees to low-temperature exposure.

Developing insects are susceptible to cold snaps, the frequency of which is expected to increase with climate change. Therefore, the developmental stage at which an insect experiences a spring cold snap could make a difference between life and death. Cold tolerance is difficult to measure, because it requires a body of evidence that allow us to make inferences about an insect's overall tolerance to cold. One way to examine cold tolerance is by measuring thermal thresholds such as CTmin or SCP. In chapter 4, I characterized thermal thresholds and the effects of stress on adult bees. Together these data are a robust assessment of cold tolerance. Knowing what the thresholds are can aid in making predictions about what types of environments or ranges where an insect can live. A study examining SCP and CTmin across species of *Drosophila* found these two measures were the best predictors of cold distribution limits (Andersen et al. 2015). Thus, these measures combined with actual responses to low-temperature are important to characterize overall cold tolerance of a species. We must continue to examine cold physiology in pollinators to better understand the repercussions of spring cold snaps.

Characterizing cold tolerance is especially important for *M. rotundata* because of their widespread use in pollination of alfalfa (*Medicago sativa* L.) for seed (Pitts-Singer & Cane 2011). We found cold tolerance varies across development, and depending on the timing of low-temperature exposure, it can result in injury or death. Farmers can use this information to make decisions about the timing of low temperature storage. In addition, knowledge of low

temperature thresholds can be useful for farmers if a spring cold snap is predicted to drop below the SCP of *M. rotundata*. For example, farmers could move pre-emergent bees indoors if they had already been placed in field domiciles. Furthermore, knowledge of cold tolerance is critical for designing low-temperature storage treatments that are less likely to be lethal and sub-lethal to adult bees. Examining the presence of sub-lethal effects are not currently considered in management decisions. Because *M. rotundata* is the world's most intensively managed solitary bee species, this knowledge will undoubtedly improve the health of agriculturally managed bees and could be applied to other bee species.

Wild bee species are in decline and climate change is expected to increase the rate of decline (Memmott et al. 2007; Brown & Paxton 2009; Forrest 2015). This study is an important contribution to the field of cold physiology, because studies examining the cold tolerance of bee species are lacking. It is crucial that we begin to understand the cold tolerance of bee species because this knowledge can aid in making predictions about population responses to climate change. Because *M. rotundata* exist across many latitudes, their thermal physiology may be plastic, thus future studies should examine the plasticity of the thermal performance in *M. rotundata*. Future conservation efforts of solitary bees can also benefit from this research because artificial rearing of bees may be an option for declining populations. Characterizing thermal physiology is necessary to make predictions about responses of wild bees to climate change and maintaining healthy pollinators under artificial settings.

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