SHORT- AND LONG-TERM EFFECTS OF ENVIRONMENTAL VARIABILITY ON THE ECOPHYSIOLOGY OF A SOLITARY BEE

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

By

Kayla Nicole Earls

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

Major Department:
Biological Sciences

March 2022

Fargo, North Dakota

North Dakota State University
Graduate School

Title

SHORT- AND LONG-TERM EFFECTS OF ENVIRONMENTAL VARIABILITY ON THE ECOPHYSIOLOGY OF A SOLITARY BEE

By

Kayla Nicole Earls

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

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Dr. Kendra Greenlee
Chair

Dr. Joseph Rinehart

Dr. Ned Dochtermann

Dr. Deirdre Prischmann-Voldseth

Approved:

03/28/2022	Dr. Kendra Greenlee
Date	Department Chair

ABSTRACT

Environments can vary across seasons. One characteristic of spring in temperate regions is fluctuations in temperature. Sudden bouts of low temperature can have lasting effects on ectotherms that rely on ambient temperature. For developing insects, being unable to avoid or manage these low temperatures puts them further at risk. For example, *Megachile rotundata* undergo active development in the spring. Exposure to low temperatures as pupae for short periods does not decrease survival but does affect adult morphology. Pollination services may also be affected if temperatures are too low to promote flight in adults. The overall question is what are the short- and long-term physiological effects of temperature in pupae and adults? The first objective of this dissertation is to investigate the long-term effects of low temperature stress by measuring flight performance, reproductive output, and offspring characteristics. After receiving a constant low temperature for a week, females were less likely to nest and changed their offspring investment. Additionally, offspring of bees exposed to fluctuating low temperature stress were more likely to enter diapause despite being early in the season. The second objective was to test if oxygen consumption across temperatures generated a thermal performance curve as a short-term effect in *M. rotundata* pupae. Results indicate that oxygen consumption scales non-linearly like a thermal performance curve; however, the negative slope at high temperatures was not observed. The third objective was to determine how microclimate conditions affect flight initiation in *M. rotundata* adults. Two different activity boxes were designed to measure environmental variables and to manipulate cavity temperature. The first activity box measured several environmental variables and showed that even at very close proximities, *M. rotundata* experience different temperature conditions. In the other activity box design, internal cavity temperature was manipulated to increase early in the morning. Results show that increasing the cavity temperature promoted earlier flight and at lower ambient temperatures. This dissertation shows that the life stage an insect experiences a thermal stress can affect short- and long-term effects on physiology and life history in *M. rotundata*.

ACKNOWLEDGEMENTS

This research would not have been possible without an amazing support system. My advisor, Kendra Greenlee, not only took a chance on me, but guided and encouraged me throughout all the strenuous processes. Joe Rinehart is another mentor that has been a founding fixture in my success and perseverance. I would also like to thank Jacob Campbell for helping with respirometry experiments. Nyle Jonason was essential for the field experiments, especially organizing and constructing the field tents. I would like to thank George Yocum and Julia Bowsher for all the exciting conversations about statistics and R. There have been many people that have come and gone throughout the insect group, Greenlee and Bowsher labs that have provided encouragement and constructive feedback. I had the pleasure of mentoring nine undergraduate students that were influential in data collection and my career. Those undergraduates were Jessica Kohntopp, Monique Porter, Jennifer Boncomo, Annika Mogck, Alicia Fischer, Carl Hayes, Twyla Gross, Abbie Dockey, and Julie Cruz. These students made research worthwhile and I wish them all the success wherever their path takes them.

DEDICATION

As a first-generation college student, I have to firstly dedicate this dissertation to my family (Earls, Goodnow) and stepfamily (Green). It is also dedicated to my past and future students. I have learned the most from their experiences and lives when developing my teaching and mentoring style and how to generally be an empathic person. Additionally, this dissertation is dedicated to all my friends that have been extremely supportive throughout the process. The Whine and Wine crew (Amy Hughes, Jenna D'Angelo, Katherine Nussbaumer, and Emily Wood) have been supporting me since fourth grade and every Friday night for the past two years. Through a turn of random events, I found myself with another amazing friend group at the start of the pandemic. The Quaranteam (Caitlin Anderson, Brian Anderson, Brian Springall, Kat Dragos, Pete Lindmark, and Wil Falkner) made surviving through the pandemic and final stages of the dissertation process bearable. Between these two friend groups, I have learned to embrace myself, the world, and the chaos we all need. They are two groups that I look forward to future laughs and adventures with, including board games, movies, video games, and tabletop roleplaying games. Lastly, this dedicated to all the important people that are no longer alive to see me finish this degree: Guy Earls III (father), Greg Goodnow (uncle), Jane Dowdell (aunt), Betty Greenier (grandmother), and Daniel Emerson (friend).

"I think this is where we are supposed to be, and this is where we're supposed to do our part for our world. […] I'm proud to die with you and I will be even prouder to survive with all of you."

-Caduceus Clay (Taliesin Jaffe), *Critical Role*, Campaign 2 Episode 139

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Environmental Variability

Local and global environments naturally vary from day to day and across years. To survive this environmental variation, including changes in temperature, organisms have evolved a variety of mechanisms, such as phenotypic plasticity (Bowler and Terblanche, 2008; Mallard et al., 2020; Schlichting, 1986). However, as the climate becomes more variable with increased likelihood of extreme weather events, the efficacy of those mechanisms is being tested (Harvey et al. 2020). In temperate regions, cold snaps or freeze-thaw events pose sudden bouts of low temperature that can be detrimental (Kellomäki et al., 1995; Roitberg and Mangel, 2016; Sambaraju et al., 2012; Stroud et al., 2020). Ectotherms in particular are sensitive to large shifts in weather, especially temperature (Deutsch et al., 2008; Kingsolver et al., 2011). Temperature is an essential cue for timing of life history events and a critical factor affecting physiological processes, including metabolism and development.

Insects are ectotherms that rely on ambient temperature to drive biological processes. Additionally, they are essential components of both food chains and agriculture that benefit humans (Redhead et al., 2020; Schoenly et al., 1991). Insects are highly diversified; therefore, their response to climate change can be variable among species and orders (Deutsch et al., 2008). A species' life history can affect how an organism can tolerate unfavorable or lethal conditions (Hutchinson and Bale, 1994; Kingsolver et al., 2011). The life stage of an insect (e.g., larval or adult) when it experiences unfavorable environmental temperatures can be protective or increase vulnerability to long-term effects. For instance, diapausing insects may be more protected from extreme low temperature events because of the evolutionary physiological adaptations, such as production of cryoprotectants (Bale and Hayward, 2010; Hahn and Denlinger, 2007; Hahn and

Denlinger 2011). In contrast, actively developing insects may not have those protections, making them susceptible to chill injury. There are many factors to consider when looking at the effects of temperature stress, including insect life stage and the intensity, duration, and frequency of the stressor (Hutchinson and Bale, 1994; Kingsolver et al., 2011; Marshall and Sinclair, 2015).

Small-scale changes in temperature (i.e., microclimate) may be more accurate in understanding how insects respond to variations in temperature. Microhabitats may help protect insects from unfavorable macroclimate (weather) conditions, such as low ambient temperature. Substrates can either amplify or buffer the macroclimate, creating a mosaic of thermal profiles (Woods et al., 2015). Temperature can vary across the landscape from soil to trees and even from one side of a leaf to another (Gols et al., 2021; Pincebourde et al., 2020; Potter et al., 2009; Rebaudo et al., 2016). For example, the underside of Jimsonweed (*Datura wrightii*) leaves provides a refuge for developing *Manduca sexta* eggs when temperatures are high, with smaller leaves providing areas of lower temperature (Potter et al. 2009). Flowers can even create a thermal refuge for flying pollinators in unfavorable low temperature conditions (Dyer, et al., 2006; Herrera, 1995a,b). Inside the early-blooming daffodil (*Narcissus longispathus*), the plant reproductive parts early in the season can be as much as 2°C warmer than air temperature (Herrera, 1995). Nesting preferences can also be influenced by microclimate. For instance, the alfalfa leafcutting bee (*Megachile rotundata*), when presented with nesting cavities in four cardinal directions, prefers to nest in cavities facing northern directions that experience lower temperature averages (Wilson et al., 2020). Microhabitat choices may have serious physiological implications. For example, metabolic rates in small carpenter bees (*Ceratina calcarata*) scaled with temperature but were higher in bees that nested in direct sunlight than the shade, suggesting that they may need to forage more to supply metabolic substrates (Richards et al., 2020). Being able to measure temperature conditions inside and outside nests would provide a clearer picture of how changes in microclimate affect cavity-nesting insects.

Insect responses to changes in temperature are often plotted against temperature, referred to as a thermal performance curve. The generated graph follows Jensen's inequality, which describes the relationship between performance and temperature as asymmetric and non-linear, with a gradual increase at lower temperatures and a steeper decline at high temperatures (Deutsch et al., 2008, Jensen, 1906; Martin and Huey, 2008, Sinclair et al., 2012). The thermal performance curve can be divided into four regions: lethal, suboptimal, optimal, and supraoptimal (Martin and Huey, 2008). Dependent variables that have been used to measure insect performance with changes in temperature include metabolic rate, locomotion, growth rate, development rate, and reproductive fitness (Blanckenhorn et al. 2021; Martin and Huey, 2008; Shah et al., 2021). Metabolic rate, as measured by oxygen consumption, has been used to show how latitude and elevation affect thermal performance curves of mayflies (Baetidae; Shah et al., 2021). Because the thermal response curves vary depending on the trait measured and the time scale used, measuring various performance traits across temperatures will aid in the understanding of how insects respond to variable environments (Kellermann et al., 2019).

Life History of Insects

Complete metamorphosis is one of the main life history and physiological differences between holometabolous and hemimetabolous insects (Klowden, 2013; Nation, 2015). During complete metamorphosis, insects undergo major morphological and physiological changes from the larval form. Also, during this complex process insects may be vulnerable to changes in the environment. Insects undergoing metamorphosis may be immobile and, therefore, unable to seek a more favorable microclimate, putting them at risk for damage, including chill injury (Bennett et al., 2015; Kingsolver et al., 2011).

One life history trajectory that has evolved to avoid the negative effects of low temperatures during the winter is diapause. Diapause is a state of arrested development, characterized by having a decreased metabolic rate (Denlinger, 2002; Denlinger, 2008). Insects that undergo diapause during the winter may also produce cryoprotectants to prevent chill injury (Bale and Hayward, 2010; Hahn and Denlinger, 2007; Hahn and Denlinger 2011). The life stage and season in which insects undergo diapause differs among species (Denlinger, 2009). For facultative diapausing insects, those that are not required to go through diapause to proceed to the adult stage, individuals may enter diapause depending on maternal effects when the egg was laid and/or by changes in environmental conditions the individual experiences, such as photoperiod or thermoperiod (Coleman et al., 2014; Denlinger, 2002; Mousseau and Dingle, 1991; Saunders, 1966; Simmonds, 1948; Tougeron et al., 2020; Vaghina et al., 2007). Those individuals are not required to experience diapause to continue to adult development (Hobbs and Richards, 1976; Rank and Rank, 1989). In temperate regions, insects that develop earlier in the season may bypass diapause and continue direct development to the adult stage, while those developing later in the season will enter diapause and resume active development the following spring (Mousseau and Dingle, 1991; Wilson et al., 2021). If individuals were to enter or not enter diapause at the incorrect time, they could be exposed to stressful conditions for which they are ill-prepared (CaraDonna et al., 2018; Tougeron et al., 2020; Tuljapurkar and Istock, 1993). The consequences of phenological mismatch have been understudied in regard to diapause. Future studies should investigate the physiological and ecological consequences of entering diapause at the wrong time.

In addition to diapause, insects can respond to unfavorable environmental conditions with behavioral and physiological changes. On an individual scale, insects can physiologically increase their body temperature in low temperature environments by activating their flight muscles prior to flight (Kingsolver et al., 2011; Heinrich, 1975; Heinrich and Esch, 1994; Heinrich and Vogt, 1993), basking in the sun (Heinrich and Pantle, 1975), or using flowers as a heat source (Dyer, et al., 2006; Herrera, 1995a,b). Intermittent warming through shivering has also been described in a few insect orders, including Hymenoptera, Diptera, Odonata, and Lepidoptera, to increase body temperature (Ducatez and Baguette, 2016; Heinrich, 1974; Heinrich and Pantle, 1975; Stoks et al., 1996). To protect developing brood from experiencing thermal stress, many social insects thermoregulate their nests. For example, honey bees, thermoregulate hive temperatures by aggregating together in low temperatures or fanning their wings to perform heat shielding to protect brood in sub- or supra-optimal temperatures (Bonoan et al., 2014; Cook et al., 2016; Eskov and Toboev, 2011; Jones and Oldroyd, 2007; Westhus et al., 2013). Similarly, acorn-nesting ants (*Temnothorax curvispinosus*) will move their brood inside the acorn to locations with more favorable temperatures (Karlik et al., 2016). Insects have evolved various methods to deal with unfavorable temperature conditions; however, unexpected shifts in temperature can put immobile insects at risk.

Temperature Stress

Stressors at low levels may trigger a hormetic response, in which the stress improves survival of a second exposure to the same or different stressor. However, increasing severity of a stressor is often detrimental, resulting in both short- and long-term effects (Mattson, 2008). Insect quality post-stress exposure is often measured by whether the individual survived or not. However, even if an insect survives exposure to a stressor, they may have sub-lethal effects

(Earls et al., 2021). Exposure to low temperature stress can slow development rate, lower metabolic rate, decrease fecundity, and contribute to morphological deformities (Colinet et al., 2018; Earls et al., 2021; Hedgekar, 1971; Huang et al., 2007; Hutchinson and Bale, 1994; Kelty et al., 1996; Laloutte et al., 2011; Marshall and Sinclair, 2009; Rinehart et al., 2000; Turnock et al., 1983). Physiologically, individuals may not be able fly and exhibit muscle and neurological degradation (Hedgekar, 1971; Kelty et al., 1996). Ecologically, limited mobility or inability to fly for a volant species results in little to no dispersal, ability to pollinate or forage, and/or find mates (Bennett et al., 2015; Earls et al., 2021). Therefore, survival does not adequately measure insect quality, because many aspects of an individual's physiology and ecology may be negatively affected.

Long-term effects, those affecting future generations, may also be induced by stressors, such as pathological outcomes or through trade-offs. Trade-offs are defined as investment in one trait at the cost of another. A parent that experiences stress may alter offspring investment in response. In terms of offspring investment, a trade-off between number and size of offspring resulting in parents either having fewer, larger offspring or having many, smaller offspring (Stearns, 1989). Larger offspring may have received more parental investment and, therefore, be more likely to survive. Additionally, individuals may invest more in their own survival over future reproduction (Stearns, 1989). For example, adult fruit flies (*Drosophila melanogaster*) that received multiple low temperature stressors invested more in survival than in offspring production by reallocating resources (Marshall and Sinclair, 2009). Similarly, when exposed to repeated freezing, goldenrod gall flies (*Eurosta solidaginis*) did not have reduced survival but had fewer offspring and increased investment in cryoprotectants (Marshall and Sinclair, 2018).

Parental condition, such as decreased quality due to stress or age, could also impact whether a trade-off occurs.

Individuals experiencing stress due to aging, infection, or physiological impairment increase their investment into reproduction to better prepare offspring for future stressors, as explained by the terminal investment hypothesis (Clutton-Brock, 1984). This hypothesis has been supported in taxa across the animal kingdom, including mammals, reptiles, amphibians, and insects. For example, older burying beetles (*Nicroplorus orbicollis*) allocated more resources for their offspring instead of themselves allowing older beetles to have larger broods than the younger beetles (Creighton et al., 2009). In another burying beetle species (*Nicrophorus vespilloides*), a similar response with increased offspring investment was observed when females were imposed with different types of stress, including additional weight, an activated immune system, or a delay in the age when a female first mates (Cotter et al., 2011; Ratz et al., 2020; Ratz and Smiseth, 2018). Male giant water bugs (*Belostoma flumineum*) provide parental care by carrying eggs on their backs until they hatch. Early in the season, they may discard the egg load to seek other mating attempts. When faced with lower temperatures mimicking the end of the mating season, males that had a smaller egg load were less likely to discard their eggs compared to males manipulated to be encumbered with eggs based on back size (Kight et al., 2000). Changes in investment may be due to parental stress and/or interpreting their environment differently in individuals providing care.

Study System: *Megachile rotundata*

Megachile rotundata is a solitary bee that nests gregariously. Adult females create their own nests inside natural or artificial cavities (Pitts-Singer and Cane, 2011). Nests are comprised of individual brood cells created by cut leaf pieces that are glued together with nectar and saliva

(Trostle and Torchio, 1994). Inside the brood cell, a female will place a provision consisting of pollen and nectar and then lay an egg on top (Cane et al., 2011; Klostermeyer et al., 1973; Trostle and Torchio, 1994). The amount of provision is determined by the female and depends on available resources (Peterson and Roitberg, 2006a,b). Provision quantity and quality determine adult body size, because this is the only food resource offspring have until they emerge as adults (Trostle and Torchio, 1994). Larvae feed on the provisions during the larval stages until they become prepupae, at which time they will either continue direct development or undergo diapause (Tepedino and Parker, 1986). Depending on latitude, offspring oviposited early in the season will continue direct development, while offspring oviposited later in the season will enter diapause (Hobbs and Richards, 1976; Krunic, 1972; Tasei and Masure, 1978).

In spring, *M. rotundata* resume development. During this time, they may be exposed to large temperature fluctuations. *Megachile rotundata* are holometabolous and immobile during the pupal stage before completing melanization and the final molt. Once bees finish development to the adult stage, individuals chew their way out of the brood cell. If bees emerge before the sibling in front of them, they may choose to circumnavigate the other bee or commit fratricide (Tepedino and Frohlich, 1984). Typically, males emerge before females as they are oviposited last, in the front of the nesting cavities (Tepedino and Frohlich, 1984; Figure 1).

Figure 1. Nest structure created by female *M. rotundata*. The first offspring are oviposited at the back of the cavity and are predominately female. Males are oviposited closer to the front or entrance of the cavity.

Megachile rotundata are also used for agricultural pollination, mainly of alfalfa (Pitts-Singer and Cane, 2011). The majority of *M. rotundata* are produced in Canada and shipped throughout the United States due to low seasonal and annual return rates (Pitts-Singer and James, 2005; Pitts-Singer and James, 2008; Pitts-Singer, 2008). Artificial nest boxes made of Styrofoam with cylindrical cavities are provided to bees in the field (Pitts-Singer and James, 2005). In addition to naturally occurring temperatures, *Megachile rotundata* experience low temperature stress through some bee management practices. For instance, developing bees can be placed at low temperatures to slow development with the goal of timing adult emergence with peak alfalfa bloom (Rinehart et al., 2011; Pitts-Singer and Cane, 2011; Yocum et al., 2010). At short durations (< 2 weeks), exposure to constant low temperature does not affect survival, but exposures lasting longer than two weeks result in increased mortality (Rinehart et al., 2011). The cause of mortality is unknown, but one explanation is that bees use up energy reserves during the temperature stress or during the re-warming. Even though survival does not decrease with short exposures, reproduction and offspring characteristics are negatively affected (Earls et al., 2021). The specific temperatures used by farmers vary (Pitts-Singer and Cane, 2011); therefore, measuring the effects of a wide range of temperatures is important for understanding the larger impact of the practice of using low temperature exposure to delay development. Additionally, natural environmental conditions, such as low ambient temperatures early in the season, restrict the use of adult *M. rotundata* for pollination of lowbush blueberry (Javorek, 1996; Javorek et al., 2002). Lowbush blueberry blooms one month earlier than alfalfa, when ambient temperatures may dip below 0°C, making *M. rotundata* susceptible to chill injury and decreasing flight propensity (Sheffield, 2008). Measuring changes in metabolic rate could provide insight on how developing *M. rotundata* respond to their environment and how changes in energy use may contribute to detrimental effects.

Megachile rotundata is the ideal system in which to study the effects of temperature on ecophysiology and life history. For instance, many studies using *M. rotundata* focused on the short-term effects of temperature stress, including measuring development rate and flight performance in adults; however, other qualitative measurements have yet to be conducted to determine long-term effects, such those affecting subsequent generations. In this dissertation, short-term effects of temperature were measured through changes in metabolic rate and flight behavior, while long-term effects included changes in reproduction and offspring investment. Because females individually construct nests and provide resources for their offspring, changes in bee quality due to temperature stress are easily measurable. Additionally, with more sensitive equipment, measuring oxygen consumption in vulnerable development stages helps provide insight on how metabolism changes in response, which may be indicative of a stress response. Gregarious nesting opens up the opportunity to study individual females working independently in one location sharing a similar microhabitat.

Objectives

Objective 1: Determine the effects of low temperature stress during pupal development on reproductive fitness and offspring characteristics

Previous research has shown that *Megachile rotundata* pupae are vulnerable to sudden changes in temperature. The purpose of this Chapter was to determine the long- and short-term effects of low temperature exposure during the pupal stage. Developing *Megachile rotundata* pupae were exposed to either a constant or fluctuating low temperature stress for one week to mimic a spring cold snap. Controls were allowed to continue developing at a constant 29°C rearing temperature. Males and individually marked females were released in field cages to mate, and I measured individual reproductive output, including number of nests, total number of brood cells, and likelihood of nesting. Offspring characteristics, including percent viability, diapause incidence, dry weight, and sex ratios, were measured to determine long-term effects of low temperature stress. Based on previous laboratory studies, I predicted that females exposed to either of the low temperature treatments would have lower reproductive fitness than controls with constant exposure having the lowest. Also, I predicted that offspring characteristics, such as viability and dry weight, would be lower than those in control bees due to changes in parental investment.

Objective 2: Determine how oxygen consumption changes in response to changing temperatures in developing *Megachile rotundata* pupae

For ectotherms, such as *Megachile rotundata*, body temperature is heavily reliant on ambient temperature. Changes in ambient and thus body temperature influence an insect's metabolic rate. The purpose of this Chapter was to determine how the rate of oxygen consumption changes in response to a wide range of temperatures at the pupal stage. Oxygen consumption was measured using a closed-respirometry system with an optical oxygen sensor at 13 temperatures ranging from 6-48°C, temperatures that developing bees may experience in the field. I predicted that the response slope would be non-linear and asymmetric as defined by a thermal performance curve and Jensen's Inequality. I predicted that at lower temperatures, the slope would be more gradual compared to the higher temperatures, and there should be a dramatic drop in oxygen consumption at temperatures higher than 40°C.

Objective 3: Determine the effects of microclimate variables on flight initiation in *Megachile rotundata*

Environmental conditions at the adult stage impact life history traits and behaviors. Unfavorable conditions limit flight bouts for mating, foraging, and offspring provisioning. Conditions that an insect experiences vary greatly depending on location and daily weather fluctuations. The purpose of this Chapter was to determine how various microclimate variables influence flight initiation in *Megachile rotundata*. I predicted that when comparing conditions early and late in the season, abiotic environmental conditions will be significantly different at first flight. The second objective was to determine if manipulating nest box temperature alters flight initiation. I predicted that increasing the nest box temperature would promote flight when ambient temperatures are lower by providing a more favorable microclimate.

References

Bale, J. S., Hayward, S. A. L. (2010) Insect overwintering in changing climate. *J. Exp. Biol.* **213**, 980–994.

Bennett, M. M., Cook, K. M., Rinehart J. P., Yocum, G. D., Kemp, W.P. and Greenlee, K. J. (2015) Exposure to suboptimal temperatures during metamorphosis reveals a critical developmental window in the solitary bee, *Megachile rotundata*. *Physiol. Biochem. Zool.* **88**, 508–520.

Bonoan, R. E., Goldman, R. R., Wong, P. Y., Starks, P. T. (2014) Vasculature of the hive: heat dissipation in honey bee (*Apis mellifera*) hive. *Naturwissenschaften* **101**, 459-465.

Bowler, K., Terblanche, J. S. (2008) Insect thermal tolerance: what is the role of ontogeny, ageing and senescence? *Biol. Rev.* **83**, 339-355.

Cane, J. H., Gardner, D., Harrison, P. (2011) Nectar and pollen sugars constituting larval provisions of the alfalfa leaf-cutting bee (*Megachile rotundata*) (Hymenoptera: Apiformes: Megachilidae). *Apidologie* **42**, 401-408.

CaraDonna, P. J., Cunningham, J. L., Iler, A. M. (2018) Experimental warming in the field delays phenology and reduces body mass, fat content, and survival: implications for the persistence of a pollinator under climate change. *Funct. Ecol.* **32**, 2345–2356.

Clutton-Brock, T. H. (1984) Reproductive effort and terminal investment in iteroparous animals. *Am. Nat.* **123**, 212-229.

Coleman, P. C., Bale, J. S., Hayward, S. A. L. (2014) Cross-generation plasticity in cold hardiness is associated with diapause, but not the non-diapause development pathway, in the blow fly *Calliphora vicina*. *J. Exp. Biol.* **217**, 1454–1461.

Cook, C. N, Kaspar, R. E., Flaxman, S. M., Breed, M. D. (2016) Rapid changing environment modulates the thermoregulatory fanning response in honeybee groups. *Anim. Behav.* **115**, 237-243.

Cotter, S. C., Ward, R. J. S., Kilner, R. M. (2010) Age-specific reproductive investment in female burying beetles: independent effects of state and risk of death. *Funct. Ecol.* **25**, 652–660.

Creighton, J. C., Heflin, N. D., Belk, M. C. (2009) Cost of reproduction, resource quality, and terminal investment in a burying beetle. *Am. Nat.* **174**, 673-684.

Denlinger, D. L. (2002) Regulation of diapause. *Annu. Rev. Entomol.* **47**, 93-122.

Denlinger, D. L. (2008) Why study diapause? *Entomol. Research* **38**, 1-9.

Denlinger, D. L. (2009) Diapause. In *Encyclopedia of Insects* (pp. 267-271). 2nd edition. Academic Press

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C., Martin, P. R. (2008) Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci.* **105**(18), 6668-6672.

Dyer, A. G, Whitney, H. M., Arnold, S. E. J., Glover, B. J., Chittka, L. (2006) Bees associate warmth with floral colour. *Nature* **442**, 525-525

Ducatez, S., Baguette, M. (2016) Inter-individual variation in shivering behaviour in the migratory painted lady *Vanessa cardui*. *Ecol. Entomol.* **41**(2), 131-137.

Earls, K. E., Porter, M. S., Rinehart, J. P., Greenlee, K. J. (2021) Thermal history of alfalfa leafcutting bees affects nesting and diapause incidence. *J. Exp. Biol.* **224**(22), jeb243242.

Eskov, E. K., Toboev, V. A. (2011) Seasonal dynamics of thermal processes in aggregations of wintering honey bees (*Apis mellifera*, Hymenoptera, Apidae). *Entomol Review* **91**, 354-359.

Gols, R., Ojeda-Prieto, L. M., Li, K., van der Putten, W. H., Harvey, J. A. (2021) Within-patch and edge microclimates vary over a growing season and are amplified during a heat wave: Consequences for ectothermic insects. *J. Therm. Biol.* **99**, 103006.

Hahn, D. A., Denlinger, D. L. (2007) Meeting the energetic demands of insect diapause: nutrient storage and utilization. *J. Insect Physiol.* **53**, 760–773.

Hahn, D. A., Denlinger, D. L. (2011) Energetics of insect diapause. *Annu. Rev. Entomol.* **56**, 103–121.

Harvey, J. A., Heinen, R., Gols, R., Thakur, M. P. (2020) Climate change-mediated temperature extremes and insects: from outbreaks to breakdowns. *Glob. Change Biol.* **26**, 6685-6701.

Hegdekar, B. M. (1971) Wing aberrations induced by precooling pharate adults of the fly *Pseudosarcophaga afinis*. *Can. J. Zool.* **49**, 952.

Heinrich, B. (1974) Thermoregulation in endothermic insects. *Science* **185**(4153), 747-756.

Heinrich, B., Esch, H. (1994) Thermoregulation in bees. *Am. Sci.*, **82**, 164-170.

Heinrich, B., Pantle, C. (1975) Thermoregulation in small flies (*Syrphus* sp.): Basking and shivering. *J. Exp. Biol.* **62**, 599-610.

Heinrich, B. and Vogt, F. D. (1993) Abdominal temperature regulation by arctic bumblebees. *Physiol. Zool.* **66**, 257-269.

Herrera, C. M. (1995a) Floral biology, microclimate, and pollination by ectothermic bees in an early-blooming herb. *Ecol.* **76**, 218-228.

Herrera, C. M. (1995b) Microclimate and individual variation in pollinators: Flowering plants are more than their flowers. *Ecol.* **76**, 1516-1524.

Hobbs, G. A., Richards, K. W. (1976) Selection for a univoltine strain of *Megachile* (Eutricharaea) *pacifica* (Hymenoptera: Megachilidae). *Can. Entomol.* **108**, 165-167.

Huang, L-H., Chen, B. and Kang, L. (2007) Impact of mild temperature hardening on thermotolerance, fecundity, and Hsp gene expression in *Liriomyza huidobrensis*. *J. Insect Physiol.* **53**, 1199–1205.

Hutchinson, L. A., Bale, J. S. (1994) Effects of sublethal cold stress on the aphid *Rhopalosiphum padi*. *J. Appl. Ecol.* **31**: 102-108.

Javorek, S. K. (1996) The potential of the alfalfa leafcutting bee *Megachile rotundata* F. (Hymenoptera: Megachilidae) as a pollinator of lowbush blueberry (*Vaccinium angustifolium* Ait. *V. mytilloides* Michx.). M.Sc. thesis, Acadia University, Wolfville, Nova scotia.

Javorek, S. K., Mackenzie, K. E., Vander Kloet, S. P. (2002) Comparative pollination effectiveness among bees (Hymenoptera: Apoidae) on lowbush blueberry (Ericaceae: *Vaccinium angustifolium*). *Ann. Entomol. Soc. Am.* **95**(3), 345-351.

Jensen, J. L. (1906) Sur les fonctions convexes et les intelualites entre les valeur moyenes. *Acta. Main.* **30**, 175-198.

Jones, J. C., Oldroyd, B. P. (2007) Nest thermoregulation in social insects. *Adv. Insect Physiol.* **33**, 154-191.

Karlik, J., Epps. M. J., Dunn, R. R., Penick, C. A. (2016) Life inside an acorn: How microclimate and microbes influence nest organization in *Temnothorax* ants. *Ethology* **122**, 790-797.

Kellermann, V., Chown, S. L., Schou, M. F., Aitkenhead, I., Janion-Scheepers, C., Clemson, A., Scott, M. T., Sgrò, C. M. (2019) Comparing thermal performance curves across traits: how consistent are they? *J. Exp. Biol.* **222**, jeb193433.

Kellomäki, S., Hänninen, H., Kolström, M. (1995) Computations on frost damage to scots pine under climatic warning in boreal conditions. *Ecol. Appl.* **5**, 42-52.

Kelty, J. D., Killian, K. A., Lee, R. E. Jr. (1996) Cold shock and rapid cold-hardening of pharate adult flesh flies (*Sacrophaga crassipalpis*): effects on behaviour and neuromuscular function following eclosion. *Physiol. Entomol.* **21**, 283-288.

Kight, S. L., **Batino, M., Zhang, Z.** (2000) Temperature-dependent parental investment in the giant waterbug *Belostoma flumineum* (Heteroptera: Belostomatidae). *Ann. Entomol. Soc. Am.* **93**, 340–342.

Kingsolver, J. G., Woods, H. A., Buckley, L. B., Potter, K. A., Maclean, H. J., Higgins, J. K. (2011) Complex life cycles and the responses of insects to climate change. *Integr. Comp. Biol.* **51**, 719–732.

Klostermeyer, E. C., Mech, S. J. Jr., Rasmussen, W. B. (1973) Sex and weight of *Megachile rotundata* (Hymenoptera: Megachilidae) progeny associated with provision weights. *J. Kansas Entomol. Soc.* **46**, 536-548.

Klowden, M. J. (2013) Physiological systems in insects. Academic press.

Krunic, M. D. (1972) Voltinism in *Megachile rotundata* (Megachilidae: Hymenoptera) in southern Alberta. *Can. Entomol.* 104, 185-188.

Lalouette, L., Williams, C. M., Hervant, F., Sinclair, B. J., Renault, D. (2011) Metabolic rate and oxidative stress in insects exposed to low temperature thermal fluctuations. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **158**(2), 229-234.

Mallard, F., Nolte, V., Schlötterer, C. (2020) The evolution of phenotypic plasticity in response to temperature stress. *Genome Biol. Evol.* **12**(12), 2429-2440.

Marshall, K. E., Sinclair, B. J. (2009) Repeated stress exposure results in a survival-reproduction trade-off in *Drosophila melanogaster*. *Proc. R. Soc. B Biol. Sci.* **277**, 963–969.

Marshall, K. E., Sinclair, B. J. (2015) The relative importance of number, duration and intensity of cold stress events in determining survival and energetics of an overwintering insect. *Funct. Ecol.* **29**, 357–366.

Marshall, K. E., Sinclair, B. J. (2018) Repeated freezing induces a trade-off between cryoprotection and egg production in the goldenrod gall fly, *Eurosta solidaginis*. *J. Exp. Biol.* **221**(15), jeb177956.

Martin, T. L., Huey, R. B. (2008) Why "suboptimal" is optimal: Jensen's inquality and ectotherm thermal preferences. *Am. Nat.* **171**(3), E102-E118.

Mattson, M. P. (2008) Hormesis defined. *Ageing Res. Rev.* **7**, 1–7.

Mousseau, T. A. and Dingle, H. (1991) Maternal effects in insect life histories. *Annu. Rev. Entomol.* **36**, 511–534.

Nation, J. L. (2015) Insect physiology and biochemistry. CRC Press.

Peterson, J. H., Roitberg, B. D. (2006a) Impact of flight distance on sex ratio and resource allocation to offspring in the leafcutter bee, *Megachile rotundata*. *Behav. Ecol. Sociobiol.* **59**, 589–596.

Peterson, J. H., Roitberg, B. D. (2006b) Impact of resource levels on sex ratio and resource allocation in the solitary bee, *Megachile rotundata*. *Environ. Entomol.* **35**, 1404–1410.

Pincebourde, S., Dillon, M. E., Woods, H. A. (2021) Body size determines the thermal coupling between insects and plant surfaces. *Func. Ecol.* **35**, 1424-1436.

Pitts-Singer, T. L. (2008) Past and present management of alfalfa bees. In *Bee Pollination in Agricultural Ecosystems*, ed. RR James, TL Pitts-Singer, **7**, 105-23. New York: Oxford Univ. Press. 232 pp.

Pitts-Singer, T. L. and Cane, J. H. (2011) The alfalfa leafcutting bee, *Megachile rotundata*: The world's most intensively managed solitary bee. *Annu. Rev. Entomol.* **56**, 221–237.

Pitts-Singer, T. L., James, R. R. (2005) Emergence success and sex ratio of commercial alfalfa leafcutting bees from the United States and Canada. *J. Econ. Entomol.* **98**(6), 1785-1790.

Pitts-Singer, T. L., James, R. R. (2008) Do weather conditions correlate with findings in failed, provision-filled nest cells of *Megachile rotundata* (Hymenoptera: Megachilidae) in Western North America? *J. Econ. Entomol.* **101**(3), 674-685.

Potter, K., Davidowitz, G., Woods, H. A. (2009) Insect eggs protected from high temperatures by limited homeothermy of plant leaves. *J. Exp. Biol.* **212**, 3448-3454.

Rank, G. H. and Rank, F. P. (1989) Diapause intensity in a French univoltine and a Saskatchewan commercial strain of *Megachile rotundata* (Fab.). *Can. Entomol.* **121**, 141-148.

Ratz, T., Nichol, T. W. and Smiseth, P. T. (2020) Parental responses to increasing levels of handicapping in a burying beetle. *Behav. Ecol.* **31**, 73-80.

Ratz, T., Smiseth, P. T. (2018) Flexible parents: joint effects of handicapping and brood size manipulation on female parental care in *Nicrophorus vespilloides*. *J. Evol. Biol.* **31**, 646-656.

Rebaudo, F., Faye, E., Dangles, O. (2016) Microclimate data improve predictions of insect abundance models based on calibrated spatiotemporal temperatures. *Front. Physiol.* **7**, 139.

Redhead, J. W., Powney, G. D., Woodcock, B. A., Pywell, R. F. (2020) Effects of future agricultural change scenarios on beneficial insects. *J. Environ. Manage.* **265**, 110550.

Richards, M. H., Cardama Garate, A., Shehata, M., Groom, D., Tattersall, G., Welch, K. (2020) Effect of nest microclimate temperatures on metabolic rates of small carpenter bees, *Ceratina calcarata* (Hymenoptera: Apidae). *Can. Entomol.* **152**(6), 772-782

Rinehart, J. P., Yocum, G. D. and Denlinger, D. L. (2000) Thermotolerance and rapid cold hardening ameliorate the negative effects of brief exposures to high or low temperatures on fecundity in the flesh fly, *Sacrophaga crassipalpis*. *Physiol. Entomol.* **25**, 330–336.

Rinehart, J. P., Yocum, G. D., West, M., Kemp, W. P. (2011) A fluctuating thermal regime improves survival of cold-mediated delayed emergence in developing *Megachile rotundata* (Hymenoptera: Megachilidae). *J. Econ. Entomol.* **104**, 1162–1166.

Roitberg, B. D. and Mangel, M. (2016) Cold snaps, heatwaves, and arthropod growth. *Ecol. Entomol.* **41**, 653–659.

Sambaraju, K. R., Carroll, A. L., Zhu, J., Stahl, K., Moore, R. D. and Aukema, B. H. (2012) Climate change could alter the distribution of mountain pine beetle outbreaks in western Canada. *Ecography (Cop.).* **35**, 211–223.

Saunders, D. S. (1966) Larval diapause of maternal origin – II. The effect of photoperiod and temperature on *Nasonia vitripennis*. *J. Insect Physiol.* **12**, 569-581.

Schlichting, C. D. (1986) The evolution of phenotypic plasticity in plants. *Ann. Rev. Ecol. Syst.* **17**, 667-693.

Schoenly, K., Beaver, R. A., Heumier, T. A. (1991) On the trophic relations of insects: A food-web approach. *Am. Nat.* **137**(5), 597-638.

Shah, A. A., Woods, H. A., Havird, J. C., Encalada, A. C., Flecker, A. S., Funk, W. C., Guayasamin, J. M., Kondratieff, B. C., Poff, N. L., Thomas, S. A., Zamudio, K. R., Ghalambor, C. K. (2021) Temperature dependence of metabolic rate in tropical and temperate aquatic insects: support for the climate variability hypothesis in mayflies but not stoneflies. *Glob. Change Biol.* **27**, 297-311.

Sheffield, C. S. (2008) Summer bees for spring crops? Potential problems with *Megachile rotundata* (Fab.) (Hymenoptera: Megachilidae) as a pollinator of lowbush blueberry (Ericaceae). *J. Kansas Entomol. Soc.* **81**(3), 276-287.

Simmonds, F. J. (1948) The influence of maternal physiology on the incidence of diapause. *Philos. Trans. R. Soc. Lond. B Biol. Sci*. **233**, 385-414.

Sinclair, B. J., Williams, C. M., Terblanche, J. S. (2012) Variation in thermal performance among insect populations. *Physiol. Biochem. Zool.* **85**(6), 594-606.

Stearns, S. C. (1989) Trade-offs in life-history evolution. *Funct. Ecol.* **3**, 259-268.

Stoks, R., Santens, M., De Bruyn, L., Matthysen, E. (1996) Pre-flight warming up of maturing *Aeshna mixta* Latreille (Anisoptera: Aeshnidae). *Odonatologica* **25**(3), 307-311.

Stroud, J. T., Mothes, C. C., Beckles, W., Heathcote, R. J. P., Donihue, C. M., Losos, J. B. (2020) An extreme cold event leads to community-wide convergence in lower temperature tolerance in a lizard community. *Biol. Lett.* **16**, 20200625.

Tasei, J. N., Masure, M. M. (1978) Sur quelques facteurs influencant le développement de *Megachile pacifica* Panz. (Megachilidae). *Apidologie* **9**, 273-290.

Tepedino, V. J. and Frohlich, D. R. (1984) Fratricide in *Megachile rotundata*, a non-social Megachilid bee: impartial treatment of sibs and non-sibs. *Behav. Ecol. Sociobiol.* **15**, 19-23.

Tepedino, V. J., Parker, F. D. (1986) Effect of rearing temperature on mortality, second-generation emergence, and size of adult in *Megachile rotundata* (Hymenoptera: Megachilidae). *J. Econ. Entomol.* **79**(4), 974-977.

Tougeron, K., Brodeur, J., Le Lann, C., Van Baaren, J. (2020) How climate change affects the seasonal ecology of insect parasitoids. *Ecol. Entomol.* **45**, 167-181.

Trostle, G., Torchio, P. F. (1994) Comparative nesting behavior and immature development of *Megachile rotundata* (Fabricius) and *Megachile apicalis* Spinola (Hymenoptera, Megachilidae). *J. Kans. Entomol. Soc.* **67**, 53-72.

Tuljapurkar, S. and Istock, C. (1993) Environmental uncertainty and variable diapause. *Theor. Popul. Biol.* **43**, 251-280.

Turnock, W. J., Lamb, R. J., Bobnaryk, R. P. (1983) Effects of cold stress during pupal diapause on the survival and development of *Mamestra configurata* (Lepidoptera: Noctuidae). *Oecologia* **56**, 185-192.

Vaghina, N. P., Voinovich, N. D., Reznik, S. Y. (2014) Maternal thermal and photoperiodic effects on the progeny diapause in *Trichogramma telengai* Sorokina (Hymenoptera: Trichogrammatidae). *Entomol. Sci.* **17**, 198-206.

Westhus, C., Kleineidam, C. J., Roces, F., Weidenmüller, A. (2013) Behavioural plasticity in the fanning response of bumblebee workers: impact of experience and rate of temperature change. *Animal Behaviour* **85**, 27-34.

Wilson, E. S., Murphy, C. E., Rinehart, J. P., Yocum, G. D., Bowsher, J. H. (2020) Microclimate temperatures impact nesting preference in *Megachile rotundata* (Hymenoptera: Megachilidae). *Environ. Entomol.* **49**, 296-303.

Wilson, E. S., Murphy, C. E., Wong, C., Rinehart, J. P., Yocum, G. D., Bowsher, J. H. (2021) Environmental impacts on diapause and survival of the alfalfa leafcutting bee, *Megachile rotundata*. *PLoS ONE* **16**(8), e0254651.

Woods, H. A., Dillon, M. E., Pincebourde, S. (2015) The roles of microclimatic diversity and of behavior in mediating the response of ectotherms to climate change. *J. Thermal Biol.* **54**, 86-97.

Yocum, G. D., Rinehart, J. P., West, M. and Kemp, W. P. (2010) Interrupted incubation and short-term storage of the alfalfa pollinator *Megachile rotundata* (Hymenoptera: Megachilidae): A potential tool for synchronizing bees with bloom. *J. Econ. Entomol.* **103**, 234–241.

CHAPTER 2: THERMAL HISTORY OF ALFALFA LEAFCUTTING BEES AFFECTS NESTING AND DIAPAUSE INCIDENCE

Abstract

Variable spring temperatures may expose developing insects to sublethal conditions, resulting in long-term consequences. The alfalfa leafcutting bee, *Megachile rotundata*, overwinters as a prepupa inside a brood cell, resuming development in spring. During these immobile stages of development, bees must tolerate unfavourable temperatures. In this study, we test how exposure to low temperature stress during development affects subsequent reproduction and characteristics of the F1 generation. Developing male and female *M. rotundata* were exposed to either constant (6°C) or fluctuating (1 h/day at 20°C) low temperature stress for one week, during the pupal stage to mimic a spring cold snap. Treated adults were marked and released into field cages, and reproductive output was compared to that of untreated control bees. Exposure to low temperatures during the pupal stage had mixed effects on reproduction and offspring characteristics. Females treated with fluctuating low temperatures were more likely to nest compared to control bees or those exposed to constant low temperature stress. Sublethal effects may have contributed to low nesting rates of bees exposed to constant low temperatures. Females from that group that were able to nest had fewer, larger offspring with high viability, suggesting a trade-off. Interestingly, offspring of bees exposed to fluctuating low temperatures were more likely to enter diapause, indicating that thermal history of parents, even during development, is an important factor in diapause determination.

Introduction

In temperate regions, insects are exposed to fluctuating temperatures throughout the seasons. At some latitudes in winter, ambient temperature fluctuates around temperatures that may be lethal to some species. Suboptimal microclimates during the winter can be metabolically costly and have long-term negative effects on reproduction (Irwin and Lee, 2003; Turnock and Fields, 2005). During winter, insects survive extreme low temperatures in a state called diapause through various physiological mechanisms, including metabolic depression and production of cryoprotectants (Bale and Hayward, 2010; Hahn and Denlinger, 2007; Hahn and Denlinger 2011). However, such physiological protections may be absent during the spring, when insects are actively developing (Chown and Sinclair, 2010; Huey, 2010).

Unfavorable spring weather events can include sudden bouts of low temperatures in the form of cold snaps or freeze-thaw events (Kellomäki et al., 1995; Roitberg and Mangel, 2016; Sambaraju et al., 2012). Abrupt temperature changes may be too severe to induce a hormetic response (Mattson, 2008) or may expose insects to suboptimal conditions that could lead to detrimental effects, because they no longer have the physiological protections afforded during diapause (Lee et al., 1987). In general, insects may seek to limit thermal stress by physiological and/or behavioral methods, such as escaping to a more favorable microclimate and activating flight muscles (Kingsolver et al., 2011; Heinrich, 1975; Heinrich and Esch, 1994; Heinrich and Vogt, 1993). However, options for behavioral thermoregulation are limited for insects during immobile life stages, such as those undergoing metamorphosis within brood cells or cocoons that must survive the temperatures to which they are exposed (Kingsolver et al., 2011). Experiencing sublethal thermal stress during metamorphosis could have negative, long-term consequences that persist into the next generation.

Many parameters of a thermal stress event are important to consider, such as the duration of exposure, frequency, and magnitude (Marshall and Sinclair, 2015). Insects that survive a stressor may have changes in offspring quantity and/or quality (Stearns, 1989). Downstream effects of stress are not ubiquitous and may differ across taxa. Furthermore, responses of some offspring characteristics, such as mortality, sex ratios, and diapause incidence, may be more affected than others depending on species (Mousseau and Fox, 1998). For instance, aphids (*Rhopalosiphum padi*) had reduced fecundity and increased offspring mortality after they were exposed to low temperatures as either first instar larvae or newly molted adults (Hutchinson and Bale, 1994). In contrast, adult fruit flies (*Drosophila melanogaster*) that experienced repeated low temperature stress versus one long continuous stress had increased survival, decreased fecundity, and altered offspring sex ratios, suggesting a trade-off and reallocation of diminished energetic stores (Marshall and Sinclair, 2009). After experiencing a low temperature stress as adults, pea leafminers (*Liriomyza huidobrensis*) produced fewer eggs, although the viability and sex ratios of the offspring were not affected (Huang et al. 2007). The wide range of responses suggests there may not be a universal pattern. Therefore, future studies need to incorporate more non-model species to understand and develop more robust patterns.

Whether an insect enters diapause or continues direct development has been found to be maternally regulated in a variety species (Coleman et al., 2014; Denlinger, 2002; Mousseau and Dingle, 1991; Simmonds, 1948). For many of these species, offspring are more likely to enter diapause when their parents are reared under low temperatures and short-day lengths (Saunders, 1966; Tougeron et al., 2020; Vaghina et al., 2007). Also, the sensitive stage of mothers for thermoperiod effects can differ among species, which makes testing the effects of stress on multiple life stages important (Mousseau and Dingle, 1991). Long-term effects on offspring diapause from temperature stress of the parental generation has not been studied.

Changes in offspring diapause incidence could indicate that a female may be interpreting environmental cues differently than predicted (Mousseau and Dingle, 1991). Entering diapause at the correct time is important for offspring to avoid exposure to an array of detrimental stressors that could negatively affect their survival and the overall population composition (Tougeron et al., 2020; Tuljapurkar and Istock, 1993). For instance, summer weather can expose immobile insects to lethal heat stress (Pitts-Singer and James, 2008). The effects of timing on diapause termination has been well studied in terms of physiological mechanisms, ecological and applied agricultural importance (Danforth, 1999; Denlinger, 2008; Forrest and Miller-Rushing, 2010, Menu et al., 2000; Rajon et al., 2014). However, the consequences of poorly timed diapause induction have been vastly understudied.

Megachile rotundata, commonly known as the alfalfa leafcutting bee, is a solitary bee that has limited mobility during overwintering and subsequent development to adult. The female parent determines offspring sex, body size, and potentially whether the offspring enters diapause (Krunic, 1972; Hobbs and Richards, 1976). Alterations in offspring quality and quantity occur through parental care provided to offspring. Female parents construct brood cells made of leaf pieces glued together with nectar and saliva and provisioned with pollen and nectar inside (Cane et al., 2011; Trostle and Torchio, 1994). At lower latitudes *M. rotundata* is bivoltine with non-diapausing bees that are predominantly female, compared to the overwintering cohort, emerging in the summer (Tepedino and Parker, 1998). The sex ratio of overwintering bees is skewed toward males, which require less of an energetic investment, receiving 17% less provisions compared to female siblings (Klostermeyer et al., 1973). Offspring investment by female *M. rotundata* can be altered by environmental stressors, including increased flight distance to forage and reduced resource levels (Peterson and Roitberg, 2006a,b; O'Neill et al., 2010). However, it is unclear whether temperature stress can also cause changes in female offspring investment.

Due to extensive research on the effects of low temperatures for its long-term and short-term storage, *M. rotundata* is an excellent model for understanding how insects respond to thermal stress during development (Rinehart et al., 2011, 2013, 2016; Torson et al., 2019; Yocum et al., 2010). For instance, *M. rotundata* exposed to low temperatures for durations as short as one week (both constant and fluctuating) during development do not differ in survival from controls (Bennett et al., 2015; Rinehart et al., 2011; Yocum et al., 2010). However, those exposed to either constant or fluctuating low temperature stress had delayed adult emergence, and males had reduced longevity (Bennett et al., 2015). Adults that survived had reduced flight ability and wing deformities (Bennett et al., 2015). Thermal stress may also negatively affect reproduction or reduce the quality and/or quantity of offspring. The effects of low temperature stress during development on future offspring production in *M. rotundata* is currently unknown.

In this study, we test the hypothesis that exposure to low temperature stress during development alters offspring characteristics. To test this hypothesis, pupal *M. rotundata* undergoing metamorphosis were exposed to two different types of low temperature stress (fluctuating or constant) for one week. A constant temperature was chosen as a baseline stressor that may be relevant for individuals buffered in their microhabitat (Irwin and Lee, 2003), while fluctuating low temperature stress would be representative of potential spring temperature changes. All bees were allowed to resume development, and emerging adult bees were transported to tents in an alfalfa field to track offspring production. Nests containing offspring were brought back to the laboratory to determine the number of offspring produced and their characteristics, including sex, dry weight, and diapause state. Changes in the number of offspring and their characteristics would show a shift in parental investment due to a long-term response to low temperature stress.

Materials and Methods

Megachile rotundata Rearing

Diapausing prepupae, of Canadian origin, were purchased from JWM Leafcutters (Nampa, ID, USA) as single, loose brood cells. *Megachile rotundata* prepupae were kept in a 6°C incubator (Percival, Perry, IA, USA) in constant darkness. To initiate development, brood cells were moved to a 29°C incubator in 16oz plastic containers covered with a fine mesh lid allowing proper air flow throughout the container.

Low Temperature Treatments

After 14 days at 29°C, when the majority of developing *M. rotundata* are at the eye-pigmented stage (Bennett et al., 2015; Yocum et al., 2010), brood cells were randomly placed into one of three treatment groups: constant low temperature stress, fluctuating low temperature stress, and control. The constant low temperature stress was a constant 6°C, while the fluctuating low temperature stress has a baseline of 6°C with a daily pulse at 20°C for one-hour with one hour ramps up and down, as previously described (Rinehart et al., 2011). We chose these temperature treatments based on previous work showing that exposure to fluctuating low temperature stress reduces mortality (Rinehart et al., 2011) and some sublethal effects (Bennett et al., 2015) compared to constant low temperature stress. Constant and fluctuating bees remained in their respective treatments for 7 days. At day 21, both low temperature treatment groups were returned to the 29°C incubator until adults emerged. Bees designated for the control group were kept in the 29°C incubator to allow for continuous development with adult emergence timed to coincide with the other treatments. All bees finished development and emerged as adults in the 29°C incubator before being marked and released.

Field Design

Each treatment was assigned three replicate tents (6.1 x 6.1 x 2.4-meters), totaling 9 tents in a blooming alfalfa field. Tents were placed 6 meters from each other in a repeating pattern to ensure field uniformity. The experiment ran from June 15th, 2019 until July 6th, 2019 in Fargo, North Dakota (USA, 46.9183038, -96.8536053). In each tent, a nest box was attached to a pole in the center, facing southeast. Nest boxes were constructed from a commercial Styrofoam blocks (Beaver Plastics, Acheson, Alberta, Canada) with 7mm holes each lined with a paper straw in a 12 x 12 grid (144 total holes).

Twenty female and 10 male bees were haphazardly assigned to each tent to ensure that resource availability would not be limiting (Pitts-Singer and Bosch, 2010). The ratio of 2 females to 1 male was used to allow the females to optimize their fitness without male harassment (Rossi et al., 2010). Prior to release in the field, females were marked on the thorax with a unique color pattern (Sharpie® Oil-Based Paint Markers, Atlanta, Georgia). A preliminary test was done in the greenhouse to determine which combinations would be possible in the field. Seven different colors were distinguishable under field conditions. Out of the 20 released bees, the first 7 had a single dot on their thorax, while the remaining bees had two dots that were discernable from each other.

Female *M. rotundata* are known to return to the same hole each time after foraging until the nest is completed (Kloystermeyer et al., 1973; McCorquodale and Owens, 1997). Therefore, a completed nest can be attributed to a known female. Bees were observed daily in the field for three weeks to determine when straws were completed, and which female created each nest. Straws were identified as complete when they were capped with visible leaf pieces protruding out of the straw (Pitts-Singer and Cane, 2011). Once completed, we removed each straw from the cavity using forceps and placed into a 29°C incubator to continue offspring development under the controlled conditions to minimize any effect of environmental variation that occurred in the field.

Offspring Measurements

To determine the number of brood cells in each straw and their contents, we x-rayed each straw using a Faxitron Bioptics, LLC X-Ray (Tucson, Arizona) for 4 seconds at 28kV and visualized using AllPro Imaging by Air Techniques (Melville, New York). From the x-ray images, we determined if brood cells contained offspring, parasites, or pollen balls (brood cells without offspring). Leaves were allowed to dry for a week before individual brood cells were removed from straws and placed in a 24-well plate according to their cell position within the straw. Brood cells were x-rayed again after bees stopped emerging to determine which had overwintering offspring and offspring that had died during development. If the brood cells were destined for diapause, indicated by the presence of a prepupa, they were moved to the 6°C incubator for 5 months for overwintering. Diapausing bees were then moved to the 29°C incubator to continue development into adults.

For offspring that continued direct development, upon adult emergence, bees were placed in new 24 well plates without leaf matter, transported to a 50°C oven (Techne Hybridiser HB-1D, Princeton, New Jersey) and dried to a constant weight. After one week, offspring were weighed to the nearest 0.01 mg on an analytical balance (Mettler AE100, Hightstown, New Jersey). Brood cells containing dead offspring were dissected to determine at which life stage the offspring died. The reproductive fitness of each female was measured by the total number of brood cells from each individual and the total number of viable offspring.

Flight Test

Upon releasing treated adult bees into the field cages, we discovered that some individuals were unable to fly. To quantify the prevalence of this defect, 50 male and 50 female bees per treatment were randomly selected for a drop test. Bees were individually placed in small petri dishes (60mm diameter) and released at the top of a wide acrylic tube (height: 1m, diameter: 0.25m). Bees were categorized by their response to being dropped as 1) flight (bees flew without hitting the bottom of the tube), 2) recovery (bees flew out after hitting the bottom of the tube), and 3) no flight and no recovery (bees fell to the bottom and did not fly out).

Statistical Analysis

Data was analyzed using JMP Pro (Version 14, SAS Institute, Cary, North Carolina), IBM SPSS (Statistics 24, Armonk, New York), and R (Version 1.1.423) (R Core Team, 2019). All data were assessed for normality and equal variance. Analysis of variance (ANOVA) was used to determine differences in the number of brood cells using temperature treatment as a factor. A binomial logistic regression was used for a multitude of tests, including flight performance of treated adults (likelihood of flight, offspring viability, sex ratios, and diapause incidence). For dry weight comparisons, Kruskal-Wallace tests were performed due to unequal variances in non-diapausing offspring; therefore, not meeting the requirements of an ANOVA. An ANCOVA was used to determine the relationship between the number and weight of offspring produced by individual females. Then, a regression was used to compare the response across treatments. All means are presented ± standard error mean (S.E.M.) and significance is determined by $p < 0.05$.

Results

Treated bees fell into three categories: those having obvious morphological deformities (Figure 2A,C), those with subtle deformities (Figure 2B), and those with no deformities. In bees that had no obvious morphological defects, flight ability was significantly affected by temperature treatment ($\chi^2 = 46.1$, $p < 0.0001$; Figure 2D). Less than half of bees treated with constant low temperature stress flew in the flight test (40% of females and 44% of males flew) a lower percent than observed for those exposed to fluctuating low temperature stress (54% females, 76% males) and control bees (88% females, 98% males).

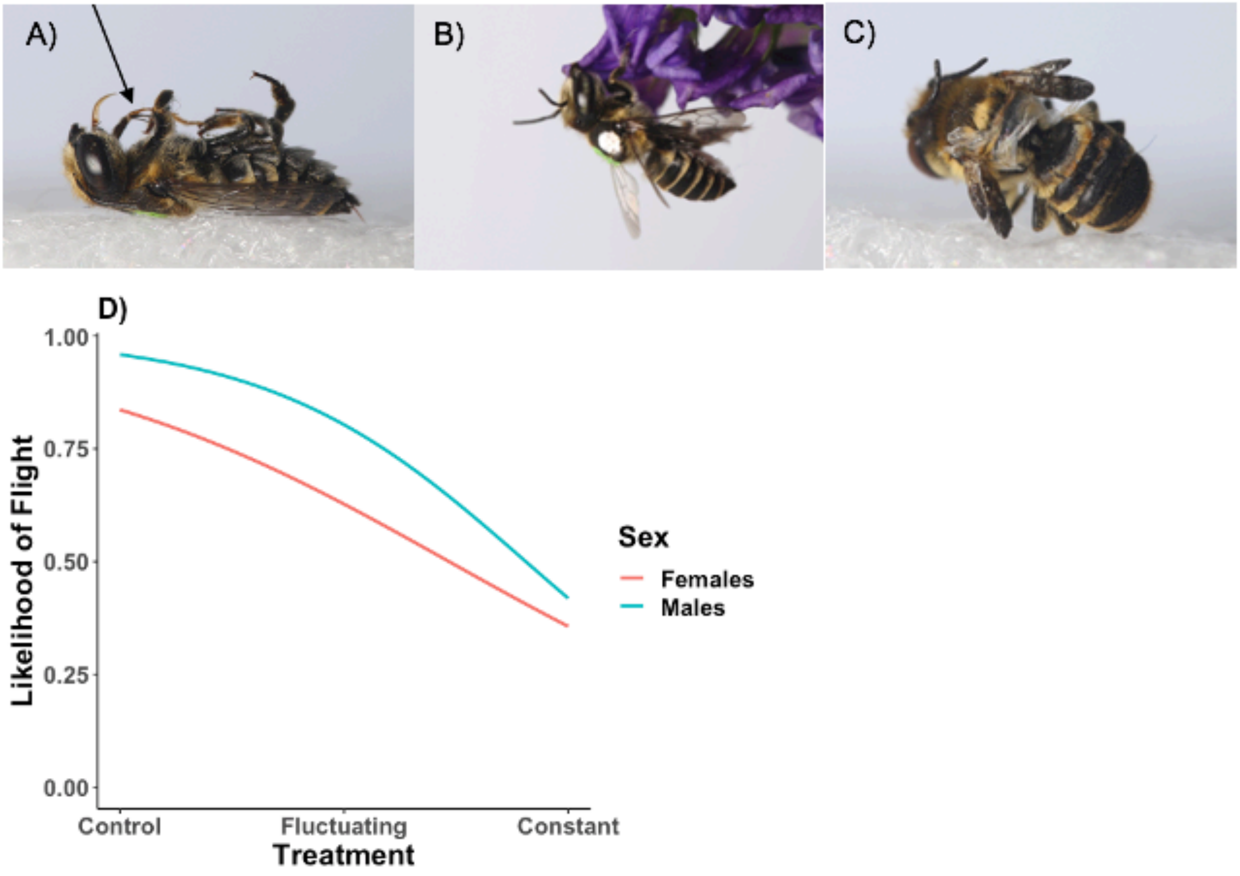

Figure 2. Observed deformities in low temperature treatments. **A)** Proboscis permanently extended in females treated with fluctuating temperatures. **B)** Inability to fly and limited forewing control in constant temperature stress females. **C)** Unexpanded wings in constant temperature treated males. **D)** The likelihood of flight for females (n = 50) and males (n = 50) that experienced a low temperature stress during development.

Of bees released in the field, females exposed to constant low temperature stress were significantly less likely to nest (10.0% ± 7.64; $\chi^2 = 11.3$, $p < 0.004$) compared to those exposed to fluctuating low temperature stress (32.7% ± 6.00). Control females (25.0% ± 5.77) nested with a similar likelihood as constant females. Females exposed to constant low temperatures failed to establish in one tent, while another tent had one nest with 2 brood cells. The third tent with constant-treated bees produced 5 nests containing a total of 36 brood cells. Despite low establishment, the average number of brood cells produced per tent was not statistically different among treatments: control, 57.0 ± 16.9; fluctuating, 87.7 ± 30.9; constant, 12.7 ± 11.7 ($F_{2,35} = 1.40$, $p = 0.261$; Figure 3B). In addition, constant low temperature females were more likely to have brood cells with viable offspring (97.2% ± 2.78) compared to controls (69.4% ± 2.37; $\chi^2 = 6.20$, $p < 0.05$; Figure 4A).

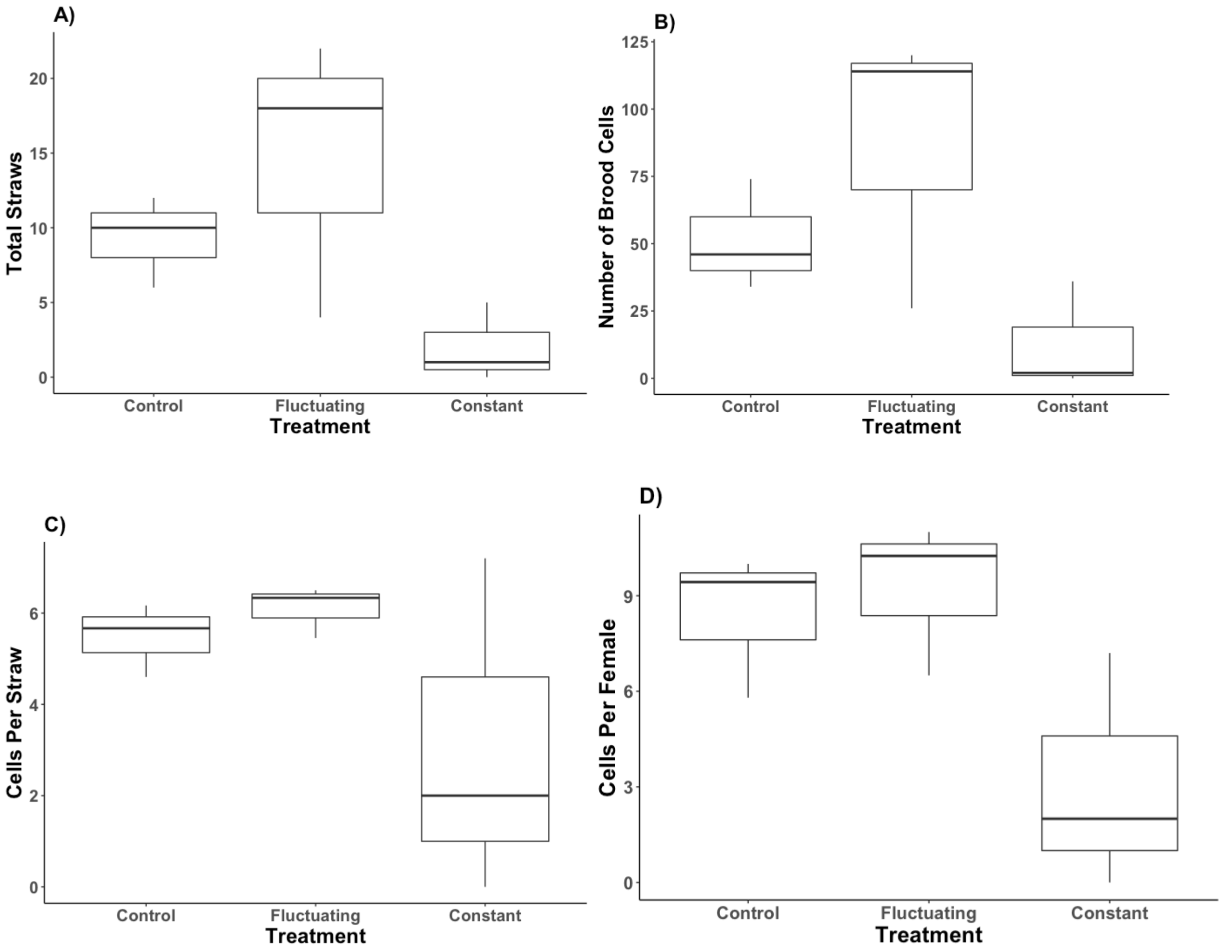

Figure 3. A) Average number of straws, **B)** average number of brood cells, **C)** average brood cells per nest, and **D)** average number of brood cells produced per female in response to low temperature stress during pupal development. Boxes represent the 1st to 3rd quartile, while the lines extending from the box show the 25th and 75th percentiles of the data. The median is indicated by the line in the center of the box. There were statistical differences detected among treatment groups. There were no statistical differences detected by ANOVA among treatment groups (n = 20 females per tent, 3 tents per treatment).

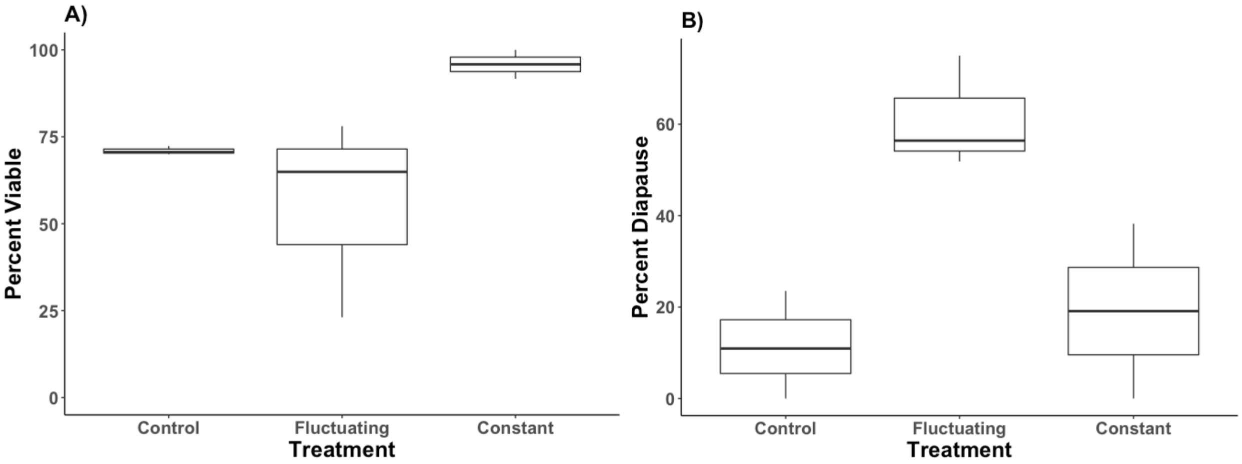

Figure 4. A) The percent of viable offspring that successfully emerged as adults. **B)** The percent of offspring that entered diapause. Boxes represent the 1st to 3rd quartile, while the lines extending from the box show the 25th and 75th percentiles of the data. The median is indicated by the line in the center of the box. Boxes with different letters are significantly different, $p < 0.05$ ($n = 20$ females per tent, 3 tents per treatment).

Diapause incidence of offspring was significantly affected by temperature treatment of parents ($\chi^2 = 46.7$, $p < 0.0001$; Figure 4B). Offspring of females treated with fluctuating temperatures were more likely to enter diapause (61.1% ± 7.08) than both the controls (11.5% ± 6.80) and constant offspring (19.1% ± 19.1%). Offspring sex varied with diapause incidence ($\chi^2 = 13.5$, $p = 0.0003$; Figure 5). Non-diapausing bees were predominately female (Control, 60.3%; fluctuating, 53.7%; constant, 69.6%) for all three treatments whereas diapausing bees were mostly males (Control, 72.7%; fluctuating, 63.4%; constant, 75%). Dry weights were analyzed between sexes and development path (diapausing or continuous development; Figure 6). Non-diapausing female offspring were at least 2 mg heavier than males across all treatments ($F_{1,206} = 80.4$, $p < 0.0001$). For diapausing offspring, offspring dry weight differed between sexes ($\chi^2 = 35.39$, df = 2, $p < 0.0001$). When analyzing sexes separately, there was no difference in dry weight among treatments of non-diapausing males ($\chi^2 = 5.36$, df = 2, $p > 0.069$; Figure 6B). For non-diapausing females, treatment affected dry weight ($\chi^2 = -32.9$, df = 2, $p < 0.008$; Figure 6A) with females produced by constant low temperature parents being larger than controls but not fluctuating temperature offspring. Diapausing male offspring produced by both constant and fluctuating low temperature treated parents were at least 1 mg heavier than controls across treatments ($\chi^2 = 7.84$, df = 2, $p = 0.020$; Figure 6B). There was no significant difference among diapausing female offspring across treatments ($\chi^2 = 4.02$, df = 2, $p = 0.134$; Figure 6A).

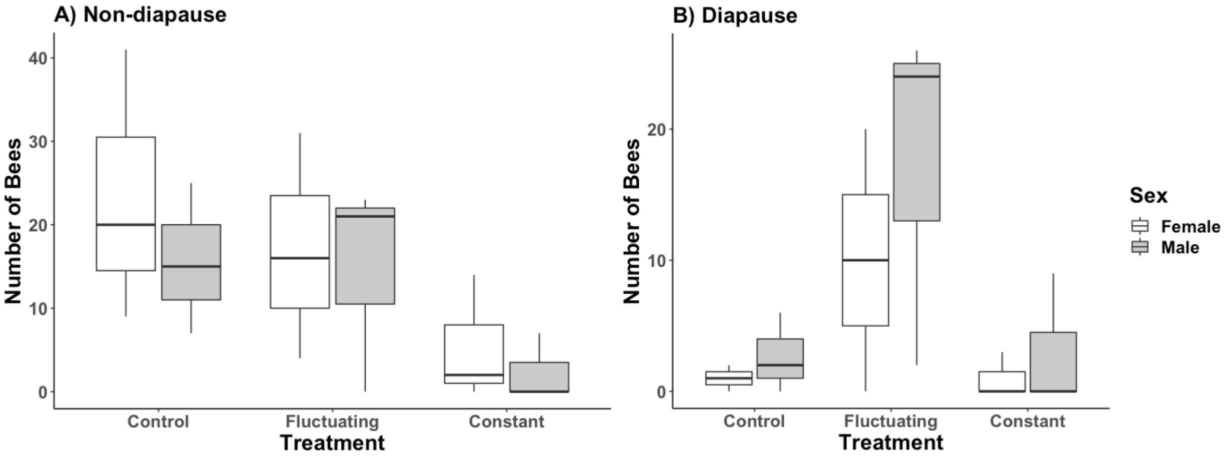

Figure 5. The number of female and male offspring produced by temperature treated bees (n = 20 females per tent, 3 tents per treatment) that continued direct development **(A)** or underwent diapause **(B)**. Boxes represent the 1st to 3rd quartile, while the lines extending from the box show the 25th and 75th percentiles of the data. The median is indicated by the line in the center of the box.

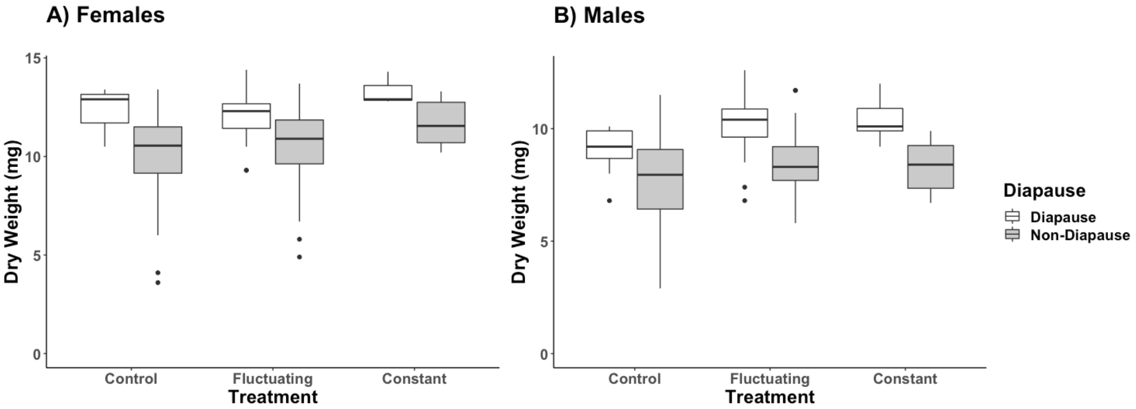

Figure 6. Dry weight (mg) of diapausing (open boxes) and non-diapausing (grey boxes) female **(A)** and male **(B)** offspring. Boxes represent the 1st to 3rd quartile, while the lines extending from the box show the 25th and 75th percentiles of the data. The median is indicated by the line in the center of the box. Boxplots that share at least one letter above them are not significantly different by treatment. * indicates significant differences within a treatment between diapausing and non-diapausing bees (n = 20 females per tent, 3 tents per treatment).

Temperature treatment significantly affected the relationship between quantity and quality of offspring ($F_{2,37} = 3.36$, $p = 0.047$; Figure 7). For fluctuating and control bees, there was no relationship between offspring average mass and the number of brood cells ($p = 0.403$ and 0.386 respectively). However, for females exposed to constant low temperatures during development, there was a negative correlation between offspring average weight and number of brood cells ($F_{1,5} = 7.72$, $p = 0.05$; Figure 6; $y = 14.1x – 0.473$, $r^2 = 0.659$).

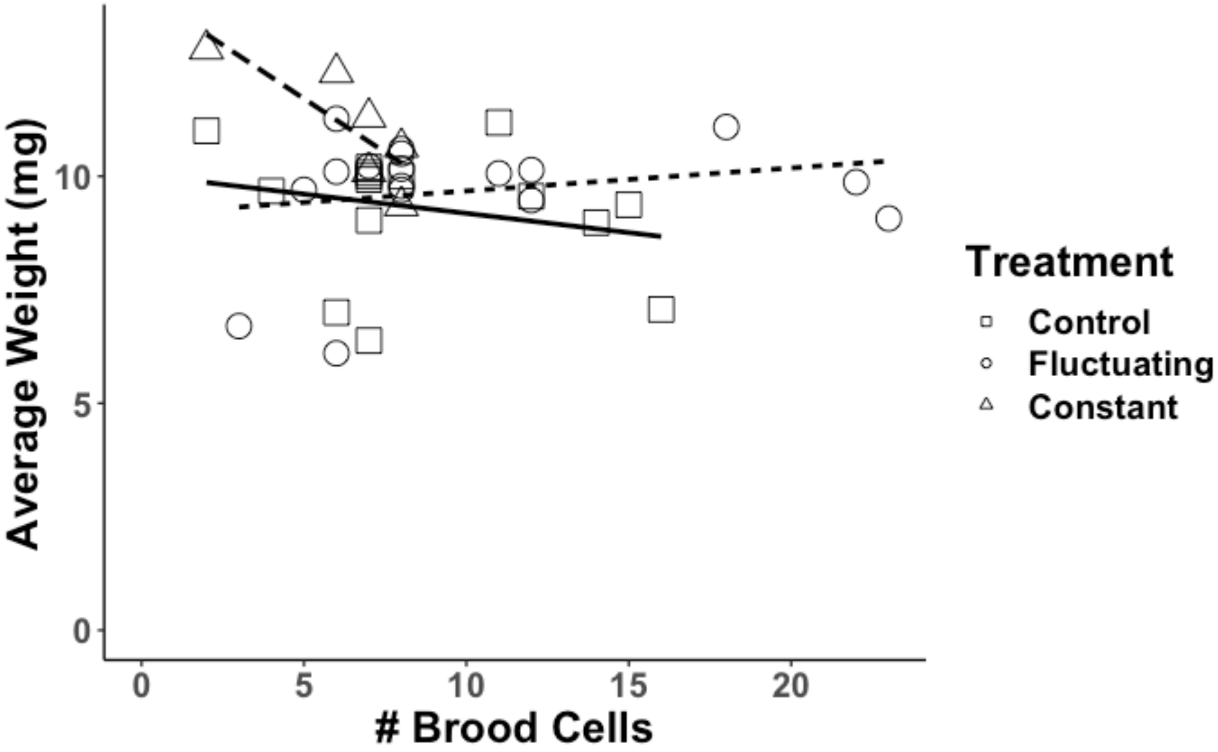

Figure 7. Each dot represents one female's reproductive effort, plotted as the average weight of the offspring (mg) versus the total number of brood cells produced by that female (n = 20 females per tent, 3 tents per treatment). The slope of the best fit line shows the change in offspring investment as females produced more offspring. A negative slope indicates decreased investment as the number of offspring increase, while a positive slope indicates increased investment as offspring number increases. There was a significant correlation between the number of brood cells and the average weight of offspring only in the constant temperature treatment (regression shown; long dashed line), but the other lines are shown for comparison. The slope of the control treatment is depicted by the solid line and fluctuating by the shorter dashed line.

Discussion

Sudden bouts of low temperature during the spring pose a substantial risk to developing insects. Insects that survive this type of stressor may have long-term effects on behavior, reproduction, and offspring characteristics. Exposure to low temperature stress during development had mixed effects on the reproductive fitness of *M. rotundata*. Bees exposed to constant low temperature stress were less likely to nest and showed a trend toward fewer brood cells per female even though the number of offspring produced among treatments did not differ significantly. The number of brood cells produced is an imprecise metric because two of the tents didn't produce any offspring. For instance, females exposed to constant temperatures during development failed to establish nests in one tent and in another tent had only two offspring. In the third tent, more females established nests and produced numbers of brood cells comparable to those in other treatments. The low nesting rate is most likely attributed to sublethal effects that prevented females from flying, as observed in this and previous studies (Bennett et al., 2015). Interestingly, females that were exposed to the fluctuating temperature treatment were more likely to nest, which may be due to a hormetic response. The use of the constant and fluctuating temperature treatments in this study are essential to creating a framework that can be built upon to understand how insects are responding in the field. Future experiments will include exposing bees to a more rigid and complex temperature profile in the laboratory with another group experiencing natural fluctuating temperatures in the field.

Our hypothesis that exposure to low temperature stress during development alters offspring characteristics was supported. This is the first study to show that the thermal history experienced by the parental generation during metamorphosis, and not adulthood, affects diapause incidence of offspring in *M. rotundata*. Also, this study is the first to show that thermal stress during spring development in the parental generation can alter offspring diapause incidence. *Megachile rotundata* undergoes facultative diapause and the number of summer generations varies with latitude (Kemp and Bosch, 2001; Krunic, 1972; Rank and Rank, 1989). Typically, early season offspring in temperate regions, such as the location of this study, are more likely to continue development and emerge as adults than late season bees, which are more likely to enter diapause and overwinter as prepupae (Hobbs and Richards, 1976; Krunic, 1972). As expected, control and constant treatments had low percentages of offspring that entered diapause, because the experiment occurred early in the season. Surprisingly, offspring produced by parents exposed to fluctuating low temperature stress were 5 times more likely to enter diapause, suggesting that offspring diapause is determined by the female parent and her thermal history. Similarly, in *Bombyx mori* (silkworm), temperature and light cues experienced during stages of parental development determines diapause incidence in offspring (Shiomi et al., 2015). The mechanisms involved in diapause determination in *M. rotundata* are unknown; however, previous research has suggested a maternal contribution (Rank and Rank, 1989). More studies are needed to determine the exact mechanism by which females control the diapause destiny of their offspring.

Entering diapause at a suboptimal time may have an impact on survival and future reproduction. *Megachile rotundata* that enter diapause early in the season may be exposed to a different suite of stressors that may contribute to prepupal mortality. Much like developing bees subjected to low temperatures in the spring, immobile diapausing bees during the hottest part of summer must endure the increasing temperatures to which they are exposed. *Osmia ribifloris* offspring exposed to increased high temperature within their nesting cavity were less likely to survive (CaraDonna et al., 2018). Also, if offspring in one nest do not all enter diapause at the same time, those that have already entered diapause risk being killed by the emerging siblings as they exit the nest cavity. Female *M. rotundata* are more likely to chew through their siblings than males, which are more likely to circumnavigate (Tepedino and Frohlich, 1984a). Fratricide has been documented in other cavity nesting bees closely related to *M. rotundata*, (*Osmia taxana, O. montana, O. californica, and O. iridis*) (Tependino and Frohlich, 1984b; Torchio and Tepedino, 1982). In our experiment, nests were returned to the lab under the same conditions to prevent environmental effects on offspring and potential fratricide. Future studies are needed to determine the impact of mistimed diapause on offspring that remain in the field.

Low establishment rates of females that were exposed to constant low temperature stress may be attributed to morphological deformities. Noticeable deformities included bees with a permanently extended proboscis (Fig. 2A) and unexpanded wings upon adult emergence (Fig. 2B). While bees with obvious deformities were excluded, those with more subtle morphological deformities may have been released in the field (Fig. 2C). Females exposed to constant temperatures during development were observed in the field crawling on alfalfa rather than flying and, when provoked, were unable to fly or control their forewings. A further test to examine flight capabilities showed that constant temperature treated bees, both male and female, were less likely to be able to fly compared to fluctuating and control bees. Similar observations were noted for bees in tethered flight, where half of males and 10% of females without obvious morphological defects were unable to fly after exposure to the same constant treatment as in this study (Bennett et al., 2015). We likely found a greater percentage of affected bees in this study, because of our larger sample size. The previous study used only 16 total constant-treated bees for the tethered flight. Female bees that are unable to fly cannot nest, provide parental care, or visit flowers to feed. Male bees that cannot fly would likely be unable to mate, which could also reduce fitness in the constant-treated bees. Over time, population growth would be limited to the few females that were able to nest. One mechanism that could contribute to morphological deformities and decreased flight performance is cold injury.

Exposure to low temperatures during diapause as pupae is known to cause cold injuries, such as unexpanded and malformed wings, seen in the adult stage of the bertha armyworm (*Mamestra configurata*) (Turnock et al., 1983). Low temperatures chosen for this study could be low enough to cause cold injury in *M. rotundata*. Sublethal effects, such as wing deformities and degradation of muscles and the nervous system, attributed to cold stress have been observed in other insects (Hedgekar, 1971; Kelty et al., 1996; Rinehart et al., 2000; Turnock et al., 1983). In flesh flies, cold injury can result in neuromuscular degradation and changes in grooming behavior and proboscis extension reflex (Kelty et al., 1996; Yocum et al., 1994). Future experiments will be conducted to explore other possible physiological consequences of thermal stress in *M. rotundata*, including immune function and metabolic rate during a low temperature stress.

Exposure to low temperatures also affected parental care in treated females. Our results showed that those constant-treated females that were able to nest invested more resources in fewer, higher quality offspring, suggesting that there is a trade-off between the number and size of offspring produced (Stearns, 1989). This suggests that females receiving a constant low temperature stress during development alter their parental care to increase the quality of the offspring they produce as opposed to increasing the quantity. Competition could have been lower in tents with constant-temperature treated bees due to decreased nesting; however, the number of released bees in this study was chosen to reduce competition and sexual harassment by males (Pitts-Singer and Bosch, 2010; Rossi et al., 2010). No trade-offs were detected in bees that were exposed to fluctuating temperatures, which could be explained by those individuals being better at acquiring or having more overall resources than the constant-temperature treated bees (King et al. 2011; van Noordwijk & de Jong, 1986).

Trade-offs in response to low temperatures have been seen in other insect species. For instance, when exposed to repeated cold exposures, *D. melanogaster* invests in current survival over future reproduction resulting in fewer offspring being produced (Marshall and Sinclair, 2009). The terminal investment hypothesis (Clutton-Brock, 1984) may explain why the constant temperature stress females invested more in their offspring compared to fluctuating and control females. According to the terminal investment hypothesis, individuals invest more into current reproduction when experiencing stress due to aging, infection, and physiological impairment (Clutton-Brock, 1984). Increased investment may better prepare offspring for future stressors and has been well-documented in mammals, reptiles, amphibians, and insects, including European earwigs, burying beetles, giant water bugs, and decorated crickets (Brannelly et al., 2016; Cotter et al., 2010; Creighton et al., 2009; Duffield et al., 2015; Kight et al., 2000; Ratz et al., 2016, 2020; Velando et al., 2006). Changes in offspring investment may indicate that the response of females exposed to sudden low temperature stress aligns with the terminal investment hypothesis.

This study is the first to show that temperature stress during development invokes terminal investment of treated *M. rotundata* and provides strong evidence for maternal control of offspring diapause incidence. Results show that experiencing stress during development has cascading effects that may include more physiological systems than reproduction. For instance, susceptibility to viruses and pathogens may increase along with morphological, neurological, and behavioral deformities. Additionally, *M. rotundata* are used in agriculture for pollination services. Farmers regularly use low temperature treatments, similar to our constant low temperature stress treatment, to delay adult emergence to align with alfalfa bloom (Pitts-Singer and Cane, 2011). The long-term consequences of this practice on reproduction and offspring, until now, have not been studied. Within the United States, prepupae destined for diapause are more likely to have increased mortality in commercial alfalfa production (Pitts-Singer and James 2005). Seasonal bee loss has financial consequences for farmers that need to purchase new bees every year to supplement decreased populations (Pitts-Singer and Cane, 2011), which may be exacerbated by changes in diapause incidence.

References

Bale, J. S., and Hayward, S. A. L. (2010) Insect overwintering in changing climate. *J. Exp. Biol.* **213**, 980–994.

Bennett, M. M., Cook, K. M., Rinehart J. P., Yocum, G. D., Kemp, W.P. and Greenlee, K. J. (2015) Exposure to suboptimal temperatures during metamorphosis reveals a critical developmental window in the solitary bee, *Megachile rotundata*. *Physiol. Biochem. Zool.* **88**, 508–520.

Brannelly, L. A., Webb, R., Skerratt, L. F. and Berger, L. (2016) Amphibians with infectious disease increase their reproductive effort: evidence for the terminal investment hypothesis. *Open Biol.* **6**.

Cane, J. H., Gardner, D. R. and Harrison, P. A. (2011) Nectar and pollen sugars constituting larval provisions of the alfalfa leaf-cutting bee (*Megachile rotundata*) (Hymenoptera: Apiformes: Megachilidae). *Apidologie* **42**, 401–408.

CaraDonna, P. J., Cunningham, J. L. and Iler, A. M. (2018) Experimental warming in the field delays phenology and reduces body mass, fat content, and survival: implications for the persistence of a pollinator under climate change. *Funct. Ecol.* **32**, 2345–2356.

Chown, S. L. and Sinclair, B. J. (2010) The macrophysiology of insect cold-hardiness. In *Low Temperature Biology of Insects* (pp. 191-222). Cambridge University Press

Clutton-Brock, T. H. (1984) Reproductive effort and terminal investment in iteroparous animals. *Am. Nat.* **123**, 212-229.

Coleman, P. C., Bale, J. S. and Hayward, S. A. L. (2014) Cross-generation plasticity in cold hardiness is associated with diapause, but not the non-diapause development pathway, in the blow fly *Calliphora vicina*. *J. Exp. Biol.* **217**, 1454–1461.

Cotter, S. C., Ward, R. J. S. and Kilner, R. M. (2010) Age-specific reproductive investment in female burying beetles: independent effects of state and risk of death. *Funct. Ecol.* **25**, 652–660.

Creighton, J. C., Heflin, N. D. and Belk, M. C. (2009) Cost of reproduction, resource quality, and terminal investment in a burying beetle. *Am. Nat.* **174**, 673-684.

Danforth, B. N. (1999) Emergence dynamics and bet hedging in a desert bee, *Perdita portalis*. *Proc. R. Soc. B Biol. Sci.* **266**, 1985–1994.

Denlinger, D. L. (2002) Regulation of diapause. *Annu. Rev. Entomol.* **47**, 93-122.

Denlinger, D. L. (2008) Why study diapause? *Entomol. Res.* **38**, 1–9.

Duffield, K. R, Hunt, J., Rapkin, J., Sadd, B. M and Sakaluk SK. (2015) Terminal investment in the gustatory appeal of nuptial food gifts in crickets. *J. Evol. Biol.* **28**, 1872–1881.

Forrest, J. and Miller-Rushing, A. (2010) Toward a synthetic understanding of the role of phenology in ecology and evolution. *Philos. Trans. R. Soc. B Biol. Sci.* **365**, 3101–3112.

Hahn, D. A. and Denlinger, D. L. (2007) Meeting the energetic demands of insect diapause: nutrient storage and utilization. *J. Insect Physiol.* **53**, 760–773.

Hahn, D. A. and Denlinger, D. L. (2011) Energetics of insect diapause. *Annu. Rev. Entomol.* **56**, 103–121.

Hegdekar, B. M. (1971) Wing aberrations induced by precooling pharate adults of the fly *Pseudosarcophaga afinis*. *Can. J. Zool.* **49**, 952.

Heinrich, B. (1975) Thermoregulation in bumblebees. *J. Comp. Physiol.* **96**, 155-166.

Heinrich, B. and Vogt, F. D. (1993) Abdominal temperature regulation by arctic bumblebees. *Physiol. Zool.* **66**, 257-269.

Heinrich, B. and Esch, H. (1994) Thermoregulation in bees. *Am. Sci.*, **82**, 164-170.

Hobbs, G. A. and Richards, K. W. (1976) Selection for a univoltine strain of *Megachile* (Eutricharaea) *pacifica* (Hymenoptera: Megachilidae). *Can. Entomol.* **108**, 165-167.

Huang, L-H., Chen, B. and Kang, L. (2007) Impact of mild temperature hardening on thermotolerance, fecundity, and Hsp gene expression in *Liriomyza huidobrensis*. *J. Insect Physiol.* **53**, 1199–1205.

Huey, R. B. (2010) Evolutionary physiology of insect thermal adaptation to cold environments. In *Low Temperature Biology of Insects* (pp. 223-241). Cambridge University Press

Hutchinson, L. A. and Bale, J. S. (1994) Effects of sublethal cold stress on the aphid *Rhopalosiphum padi*. *J. Appl. Ecol.* **31**: 102-108.

Irwin, J. T. and Lee, R. E. Jr. (2003) Cold winter microenvironments conserve energy and improve overwintering survival and potential fecundity of the goldenrod gall fly, *Eurosta solidaginis*. *Oikos* **100**, 71-78.

Kellomäki, S., Hänninen, H. and Kolström, M. (1995) Computations on frost damage to scots pine under climatic warning in boreal conditions. *Ecol. Appl.* **5**, 42-52.

Kelty, J. D., Killian, K. A. and Lee, R. E. Jr. (1996) Cold shock and rapid cold-hardening of pharate adult flesh flies (*Sacrophaga crassipalpis*): effects on behaviour and neuromuscular function following eclosion. *Physiol. Entomol.* **21**, 283-288.

Kemp, W. P. and Bosch, J. (2001) Postcocooning temperatures and diapause in the alfalfa pollinator *Megachile rotundata* (Hymenoptera: Megachilidae). *Ann. Entomol. Soc. Am.* **94**, 244–250.

Kight, S. L.**, Batino, M. and Zhang, Z.** (2000) Temperature-dependent parental investment in the giant waterbug *Belostoma flumineum* (Heteroptera: Belostomatidae). *Ann. Entomol. Soc. Am.* **93**, 340–342.

King, E. **G., Roff, D. A., Fairbairn, D. J.** (2011) Trade-off acquisition and allocation in *Gryllus firmus*: a test of the Y model. *J. Evol. Biol.* **24**, 256-264

Kingsolver, J. G., Woods, H. A., Buckley, L. B., Potter, K. A., Maclean, H. J. and Higgins, J. K. (2011) Complex life cycles and the responses of insects to climate change. *Integr. Comp. Biol.* **51**, 719–732.

Klostermeyer, E. C., Mech, S. J. Jr. and Rasmussen, W. B. (1973) Sex and weight of *Megachile rotundata* (Hymenoptera: Megachilidae) progeny associated with provision weights. *J. Kansas Entomol. Soc.* **46**, 536-548.

Krunic, M. D. (1972) Voltinism in *Megachile rotundata* (Megachilidae: Hymenoptera) in southern Alberta. *Can. Entomol.* 104, 185-188.

Lee, R. E. Jr., Chen, C-P. and Denlinger, D. L. (1987) A rapid cold-hardening process in insects. *Science* 238, 1415-1417.

Marshall, K. E. and Sinclair, B. J. (2009) Repeated stress exposure results in a survival-reproduction trade-off in *Drosophila melanogaster*. *Proc. R. Soc. B Biol. Sci.* **277**, 963–969.

Marshall, K. E. and Sinclair, B. J. (2015) The relative importance of number, duration and intensity of cold stress events in determining survival and energetics of an overwintering insect. *Funct. Ecol.* **29**, 357–366.

Mattson, M. P. (2008) Hormesis defined. *Ageing Res. Rev.* **7**, 1–7.

McCorquodale, D. B. and Owen, R. E. (1997) Allozyme variation, relatedness among progeny in a nest, and sex ratio in the leafcutter bee, *Megachile rotundata* (Fabricius) (Hymenoptera: Megachilidae). *Can. Entomol.* **129**, 211-219.

Menu, F., Roebuck, J-P. and Viala, M. (2000) Bet-hedging diapause strategies in stochastic environments. *Am. Nat.* **155**, 724–734.

Mousseau, T. A. and Dingle, H. (1991) Maternal effects in insect life histories. *Annu. Rev. Entomol.* **36**, 511–534.

Mousseau, T. A. and Fox, C. W. (1998) The adaptive significance of maternal effects. *Trends Ecol. Evol.* **13**, 403–407.

O'Neill, K. M., Pearce, A. M., O'Neill, R. P. and Miller, R. S. (2010) Offspring size and sex ratio variation in a feral population of alfalfa leafcutting bees (Hymenoptera: Megachilidae). *Ann. Entomol. Soc. Am.* **103**, 775–784.

Peterson, J. H. and Roitberg, B. D. (2006a) Impact of flight distance on sex ratio and resource allocation to offspring in the leafcutter bee, *Megachile rotundata*. *Behav. Ecol. Sociobiol.* **59**, 589–596.

Peterson, J. H. and Roitberg, B. D. (2006b) Impact of resource levels on sex ratio and resource allocation in the solitary bee, *Megachile rotundata*. *Environ. Entomol.* **35**, 1404–1410.

Pitts-Singer, T. L. and James, R. R. (2005) Emergence success and sex ratio of commercial alfalfa leafcutting bees from the United States and Canada. *J. Econ. Entomol.* **98**, 1785–1790.

Pitts-Singer, T. L. and James, R. R. (2008) Do weather conditions correlate with findings in failed, provision-filled nest cells of *Megachile rotundata* (Hymenoptera: Megachilidae) in Western North America? *J. Econ. Entomol.* **101**, 674–685.

Pitts-Singer, T. L. and Bosch, J. (2010) Nest establishment, pollination efficiency, and reproductive success of *Megachile rotundata* (Hymenoptera: Megachilidae) in relation to resource availability in field enclosures. *Environ. Entomol.*, 39(1), 149-158.

Pitts-Singer, T. L. and Cane, J. H. (2011) The alfalfa leafcutting bee, *Megachile rotundata*: The world's most intensively managed solitary bee. *Annu. Rev. Entomol.* **56**, 221–237.

R Core Team. (2019) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rajon, E., Desouhant, E., Chevalier, M., Débias, F. and Menu, F. (2014) The evolution of bet hedging in response to local ecological conditions. *Am. Nat.* **184**.

Rank, G. H. and Rank, F. P. (1989) Diapause intensity in a French univoltine and a Saskatchewan commercial strain of *Megachile rotundata* (Fab.). *Can. Entomol.* **121**, 141-148.

Ratz, T., Nichol, T. W. and Smiseth, P. T. (2020) Parental responses to increasing levels of handicapping in a burying beetle. *Behav. Ecol.* **31**, 73-80.

Ratz, T., Kramer, J., Veuille, M. and Meunier, J. (2016) The population determines whether and how life-history traits vary between reproductive events in an insect with maternal care. *Oecologia* **182**, 443-452.

Rinehart, J. P., Yocum, G. D. and Denlinger, D. L. (2000) Thermotolerance and rapid cold hardening ameliorate the negative effects of brief exposures to high or low temperatures on fecundity in the flesh fly, *Sacrophaga crassipalpis*. *Physiol. Entomol.* **25**, 330–336.

Rinehart, J. P., Yocum, G. D., West, M. and Kemp, W. P. (2011) A fluctuating thermal regime improves survival of cold-mediated delayed emergence in developing *Megachile rotundata* (Hymenoptera: Megachilidae). *J. Econ. Entomol.* **104**, 1162–1166.

Rinehart, J. P., Yocum, G. D., Kemp, W. P. and Bowsher, J. H. (2016) Optimizing fluctuating thermal regime storage of developing *Megachile rotundata* (Hymenoptera: Megachilidae). *J. Econ. Entomol.* **109**, 993–1000.

Rinehart, J. P., Yocum, G. D., West, M., Kemp, W. P. and Greenlee, K. J. (2013) A fluctuating thermal regime improves long-term survival of quiescent prepupal *Megachile rotundata* (Hymenoptera: Megachilidae). *J. Econ. Entomol.* **106**, 1081–1088.

Roitberg, B. D. and Mangel, M. (2016) Cold snaps, heatwaves, and arthropod growth. *Ecol. Entomol.* **41**, 653–659.

Rossi, B. H., Nonacs, P. and Pitts-Singer, T. L. (2010) Sexual harassment by males reduces female fecundity in the alfalfa leafcutting bee, *Megachile rotundata*. *Anim. Behav.* **79**, 165–171.

Sambaraju, K. R., Carroll, A. L., Zhu, J., Stahl, K., Moore, R. D. and Aukema, B. H. (2012) Climate change could alter the distribution of mountain pine beetle outbreaks in western Canada. *Ecography (Cop.).* **35**, 211–223.

Saunders, D. S. (1966) Larval diapause of maternal origin – II. The effect of photoperiod and temperature on *Nasonia vitripennis*. *J. Insect Physiol.* **12**, 569-581.

Shiomi, K., Takasu, Y., Kunii, M., Tsuchiya, R., Mukaida, M., Kobayashi, M., Sezutsu, H., Ichida Takahama, M. and Mizoguchi, A. (2015) Disruption of diapause induction by TALEN-based gene mutagenesis in relation to a unique neuropeptide signaling pathway in *Bombyx*. *Sci. Rep.* **5**, 1–10.

Simmonds, F. J. (1948) The influence of maternal physiology on the incidence of diapause. *Philos. Trans. R. Soc. Lond. B Biol. Sci*. **233**, 385-414.

Stearns, S. C. (1989) Trade-offs in life-history evolution. *Funct. Ecol.* **3**, 259-268.

Tepedino, V. J. and Parker, F. D. (1988) Alternation of sex ratio in a partially bivoltine bee, *Megachile rotundata* (Hymenoptera: Megachilidae). *Ann. Entomol. Soc. Am.* **81**, 467–476.

Tepedino, V. J. and Frohlich, D. R. (1984a) Fratricide in *Megachile rotundata*, a non-social Megachilid bee: impartial treatment of sibs and non-sibs. *Behav. Ecol. Sociobiol.* **15**, 19-23.

Tepedino, V. J and Frohlich, D. R. (1984b) Fatricide in a parsivoltine bee (*Osmia texana*). *Anim. Behav.* **32**, 1265-1266.

Torchio, P. F. and Tepedino, V. J. (1982) Parsivoltinism in three species of *Osmia* bees. *Psyche* **89**, 221-238.

Torson, A. S., Yocum, G. D., Rinehart, J. P., Nash, S. A. and Bowsher, J. H. (2019) Fluctuating thermal regimes prevent chill injury but do not change patterns of oxidative stress in the alfalfa leafcutting bee, *Megachile rotundata*. *J. Insect Physiol.* **118**, 103935.

Tougeron, K., Brodeur, J., Le Lann, C., Van Baaren, J. (2020) How climate change affects the seasonal ecology of insect parasitoids. *Ecol. Entomol.* **45**, 167-181.

Trostle, G. and Torchio, P. F. (1994) Comparative nesting behavior and immature development of *Megachile rotundata* (Fabricius) and *Megachile apicalis* Spinola (Hymenoptera: Megachilidae). *J. Kansas Entomol. Soc.* **67**, 53-72.

Tuljapurkar, S. and Istock, C. (1993) Environmental uncertainty and variable diapause. *Theor. Popul. Biol.* **43**, 251-280.

Turnock, W. J. and Fields, P. G. (2005) Winter climates and cold hardiness in terrestrial insects. *Eur. J. Entomol.* **102**, 561–576.

Turnock, W. J., Lamb, R. J. and Bobnaryk, R. P. (1983) Effects of cold stress during pupal diapause on the survival and development of *Mamestra configurata* (Lepidoptera: Noctuidae). *Oecologia* **56**, 185-192.

van Noordwijk, A., de Jong, G. (1986) Acquisition and allocation of resources: their influence on variation in life history tactics. *Am. Nat.* **128**, 137-142.

Vaghina, N. P., Voinovich, N. D., Reznik, S. Y. (2014) Maternal thermal and photoperiodic effects on the progeny diapause in *Trichogramma telengai* Sorokina (Hymenoptera: Trichogrammatidae). *Entomol. Sci.* **17**, 198-206.

Velando, A., Drummond, H. and Torres, R. (2006) Senescent birds redouble reproductive effort when ill: confirmation of the terminal investment hypothesis. *Proc. R. Soc. B Biol. Sci.* **273**, 1443–1448.

Yocum, G. D., Rinehart, J. P., West, M. and Kemp, W. P. (2010) Interrupted incubation and short-term storage of the alfalfa pollinator *Megachile rotundata* (Hymenoptera: Megachilidae): A potential tool for synchronizing bees with bloom. *J. Econ. Entomol.* **103**, 234–241.

Yocum, G. D., Zdarek, J., Joplin, K. H., Lee, R. E. Jr., Smith, D. C., Manter, K. D. and Denlinger, D. L. (1994) Alteration of the eclosion rhythm and eclosion behaviour in the flesh fly, *Sarcophaga crassipalpis*, by low and high temperature stress. *J. Insect Physiol.* **40**, 13–21.

CHAPTER 3: EFFECTS OF TEMPERATURE ON METABOLIC RATE DURING METAMORPHOSIS IN THE ALFALFA LEAFCUTTING BEE

Abstract

Spring conditions, especially in temperate regions, can fluctuate abruptly and drastically. Environmental variability can expose organisms to temperatures that are outside of their optimal thermal range. For ectotherms, sudden changes in temperature can cause short- and long-term physiological effects, including changes in respiration, morphology, and reproduction. During active development, insects can be exposed to changes in temperature. The alfalfa leafcutting bee, *Megachile rotundata*, resumes active development after overwintering as prepupae in the spring when temperature can be variable. Oxygen consumption of *M. rotundata* pupae were measured across a large range of temperatures (6-48°C) using a closed optical oxygen sensor system. Absolute and mass specific metabolic rates were calculated for bees that were extracted from their brood cells and those remaining in the brood cell and between sexes. Metabolic response to temperature created a non-linear slope, which is an assumption of a thermal performance curve; however, the predicted negative slope at higher temperatures was not observed. Despite sexual dimorphism in body mass, sex differences only occurred in mass-specific metabolic rates. Understanding the physiological and ecological effects of thermal environmental variability on *M. rotundata* will help to better predict their response to climate change.

Introduction

Temperatures experienced by organisms can greatly vary depending on location, season, and microhabitat. Large temperature fluctuations can have a more dramatic effect on ectotherms, such as insects, because of their reliance on ambient temperature. During the spring when temperatures are particularly variable and unpredictable, many insects are undergoing development and thus, may have to endure these wide ranges of temperatures (von Schmalensee et al., 2021). Even short, abrupt bouts of unfavorable thermal conditions can have lasting effects on developing organisms (Earls et al., 2021; Jentsch et al. 2007; Sgrò et al., 2016; Zizzari and Ellers, 2011).

Depending on its microhabitat, an insect may experience varying thermal profiles, which can differ greatly from large-scale climate data (Sheldon and Dillon, 2016; Woods et al., 2015). Location within plant communities, such as in canopies, on leaves, or in the soil, can protect insects from periods of high temperature (Gols et al., 2021; Pincebourde et al., 2020). For instance, *Manduca sexta* eggs laid on the underside of Jimsonweed leaves (*Datura wrightii*) experience lower temperatures and are protected from high ambient temperatures (Potter et al., 2009). Even the size of the leaf can alter the temperature that insects experience, with smaller leaves having lower temperatures than larger leaves (Potter et al., 2009). Mobile insects are able to move to more ideal microclimates, while immobile insects, such as those undergoing metamorphosis, have to endure temperature changes. For example, acorn-nesting ants (*Temnothorax curvispinosus*) will move brood to the upper proportion of their acorn nests where temperatures are warmer (Karlik et al., 2016). In high temperatures, social insects may begin wing fanning behavior and/or evaporative cooling inside the hive (Cook et al., 2016; Jones and Oldroyd, 2007; Westhus et al., 2013). Physiologically, insects can activate flight muscles in low temperatures to increase body temperature (Kingsolver et al., 2011; Heinrich, 1975; Heinrich and Esch, 1994; Heinrich and Vogt, 1993). However, immobile insects, especially those undergoing metamorphosis inside a coccon, are limited in their ability to avoid exposure to suboptimal temperatures (Kingsolver et al., 2011). It is unclear how oxygen consumption changes in response to temperature in small insects that are at sensitive developing stages.

One way to evaluate thermal effects on insects is by generating a thermal performance curve (TPC) that shows the relationship of insect performance across temperatures (Deutsch et al., 2008, Martin and Huey, 2008, Sinclair et al., 2012). Performance can be measured through various physiological and life history traits that are time-dependent (Schulte et al. 2011). The resulting curve is asymmetric and increases with increasing temperature up to a point. The range of temperatures can be separated into four categories based on the response of the performance metric: suboptimal, optimal, supraoptimal, and lethal. Typically, the range of ambient temperature (T_a) surrounding the top of the performance curve is considered optimal. The relationship of the performance metric gradually decreases with temperatures below the optimum, whereas above the optimum, the performance metric dramatically decreases until temperatures become lethal (Blanckenhorn et al., 2021; Martin and Huey, 2008; Schulte et al., 2011). TPC can differ between species and with the performance metric used (e.g., locomotion or metabolic rate) (Blanckenhorn et al., 2021; Martin and Huey, 2008; Schulte et al., 2011).

Many studies use metabolic rate because it is indicative of changes in energy use. Metabolic rate is the sum of all metabolic processes and indicates the amount of energy production and expenditure of an organism. For ectotherms and poikilotherms, metabolism is tightly linked to ambient temperature (Addo-Bediako et al., 2002; Chown and Gaston, 1999; Deutsch et al., 2008; Harrison and Fewell, 1995; Neven, 2000; Sgrò et al., 2016; Shah et al., 2021; Terblanche et al. 2009). Understanding the effects of unfavorable or suboptimal temperatures on metabolic rate can be important for identifying how ectotherms respond to abrupt changes in environmental conditions.

The alfalfa leafcutting bee, *Megachile rotundata*, is an example of an insect that is restricted in its ability to seek more favorable microclimates because it remains in the cavity where its mother laid it as an egg until adulthood (Trostle and Torchio, 1994). Offspring destined for diapause are exposed to a long series of changes in temperatures as they are confined within their brood cells for 6 – 9 months (Krunic, 1972; Pankiw et al., 1980, Wilson et al., 2021; Yocum et al., 2018). *Megachile rotundata* is an extensively managed solitary bee used for agricultural pollination from Canada and throughout the United States and as such experiences a wide range of temperatures (Pitts-Singer and James, 2005; Pitts-Singer and James, 2008). Previous research has mostly examined survival and delayed development of *M. rotundata* when pupae are subjected to low temperature stress with varying duration (Bennett et al., 2015; Earls et al., 2021; Kemp and Bosch, 2000; Rinehart et al., 2011; Underraga and Stephen, 1980b; Yocum et al., 2010; Yocum et al., 2019). The effects of temperature on *M. rotundata* have and continue to be investigated making it the ideal organism for which to generate a TPC, which could be used to compare the metabolic rate of multiple solitary bee species across a range of body sizes.

Our objectives using oxygen consumption ($\dot{V}O_2$) as the performance metric across temperatures were to **1)** determine if $\dot{V}O_2$ could be accurately measured while pupae remained in their brood cells and **2)** determine sex differences in *M. rotundata* pupae. Our hypothesis was that the pattern of $\dot{V}O_2$ versus temperature is a traditional TPC in male and female *M. rotundata* pupae. Other hypothesis includes that there would be no differences in $\dot{V}O_2$ between pupae left in their brood cells versus extracted. Overwintering *M. rotundata* prepupae were allowed to develop into pupae at which time they were exposed to temperatures ranging from 6-48°C. To better characterize the metabolic profile of *M. rotundata*, multiple comparisons of $\dot{V}O_2$ were made, including between sexes, pupae remaining inside their brood cells and those extracted, age, and absolute versus mass-specific metabolic rate. Measuring responses, such as metabolic rate, to temperature at different life stages can allow researchers to begin to understand how the effects of small-scale changes affect physiological process that can have long-term effects.

Methods

Optical Oxygen Sensor

We measured oxygen consumption using closed-system respirometry with an optical oxygen sensor (Loligo Microplate System ©, Viborg, Denmark). This respirometry system uses glass, 24-well microplates with individual optical fluorescence oxygen sensors attached to each gas-tight well. The resolution of each sensor is ± 0.4% O_2 at 20.9% O_2. For all of the objectives, the two microplates were allowed to equilibrate overnight to the desired temperature. The microplate system was calibrated at each temperature with a two-point calibration using room air and 100% nitrogen. Wells were left open for the room air percent calibration. Wells were individually flushed with N4.8 grade N_2 for 5 minutes at 2000 ml/minute with a mass flow controller (Sierra SideTrak840, Monterey, California, USA). Nitrogen was delivered into each well with a polypropylene adaptor that we designed and milled by a CNC from two blocks of polypropylene that were fused together. To ensure that incurrent gas was the same temperature as the incubator, gases were pushed through copper tubing submerged in a water bath set at the incubator temperature. To ensure the Loligo Microplate System © was reading the correct amount of oxygen, wells were flushed with gas mixes of 21% and 18% O_2 balance N_2 at 25°C using the same method as the N_2 calibration, and percent air saturation was recorded. Percent air saturation was converted to percent oxygen for comparisons.

Closed Respirometry System

To test the precision of the optical sensors, we used a traditional closed system respirometry setup to measure oxygen consumption (Oxzilla FC-2 Differential Oxygen Analyzer, Sable Systems International, Las Vegas, NV, USA) and carbon dioxide emission (Li-Cor-700, LI-COR Biosciences, Lincoln, NE, USA). Twenty bees were measured in both systems at 20°C at the same time. Brood cells were individually placed into gas-tight 3 ml syringes and flushed with dry, CO_2-free air (Balston Puregas system, Haverhill, MA, USA) for ~30 secs at ~200 ml min^{-1}, ensuring that the starting levels of CO_2 in the syringe were near zero. Syringes were then sealed and placed into an incubator at 20°C and left undisturbed for up to nine hours to ensure an adequate O_2 depletion necessary for accurate measurements in the oxygen analyzer. *Megachile rotundata* remained in the syringes longer than the bees in the microplate system due to the differences in vessel size (3 ml syringes versus 940 µl wells). A manual bolus (~1 ml) was injected into the system and pushed through the system with air generated by the Balston Puregas system at 100 ml min^{-1}. Drierite was used to scrub water before entering the CO_2 analyzer, and a combination of Drierite and Ascarite was used to scrub water and CO_2 before entering the O_2 analyzer. Peaks generated by the bolus injection were integrated and converted into milliliters of CO_2 produced and O_2 consumed as described in Lighton (2008), and then converted to ml hr^1 g^{-1} by dividing by the time in the sealed chamber and the wet mass of the individual.

Animal Rearing

Loose brood cells containing post-diapause quiescent *Megachile rotundata* prepupae were purchased from JWM Leafcutters (Nampa, ID, USA) in March of 2020. Brood cells were stored in an incubator (Percival, Perry, IA, USA) at 6°C in complete darkness until use in the experiment. Bee development was initiated by placing bees in a 29°C incubator in a 16 oz plastic container with a fine mesh lid. Bees were allowed to develop for 2 weeks, at which time they reached the red-eye stage, a stage characterized by the darkened eye pigment (Bennett et al., 2015). Bees in the same cohort were extracted prior to being exposed to low temperatures to ensure that they were at the correct pupal stage.

Objective 1

Objective 1 bees were oviposited by females in summer 2019 and stored at 6°C until fall 2020. Red-eye pupae in brood cells (n = 32 per temperature) were haphazardly chosen and removed from the 29°C incubator. Half of the bees (n = 16) were placed in the wells in their brood cells, while the other half were extracted out of their brood cells and placed directly into the microplate wells (940 µl). Eight wells on each plate were left empty to act as controls. Percent air saturation in each well was recorded every 30 seconds at the following temperatures: 6, 10, 14, 18, 22, 26, 29, 33, 35°C, for two hours or until oxygen levels reached 18%. Bees were weighed immediately post exposure and replaced in 29°C in humidity-controlled chambers to finish development so that we could determine sex of each individual.

Objective 2

The experiment was replicated in spring 2021 with more temperatures and younger bees that were oviposited in summer 2020 and stored at 6°C only until spring of the following year. For objective 2, red-eye pupae were left in their brood cells and the number of controls was reduced to four. Brood cells were haphazardly selected and then randomly assigned to wells by MicroResp™ (Loligo Systems, Viborg, Denmark). Percent air saturation in each well was recorded every 30 seconds at the following temperatures: 6, 10, 14, 18, 20, 22, 26, 29, 33, 35, 40, 45, 48°C (n = 40 per temperature). Bees were weighed immediately post exposure and replaced in 29°C to finish developing in humidity-controlled chambers. Sex was determined when bees emerged as adults.

Calculation of Oxygen Consumption

For both objectives 1 and 2, $\dot{V}O_2$ was calculated with the following equations:

Absolute:

$$\dot{V}O_2\left(\frac{\frac{ml}{g}}{hr}\right) = slope\ (\frac{\Delta\%\ air\ saturation}{hr}) \times \frac{0.21}{100} \times\ (940\mu l - bee\ volume - brood\ cell\ volume)\ \times \frac{1ml}{1000ul}$$

Mass specific:

$$\dot{V}O_2\left(\frac{\frac{ml}{g}}{hr}\right) = slope\ (\frac{\Delta\%\ air\ saturation}{hr}) \times \frac{0.21}{100} \times \frac{(940\mu l - bee\ volume -\ brood\ cell\ volume)}{mass\ of\ the\ bee\ (g)} \times \frac{1ml}{1000ul}$$

The slope was calculated using MicroResp™ software (Loligo Systems, Viborg, Denmark) using T_0 as 10 minutes after the wells were sealed until the averaged percent oxygen reached 18% to reduce the effects of oxygen limitation. T_0 was selected to reduce temperature effects of opening and closing the incubator door on the percent oxygen saturation readings. Q_{10} was calculated comparing absolute $\dot{V}O_2$ from 10-20°C, 20-30°C, 30-40°C, and 35-45°C (Neven, 2000).

Statistical Analysis

All statistical analyses were performed in R (Version 1.1.423) (R Core Team, 2019) using the following packages: *drc*, *nlme*, *dplyr*, *car*. Prepupae that died (indicated by no adult emergence) and pupae that were parasitized (indicated by the presence of parasitoid larvae during the pupal stage) were excluded from all analyses. ANOVAs were run for the following comparisons of metabolic rate: effects of temperature, sex, brood cell status. Sex, age, and whether bees were left in brood cells or extracted were treated as covariates. Oxygen consumption was $\log_{10}$ transformed to meet the assumptions of ANOVA. Polynomial curves were compared using ANOVA to determine the best fit. In objective 2, linear regressions were performed for each temperature to compare log mass and absolute $\dot{V}O_2$. Significant regressions were then compared using ANCOVA. T-tests were used to compare differences in $\dot{V}O_2$ between the microplate and fuel cell oxygen analyzer, bee masses between males and females, and bee masses between objectives 1 and 2. Tukey post-hoc comparisons were used to detect significant differences across temperatures and groups. All means are presented ± standard error mean (S.E.M.), and significance is determined by $p < 0.05$.

Results

Closed Respirometry Systems

There was no statistical difference between pupae measured in the microplate system (0.319 ml/g/hr ± 0.012) and the fuel cell analyzer (0.331 ml/g/hr ± 0.011) at 20°C ($t = -0.682$, $df = 25$, $p = 0.502$).

Objective 1

Megachile rotundata that remained in their brood cells and those that were extracted for measurements did not differ in either absolute ($F_{1,290} = 0.423$, $p = 0.516$) or mass-specific ($F_{1,290} = 1.29$, $p = 0.258$) $\dot{V}O_2$; therefore, the treatments were combined for further analysis. Absolute and mass specific $\dot{V}O_2$ were statistically different across temperatures (Absolute: $F_{8,290} = 909$, $p < 0.0001$, Fig 8A; Mass specific: $F_{8,290} = 1264$, $p < 0.0001$, Fig 8B). Metabolic rate increased non-linearly as temperatures increased and were best fit by a 3-degree polynomial curve (Table 1).

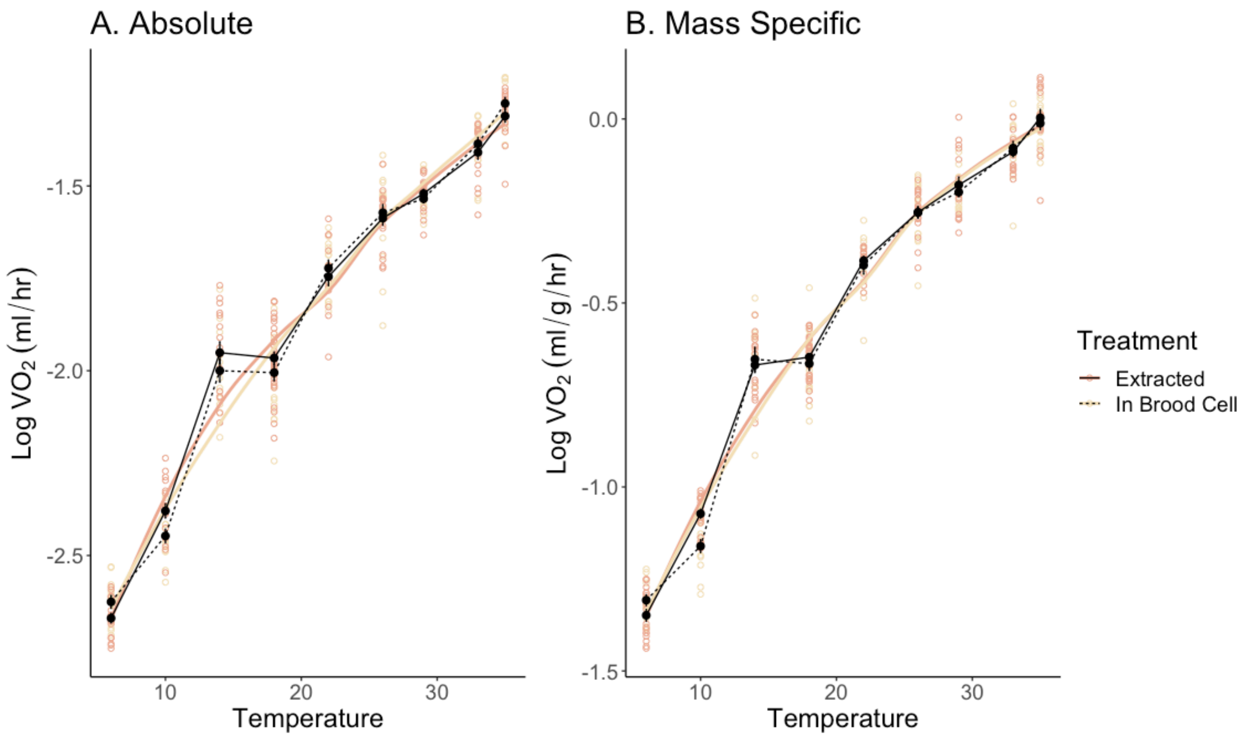

Figure 8. Log transformed $\dot{V}O_2$ for bees left in their brood cells and those that were extracted out during measurements in objective 1. No statistical differences ($p > 0.05$) were detected by ANOVA in absolute **(A)** and mass specific **(B)** rates between bees that were extracted ($n = 16$) or left in their brood cells ($n = 16$). Open circles indicate individual bee measurements, while the black circles are the mean ± SEM.

Objective 2

None of the bees measured at 48°C finished development and were excluded from the analysis and figures, because sex is only confidently measured at the adult stage. Absolute metabolic rate increased across temperatures ($F_{11,445} = 1160$, $p < 0.0001$), but did not differ between sexes ($F_{1,445} = 0.0464$, $p = 0.830$; Fig 10A). Mass as a covariate had a significant effect on absolute metabolic rate ($F_{1,445} = 35.9$, $p < 0.0001$). Males weighed less than female bees ($t = 19.808$, $df = 456$, $p < 0.0001$). Mass specific metabolic rates between males and females were significantly different, except at 14°C and 29°C. All slopes generated in this objective were best fit by a 4-degree polynomial curve (Table 1). A positive relationship between log absolute $\dot{V}O_2$ and log mass was determined in the following temperatures: 22 ($F_{1,33} = 11.7$, $p = 0.0017$, $R^2 = 0.239$), 26 ($F_{1,62} = 12.0$, $p < 0.0001$, $R^2 = 0.149$), 33 ($F_{1,36} = 13.3$, $p < 0.0001$, $R^2 = 0.250$), 40

($F_{1,35} = 5.72$, $p = 0.022$, $R^2 = 0.116$), 45 ($F_{1,35} = 6.11$, $p = 0.018$, $R^2 = 0.124$), and 48°C ($F_{1,38} = 10.2$, $p = 0.0029$, $R^2 = 0.19$; Fig 11). Based on the Tukey posthoc, all of the 6 slopes were significantly different from each other except for 33°C and 40°C ($F_{12,491} = 797$, $p < 0.0001$; Fig 11).

Absolute metabolic rates were compared between both objectives (Fig 9). Generally, objective 2 bees had higher oxygen consumption than objective 1 ($F_{1,673} = 49.3$, $p < 0.0001$). Pupae in objective 1 weighed 4 mg more than pupae in objective 2, (t= 6.55, df = 682, $p < 0.0001$). The average mass for bees in objective 1 was 0.0487 g ± 0.0005 and from objective 2, the average mass was 0.0444 g ± 0.0004.

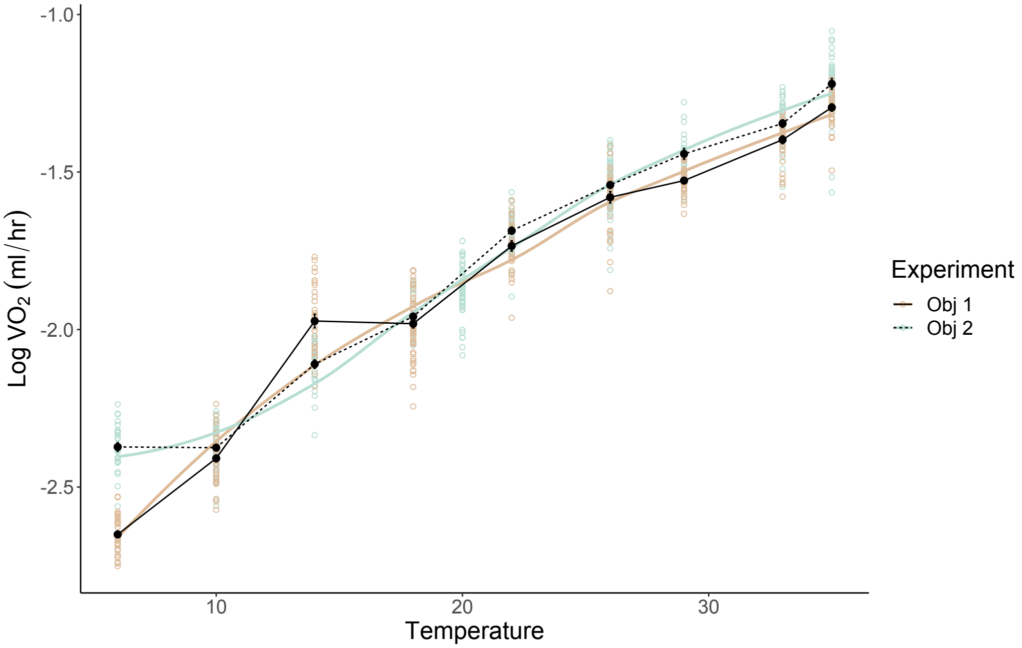

Figure 9. Comparison of log transformed absolute $\dot{V}O_2$ between the first (n = 32 per temperature) and second (n = 40 per temperature) objective. There was a significant difference in absolute $\dot{V}O_2$ between objective 1 and 2 using an ANOVA. Additionally, pupae that were in objective 1 weighed 4 mg more than pupae in objective 2. Open circles indicate individual bee measurements, while the black circles are the mean ± SEM.

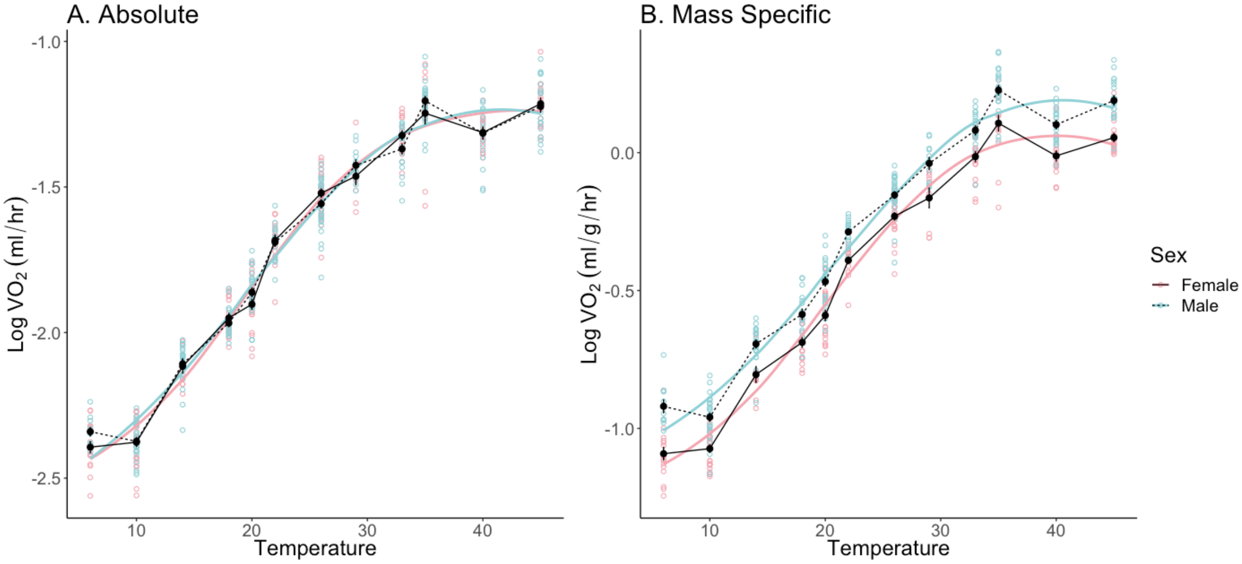

Figure 10. Log transformed $\dot{V}O_2$ for female and male bees measured by the optical oxygen sensor system in objective 2. **A.** No statistical differences ($p > 0.05$) were detected between male and female absolute $\dot{V}O_2$ using an ANOVA across temperatures ($n = 40$ bees per temperature). There was a significant difference in absolute $\dot{V}O_2$ across temperatures. **B.** Differences in mass specific $\dot{V}O_2$ between sexes was significantly different across temperatures ($n = 40$ bees per temperature). The number of males and females differed across temperatures (see methods). Open circles indicate individual bee measurements, while the black circles are the mean ± SEM.

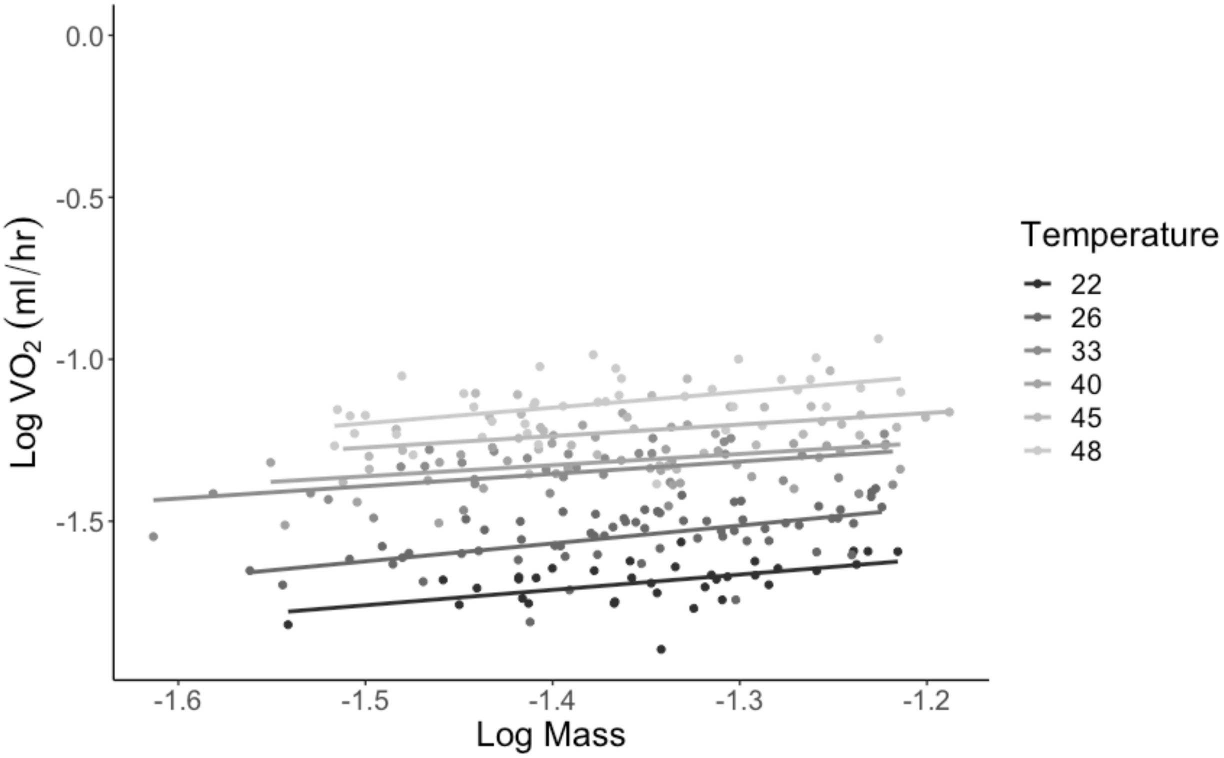

Figure 11. Significant regressions created by temperature treatments. Individual points represent a single M. rotundata pupa (n = 40 per temperature treatment). Individual regressions were performed on all the temperatures to determine which are significant ($p < 0.05$). An ANCOVA was performed on the significant regressions ($F_{5,244} = 255$, $p < 0.0001$). All slopes are significantly different except for 33°C and 40°C.

Table 1. Polynomial slopes for curves in figures 1 and 2. Best fit curves were determined by ANOVA. Reported F and p values are reported values for the regression equations. Rows in light grey are the equations for absolute $\dot{V}O_2$, while rows in white are related to mass specific $\dot{V}O_2$. Sample sizes can be found within the methods section and other figure legends. *** indicates p < 0.0001

Figure	x^4	x^3	x^2	x	Intercept	R^2	F value
1A, 2 Obj1	-	0.0000372	-0.00310	0.119	-3.27	0.944	$F_{3,296} = 1667$***
1B	-	0.0000185	-0.00209	0.104	-1.90	0.954	$F_{3,296} = 2068$***
2 Obj2	0.00000564	-0.000514	0.0163	-0.163	-5.38e-1	0.938	$F_{4,379} = 1460$***
3A	0.00000261	-0.000304	0.0114	-0.117	-2.06	0.937	$F_{4,460} = 1737$***
3B Males	0.00000183	-0.000229	0.00905	-0.0898	-0.726	0.949	$F_{4,244} = 1163$***
3B Females	0.00000257	-0.000309	0.0120	-0.129	-0.707	0.927	$F_{4,210} = 677$***

Table 2. Q_{10} values for absolute metabolic rates in objective 2. Pre-log transformed metabolic rates were used to calculate Q_{10}'s. Absolute metabolic rates for males and females were combined together because there was no statistical difference between the sexes. T = temperature, R = metabolic rate.

T_1	T_2	R_1	R_2	Q_{10}
10	20	0.00428	0.01336	3.119
20	29	0.01336	0.03663	3.067
29	40	0.03663	0.04923	1.308
35	45	0.06168	0.06144	0.9960

Discussion

Ectotherms, such as insects, rely on ambient temperature for body temperature. Previous research looking at development of *M. rotundata* have shown that temperature can affect development rate, lipid consumption, adult flight performance, and reproduction (Bennett et al., 2015; Earls et al., 2021; O'Neill et al., 2011; Yocum et al., 2010). Performance traits, such as metabolic rate, respond non-linearly to temperature generating an asymmetric thermal performance curve (Blanckenhorn et al., 2021, Martin and Huey, 2008; Schulte et al., 2011). The purpose of this study was to determine how $\dot{V}O_2$ of *M. rotundata* pupae respond to changes in temperature. Firstly, our hypothesis that $\dot{V}O_2$ would not be different between bees left in their brood cells versus being extracted was supported. The optical oxygen sensor was sensitive enough to measure $\dot{V}O_2$ while pupae remained in their brood cells. Measuring $\dot{V}O_2$ in developing insects that are highly susceptible to handling stress while in their brood cell opens many possibilities for future experiments. Also, our results show that brood cells are highly permeable for oxygen. Otherwise, results would have shown that extracted bees would have higher $\dot{V}O_2$.

Our hypothesis that $\dot{V}O_2$ across temperatures creates a traditional TPC was mostly supported. Non-linear slopes were observed in both objectives and sexes; however, the predicted negative slope was not observed. This could be due to the length of exposure to each temperature. Exposure may be too brief to see the predicted negative slope and to capture senescence. Only one hour at 40°C is enough to increase heat shock protein expression in *M. rotundata* (HSP70 and HSP90; Yocum et al., 2005). Additionally, survival to the adult stage is greatly diminished after only an hour of exposure at 45°C and no *M. rotundata* survived an hour at 50°C (Undurraga & Stephen, 1980a). None of the pupae in this study measured at 48°C completed melanization or the last molt, even though individuals showed high levels of oxygen consumption while in the optical oxygen sensor system. The sharp decline in $\dot{V}O_2$ may become apparent if the TPC was based on measurements of oxygen consumption at more or longer timepoints, including when individuals have been returned to rearing temperatures.

Typically, males are oviposited laid last at the entrance of the nest. Males have shorter development times and emerge by chewing their way out of their brood cells as adults before females (Pitts-Singer and Cane, 2011). Our results showed that males had higher mass-specific $\dot{V}O_2$ compared to females (Fig. 3B), which was unexpected because males are smaller than females. Previous research suggests that males and females have the same temperature threshold for development (Kemp and Bosch, 2000; O'Neill et al., 2011). Higher mass-specific $\dot{V}O_2$ may suggest that increased metabolic rate is a result of faster development times compared to females.

Even small increases in temperatures above optimal can have lasting effects in other insects. For instance, Asian lady beetles (*Harmonia axyridis*) that experienced high temperatures as eggs had reduced development time, adult survival, and reproduction (Zhang et al., 2014). Short exposures of 2-4 hours to high temperatures (40-45°C) as pupae negatively affected survivorship and reproduction of the almond moth (*Ephestia cautella*) and Indian meal moth (*Plodia interpunctella*). Those short exposures at 50°C also caused complete mortality in males and females of both species of moths (Arbogast, 1981). Similar patterns have been observed in treated pupae, including the red flour beetle (*Tribolium castaneum*) and tobacco hornworm (*Manduca sexta*) (Kingsolver et al., 2011; Mahroof et al., 2005). At the adult stage, high temperature exposure can cause male sterility in *Drosophila buzzatii* and reduced fecundity in *Drosophila melanogaster* (Jørgensen et al., 2006, Krebs and Loeschcke, 1994). The effects of heat stress can also vary depending on life stage exposure. Negative effects on reproduction become more dramatic in later stages of development on fecundity in the diamondback moth (*Plutella xylostella*), rose grain aphid (*Metopolophium dirhodum*) when measuring across life stages (Ma et al., 2004). Supraoptimal and suboptimal temperature ranges can both have long lasting effects on insect physiology.

Suboptimal temperature ranges experienced by developing insects may be larger before becoming lethal compared to high temperatures. However, cold injuries may be acquired during these exposures that can impact morphology and multiple physiological systems, including reproductive, muscular, and nervous systems (Earls et al., 2021; Hedgekar, 1971; Kelty et al., 1996; Rinehart et al., 2000; Turnock et al., 1983). Decreased fecundity due to a low temperature stress has been observed for a few insect species, including aphids (*Rhopalosiphum padi*), fruit flies (*Drosophila melanogaster*) and pea leafminers (*Liriomyza huidobrensis*; Huang et al. 2007; Hutchinson and Bale, 1994; Marshall and Sinclair, 2009). Neuromuscular degradation and changes in behavior and proboscis extension reflex are some cold injuries described in flesh flies (Kelty et al., 1996; Yocum et al., 1994). Insects that are exposed to low temperatures and then returned to more optimal temperatures have been found to have higher metabolic rates than individuals that did not experience low temperatures (Laloutte et al., 2011, Williams et al., 2016). An increase in metabolic rate after exposure to a low temperature could indicate a physiological recovery response (Lalouette et al., 2011; Colinet et al., 2018). This could be tested by measuring post exposure $\dot{V}O_2$ in *M. rotundata* and other insects with the optical oxygen sensor because data is generated in real time.

Clearly, changes in temperature can affect physiological and behavioral traits, such as reproduction, development times, foraging, and dispersal (Andrew et al. 2013). Previous research has shown that *M. rotundata* pupae are sensitive to large changes in temperatures that result in increased mortality, deformities, flight performance, and changes in reproductive investment (Bennett et al., 2015; Earls et al., 2021; Kemp and Bosch, 2000; Rinehart et al., 2011; Underraga and Stephen, 1980a,b; Yocum et al., 2010; Yocum et al., 2019). Constant temperatures were used for short periods of time which may mimic a short exposure to temperature stress. However, the impacts of fluctuating temperatures on metabolic rate could be essential in understanding natural conditions and could be used in predictive models for insect responses to climate change (Faye et al., 2017; Maino et al., 2016; Sheldon and Dillon, 2016). Results from this study show that $\dot{V}O_2$ changes non-linearly across temperatures and that males have higher mass-specific $\dot{V}O_2$ compared to females. Future studies could include the TPC generated in this study to help determine the likelihood of occurrence and mechanisms contributing to negative effects of temperature stress. In conclusion, the TPCs displayed in this study can be used for predictive models in response to climate change and to better understand the physiological and ecological effects of thermal stress.

References

Addo-Bediako, A., Chown, S. L., Gaston, K. J. (2002) Metabolic cold adaptation in insects: a large-scale perspective. *Funct. Ecol.* **16**, 332-338.

Andrew, N. R., Hart, R. A., Jung, M. P., Hemmings, Z., Terblanche, J. S. (2013) Can temperate insects take the heat? A case study of the physiological and behavioural responses in a common ant, *Iridomyrmex purpureus* (Formicidae), with potential climate change. *J. Insect Phsyiol.* **59**, 870-880.

Arbogast, R. T. (1981) Mortality and reproduction of *Ephestia cautella* and *Plodia interpunctella* exposed as pupae to high temperatures. *Environ. Entomol.* **10**, 708-711

Bennett, M. M., Cook, K. M., Rinehart J. P., Yocum, G. D., Kemp, W.P. and Greenlee, K. J. (2015) Exposure to suboptimal temperatures during metamorphosis reveals a critical developmental window in the solitary bee, *Megachile rotundata*. *Physiol. Biochem. Zool.* **88**, 508–520.

Blanckenhorn, W. U., Berger, D., Rohner, P. T., Schäfer, M. A., Akashi, H., Walters, R. J. (2021) Comprehensive thermal performance curves for yellow dung fly life history traits and the temperature-size-rule. *J. of Therm. Biol.* **100**, 103069.

Chown, S. L., Gaston, K. J. (1999) Exploring links between physiology and ecology at macro-scales: the role of respiratory metabolism in insects. *Biol. Rev.* **74**, 87-120.

Cook, C. N, Kaspar, R. E., Flaxman, S. M., Breed, M. D. (2016) Rapid changing environment modulates the thermoregulatory fanning response in honeybee groups. *Anim. Behav.* **115**, 237-243.

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C., Martin, P. R. (2008) Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci.* **105**(18), 6668-6672.

Earls, K. E., Porter, M. S., Rinehart, J. P., Greenlee, K. J. (2021) Thermal history of alfalfa leafcutting bees affects nesting and diapause incidence. *J. Exp. Biol.* **224**(22), jeb243242.

Faye, E., Rebaudo, F., Carpio, C., Herrera, M., Dangles, O. (2017) Does heterogeneity in crop canopy microclimates matter for pests? Evidence from aerial high-resolution thermography. *Agr. Ecosyst. Environ.* **246**, 124-133.

Gols, R., Ojeda-Prieto, L. M., Li, K., van der Putten, W. H., Harvey, J. A. (2021) Within-patch and edge microclimates vary over a growing season and are amplified during a heat wave: Consequences for ectothermic insects. *J. Therm. Biol.* **99**, 103006.

Harrison, J. F., Fewell, J. H. (1995) Thermal effects on feeding behavior and net energy intake in a grasshopper experiencing large diurnal fluctuations in body temperature. *Physiol. Zool.* **68**(3), 453-473.

Hegdekar, B. M. (1971) Wing aberrations induced by precooling pharate adults of the fly *Pseudosarcophaga afinis*. *Can. J. Zool.* **49**, 952.

Heinrich, B. (1975) Thermoregulation in bumblebees. *J. Comp. Physiol.* **96**, 155-166.

Heinrich, B. and Vogt, F. D. (1993) Abdominal temperature regulation by arctic bumblebees. *Physiol. Zool.* **66**, 257-269.

Heinrich, B. and Esch, H. (1994) Thermoregulation in bees. *Am. Sci.*, **82**, 164-170.

Jentsch, A., Kreyling, J., Beierkuhnlein, C. (2007) A new generation of climate-change experiments: events, not trends. *Front. Ecol. Environ.* **5**(7), 365-374.

Jones, J. C., Oldroyd, B. P. (2007) Nest thermoregulation in social insects. *Adv. Insect Physiol.* **33**, 154-191.

Jørgensen, K. T., Sørensen, J. G., Bundgaard, J. (2006) Heat tolerance and the effect of mild heat stress on reproductive characters in *Drosophila buzzatii* males. *J. Therm. Biol.* **31**, 280-286.

Karlik, J., Epps. M. J., Dunn, R. R., Penick, C. A. (2016) Life inside an acorn: How microclimate and microbes influence nest organization in *Temnothorax* ants. *Ethology* **122**, 790-797.

Kelty, J. D., Killian, K. A. and Lee, R. E. Jr. (1996) Cold shock and rapid cold-hardening of pharate adult flesh flies (*Sacrophaga crassipalpis*): effects on behaviour and neuromuscular function following eclosion. *Physiol. Entomol.* **21**, 283-288.

Kemp, W. P., Bosch, J. (2000) Development and emergence of the alfalfa pollinator *Megachile rotundata* (Hymenoptera: Megachilidae). *Ann. Entomol. Soc. Am.* **93**(4), 904-911.

Kingsolver, J. G., Woods, H. A., Buckley, L. B., Potter, K. A., MacLean, H. J., Higgins, J. K. (2011) Complex life cycles and responses of insects to climate change. *Integr. Comp. Biol.* **51**(5), 719-732.

Krebs, R. A., Loeschcke, V. (1994) Costs and benefits of activation of the heat-shock response *in Drosophila melanogaster*. *Funct. Ecol.* 8(6), 730-737

Lalouette, L., Williams, C. M., Hervant, F., Sinclair, B. J., Renault, D. (2011) Metabolic rate and oxidative stress in insects exposed to low temperature thermal fluctuations. *Comp. Biochem. Phys. A* **158**(2), 229-234.

Ma, C. S., Hau, B, Poehling, H. M. (2004) Effects of pattern and timing of high temperature exposure on reproduction of the rose grain aphid, *Metopolophium dirhodum*. *Entomol. Exp. Appl.* **110**, 65-71.

Maino, J. L., Kong, J. D., Hoffmann, A. A., Barton, M. G., Kearney, M. R. (2016) Mechanistic models for predicting insect responses to climate change. *Curr. Opin. Insect Sci.* **17**, 81-86.

Mahroof, R., Subramanyam, B. Flinn, P. (2005) Reproductive performance of *Tribolium castaneum* (Coleoptera: Tenebrionidae) exposed to the minimum heat treatment temperature as pupae and adults. *J. Econ. Entomol.* **98**(2), 626-633.

Martin, T. L., Huey, R. B. (2008) Why "suboptimal" is optimal: Jensen's inquality and ectotherm thermal preferences. *Am. Nat.* **171**(3), E102-E118.

Neven, L. G. (2000) Physiological responses of insects to heat. *Postharvest Biology and Technology* **21**(1), 103-111.

O'Neill, K. M., O'Neill, R. P., Kemp, W. P., Delphia, C. M. (2011) Effect of temperature on post-overwintering development and total lipid content of alfalfa leafcutting bees. *Environ. Entomol.* **40**(4), 917-930.

Owings, A. A., Yocum, G. D., Rinehart, J. P., Kemp, W. P., Greenlee, K. J. (2014) Changes in respiratory structure and function during post-diapause development in the alfalfa leafcutting bee, *Megachile rotundata*. *J. Insect Physiol.* **66**, 20-27.

Pincebourde, S., Dillon, M. E., Woods, H. A. (2021) Body size determines the thermal coupling between insects and plant surfaces. *Func. Ecol.* **35**, 1424-1436.

Pitts-Singer, T. L., James, R. R. (2005) Emergence success and sex ratio of commercial alfalfa leafcutting bees from the United States and Canada. *J. Econ. Entomol.* **98**(6), 1785-1790.

Pitts-Singer, T. L., James, R. R. (2008) Do weather conditions correlate with findings in failed, provision-filled nest cells of *Megachile rotundata* (Hymenoptera: Megachilidae) in Western North America? *J. Econ. Entomol.* **101**(3), 674-685.

Potter, K., Davidowitz, G., Woods, H. A. (2009) Insect eggs protected from high temperatures by limited homeothermy of plant leaves. *J. Exp. Biol.* **212**, 3448-3454.

R Core Team. (2019) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rinehart, J. P., Yocum, G. D. and Denlinger, D. L. (2000) Thermotolerance and rapid cold hardening ameliorate the negative effects of brief exposures to high or low temperatures on fecundity in the flesh fly, *Sacrophaga crassipalpis*. *Physiol. Entomol.* **25**, 330–336.

Rinehart, J. P., Yocum, G. D., West, M. and Kemp, W. P. (2011) A fluctuating thermal regime improves survival of cold-mediated delayed emergence in developing *Megachile rotundata* (Hymenoptera: Megachilidae). *J. Econ. Entomol.* **104**, 1162–1166.

Sgrò, C. M., Terblanche, J. S., Hoffmann, A. A. (2016) What can plasticity contribute to insect responses to climate change. *Annu. Rev. Entomol.* **61**, 433-451.

Shah, A. A., Woods, H. A., Havird, J. C., Encalada, A. C., Flecker, A. S., Funk, W. C., Guayasamin, J. M., Kondratieff, B. C., Poff, N. L., Thomas, S. A., Zamudio, K. R., Ghalambor, C. K. (2021) Temperature dependence of metabolic rate in tropical and temperate aquatic insects: support for the climate variability hypothesis in mayflies but not stoneflies. *Glob. Change Biol.* **27**, 297-311.

Sheldon, K. S., Dillon, M. E. (2016) Beyond the mean: Biological impacts of cryptic temperature change. *Integr. Comp. Biol.* **56**, 110-119.

Schulte, P. M., Healy, T. M., Fangue, N. A. (2011) Thermal performance curves, phenotypic plasticity, and the time scales of temperature exposure. *Integr. Comp. Biol* **51**(5), 691-702.

Sinclair, B. J., Williams, C. M., Terblanche, J. S. (2012) Variation in thermal performance among insect populations. *Physiol. Biochem. Zool.* **85**(6), 594-606.

Terblanche, J. S., Clusella-Trullas, S., Deere, J. A., Van Vuuren, B. J., Chown, S. L. (2009) Directional evolution of the slope of the metabolic rate-temperature relationship is correlated with climate. *Physiol. Biochem. Zool.* **82**(5), 495-503.

Trostle, G., Torchio, P. F. (1994) Comparative nesting behavior and immature development of *Megachile rotundata* (Fabricius) and *Megachile apicalis* Spinola (Hymenoptera: Megachilidae). *J. Kansas Entomol. Soc.* **67**, 53-72.

Turnock, W. J., Lamb, R. J. and Bobnaryk, R. P. (1983) Effects of cold stress during pupal diapause on the survival and development of *Mamestra configurata* (Lepidoptera: Noctuidae). *Oecologia* **56**, 185-192.

Undurraga, J. M., Stephen, W. P. (1980a) Effect of temperature on development and survival in post-diapausing alfalfa leafcutting bee prepupae and pupae (*Megachile rotundata* (F.): Hymenoptera: Megachilidae). I. High temperatures. *J. Kansas Entomol. Soc.* **53**(3), 669-676.

Undurraga, J. M., Stephen, W. P. (1980b) Effect of temperature on development and survival in post-diapausing alfalfa leafcutting bee prepupae and pupae (*Megachile rotundata* (F.): Hymenoptera: Megachilidae). I. Low temperature. *J. Kansas Entomol. Soc.* **53**(3), 677-682.

von Schmalensee, L., Gunnarsdóttir, K. H., Näslund, J., Gotthard, K., Lehmann, P. (2021) Thermal performance under constant temperatures can accurately predict insect development times across naturally variable microclimates. *Ecol. Lett.* **24**, 1633-1645.

Westhus, C., Kleineidam, C. J., Roces, F., Weidenmüller, A. (2013) Behavioural plasticity in the fanning response of bumblebee workers: impact of experience and rate of temperature change. *Animal Behaviour* **85**, 27-34.

Williams, C. M., Szejner-Sigal, Morgan, T. J., Edison, A. S., Allison, D. B., Hahn, D. A. (2016) Adaptation to low temperature exposure increases metabolic rates independently of growth rates. *Integr. Comp. Biol.* **56**, 62-72.

Wilson, E. S., Murphy, C. E., Wong, C., Rinehart, J. P., Yocum, G. D., Bowsher, J. H. (2021) Environmental impacts on diapause and survival of the alfalfa leafcutting bee, *Megachile rotundata*. *PLoS ONE* **16**(8), e0254651.

Woods, H. A., Dillon, M. E., Pincebourde, S. (2015) The roles of microclimatic diversity and of behavior in mediating the response of ectotherms to climate change. *J. Therm. Biol.* **54**, 86-97.

Yocum, G. D., Childers, A. K., Rinehart, J. P., Rajamohan, A., Pitts-Singer, T. L., Greenlee, K. J., Bowsher, J. H. (2018) Environmental history impacts gene expression during diapause development in the alfalfa leafcutting bee*, Megachile rotundata*. *J. Exp. Biol.* **221**(13), jeb173443.

Yocum, G. D., Kemp, W. P., Bosch, J., Knoblett, J. N. (2005) Temporal variation in overwintering gene expression and respiration in the solitary bee *Megachile rotundata*. *J. Insect Physiol.* **51**(6), 621-629.

Yocum, G. D., Rinehart, J. P., West, M. and Kemp, W. P. (2010) Interrupted incubation and short-term storage of the alfalfa pollinator *Megachile rotundata* (Hymenoptera: Megachilidae): A potential tool for synchronizing bees with bloom. *J. Econ. Entomol.* **103**, 234–241.

Yocum, G. D., Rinehart, J. P., Rajamohan, A., Bowsher, J. H., Yeater, K. M., Greenlee, K. J. (2019) Thermoprofile parameters affect survival of *Megachile rotundata* during exposure to low temperatures. *Integr. Comp. Biol.* **59**(4), 1089-1102.

Yocum, G. D., Zdarek, J., Joplin, K. H., Lee, R. E. Jr., Smith, D. C., Manter, K. D. and Denlinger, D. L. (1994) Alteration of the eclosion rhythm and eclosion behaviour in the flesh fly, *Sarcophaga crassipalpis*, by low and high temperature stress. *J. Insect Physiol.* **40**, 13–21.

Zhang, S., Cao, Z., Wang, Q., Zhang, F., Liu, T-X. (2014) Exposing eggs to high temperatures affects the development, survival and reproduction of *Harmonia axyridis*. *J. Therm. Biol.* **39**, 40-44

Zizzari, Z. A., Ellers, J. (2011) Effects of exposure to short-term heat stress on male reproductive fitness in a soil arthropod. *J. Insect Physiol.* **57**, 421-426.

CHAPTER 4: MICROCLIMATE AND SEASON AFFECT FLIGHT INITIATION IN THE ALFALFA LEAFCUTTING BEE

Abstract

Small-bodied organisms, such as insects, experience a mosaic of abiotic conditions as they move throughout the landscape. While weather conditions are often reported for a large area, temperature and other abiotic factors vary on a finer scale. For insects that nest in cavities, the temperatures in and around the cavity may be important for flight initiation. These cavities can expose nesting bees to conditions that differ from broad weather readings and may affect when or if bees fly. The purpose of this study is to determine which environmental factors that influence flight in early and late summer are most important and how altering internal cavity temperatures contributes to flight initiation in a cavity nesting bee, *Megachile rotundata*. Two nest boxes were created to monitor flight initiation. The first nest box had no manipulations and recorded several abiotic environmental conditions, including multiple temperatures, light, wind speed, and humidity. The second nest box measured the same conditions but was divided so that half of the cavities were heated, and half were allowed to fluctuate with ambient temperature. First flight for individual bees was recorded and measured from 7-11am daily. A hierarchical cluster analysis showed that early or late summer was an important factor for flight initiation. All the environmental variables at the time of first flight were significantly different between early and late season except for air pressure. Early in the season, temperatures inside and at the face of the box did not differ from each other but were higher than ambient readings. Heating the nest cavities promoted flight at lower ambient temperatures. Additionally, bees nesting in the heated section of the nest box flew earlier than bees in the control section. Abiotic environmental

conditions affecting first flight varies across the season and altering the microclimate by increasing cavity temperature promotes earlier flight at lower ambient temperatures.

Introduction

Abiotic environmental conditions differ across various scales. Macro- and mesoclimates consist of environmental conditions over large scales (>200 kilometers; 1-200 km), such as atmospheric conditions, while microclimate are more localized over a much smaller location (0.001-1km; Duffy et al., 2015; Pincebourde and Woods, 2020). The conditions experienced at the smaller scale (e.g., air temperature or wind speed) differ from conditions at the macroscale (Duffy et al., 2015; Pincebourde and Woods, 2020). Differences in conditions may have a larger impact on smaller organisms, such as insects, that are small and occupy smaller spaces compared to bigger organisms (Pincebourde et al., 2021). In the winter, temperatures often vary up 30°C with the bark surface of a tree much warmer than the ambient temperature (Stoutjesdijk, 1977). This pattern may be exacerbated when considering that a weather station may be kilometers away from a field site over varying topography. In high temperature conditions, *Manduca sexta* eggs experience lower temperatures on the underside of leaves where humidity is also higher, preventing water loss (Potter et al., 2009, Smith, 1978; Woods, 2010). Flower morphology also affects the microclimate pollinators experience during visitations by having higher or lower than ambient temperatures around reproductive structures (Shrestha et al., 2018). For example, when ambient temperatures are low, blooming flowers provide heat to visiting pollinators which may promote flight in otherwise unfavorable conditions (Dyer, et al., 2006; Herrera, 1995a,b). For cavity nesting insects, the microclimate of their nest may be important for flight initiation.

The alfalfa leafcutting bee, *Megachile rotundata*, is a solitary bee that nests gregariously in natural and artificial cavities. Nesting cavities are small, ranging from 5-7mm in diameter, and near forage (< 200 meters; Bradner et al., 1965; Gerber and Klostermeyer, 1970; Pankiw and Siemens, 2012; Peterson and Roitberg, 2016). Within the cavities, females construct brood cells from cut leaf pieces where they place pollen and nectar provisions along with an oviposited egg (Pitts-Singer and Cane, 2011). Microclimate conditions directly around and inside nesting cavities may also influence *M. rotundata* flight activity. For instance, temperature within the nest cavities affects nest selection in *M. rotundata* during the summer (Wilson et al., 2020). Using artificial nesting boxes facing different directions, females preferred to nest in cavities facing north, which had the lowest average temperatures (Wilson et al., 2020). Females reliably return to the same nesting cavity even when its location has been manipulated (Fauria et al., 2004), suggesting that nest selection is an important factor. Females remain in the nesting cavity during the night and emerge in the morning to bask in the sunlight presumably to increase their body temperature (Pitts-Singer and Bosch, 2010).

Ambient temperature and light are important factors for foraging activity in alfalfa fields (Lerer et al., 1982). Temperature also restricts where *M. rotundata* can be used for alfalfa pollination. For instance, *M. rotundata* has been shown to be an excellent pollinator of lowbush blueberry; however, low temperatures in northeastern North America limit flight and therefore, pollination services (Javorek, 1996; Javorek et al., 2002). In addition, low temperatures are associated with an increased likelihood of developing fungus within the brood cells increasing offspring mortality (Pitts-Singer and James, 2008). Contrarily, developing offspring that experience higher temperatures during the summer are also less likely to survive (CaraDonna et al., 2018; Pitts-Singer and James, 2008). Previous researchers used event recorders at the nest entrance to measure and describe nesting behavior while correlating it with macroclimate conditions, such as ambient temperature and light (Klostermeyer and Geber, 1969). However, it is currently unknown how the microclimate in and around the nesting cavities affects flight in *M. rotundata*.

The purpose of this study was to determine 1) which abiotic factors influence first flight and 2) whether increasing nest box temperature would affect flight. For the first objective, a Styrofoam nest box was outfitted with several sensors to measure abiotic microclimate factors and a GoPro camera to record bee flight for 4 hours every morning, both early and late in the summer. We predicted that multiple environmental factors would be important in determining first flight, such as the time of the season. For the second objective, we manipulated the temperature of the nesting box. We hypothesized that warmer microclimates in the nesting box promote flight when ambient temperatures are lower. We predicted that females in the warmer nesting box would fly earlier in the day in lower ambient temperatures compared to females in unheated nest boxes. Results from this study will be helpful for understanding the effects of microclimate on activity in *M. rotundata* and other cavity nesting insects, while using a novel design that can be adapted to other organisms to measure microclimate.

Materials and Methods

Animal Rearing

Megachile rotundata prepupae were purchased from JWM Leafcutters (Nampa, ID, USA) as loose brood cells. Prepupae were stored in constant darkness at 6°C in an incubator (Percival, Perry, IA, USA). Brood cells were moved to a 29°C incubator (Percival, Perry, IA, USA) in 16-ounce, plastic containers covered with fine mesh lids to initiate development. Adult emergence was checked daily. Bees that emerged in the morning were released later that day at one of the nesting boxes. Females were marked with unique paint patterns on their thorax, so that individuals could be identified in the videos (Sharpie® Oil-Based Paint Markers, Atlanta, Georgia).

Weather Station Activity Monitor

To monitor activity, I built a nesting box outfitted with a series of sensors to measure abiotic environmental variables and a GoPro camera to record first flight of nesting *M. rotundata* (Fig. 12). Nest boxes were constructed from commercial Styrofoam blocks (13 cm x 8 cm x 12cm; Beaver Plastics, Acheson, Alberta, Canada) with 7mm holes in a 12 x 12 grid (144 total holes). Each cavity was lined with a paper straw so that nests could be easily removed. Completed nests were removed to provide more nesting opportunities to females. The nest boxes were placed in the back center of a wooden shelter (64 centimeters x 64 centimeters x 64 centimeters) with an open front. Activity at the box was recorded with the GoPro camera (1080p at 30fps, HERO5, GoPro, San Mateo, California, USA) controlled by a BlinkX controller (CamDo Solutions Inc., Vancouver, British Columbia, Canada) powered by an external solar battery pack (Voltaic Systems, Brooklyn, New York, USA). The camera, electronics, and computing equipment were housed in a weatherproof container (MTM Molded Products Co., Dayton, Ohio, USA), which was placed on top of the wooden shelter (Fig. 12). The small section of the weatherproof container bottom was cut out and replaced with clear acrylic to create a window looking down at the nest box. The edges were sealed to keep the container weatherproof. The camera was positioned in the weatherproof container facing the front of the nest box and the BlinkX controller was programmed so that the camera recorded from 7:00 am to 11:00 am central daylight time. A digital clock was positioned in the view of the camera to record time of day because the camera did not support a timestamp on the video. Flight was recorded only in the morning because that is when the biggest increases in temperature and light occur. Also, we wanted to ensure that bee flight was only affected by environmental conditions and not by previous flight activity during the day. All video was recorded to a microSD card. Longer recording times were not possible because of battery power and data storage limits.

The weatherproof container also housed an Arduino UNO (Arduino, Ivrea, Italy) connected to several sensors measuring environmental variables (Fig. 12): 3 different temperature locations (°C), light (lux; TSL2561), humidity (percent; SHT-30 mesh-protected sensor), wind speed (meters per second), and air pressure (pascals; BMP085). All sensors were from Adafruit Industries (New York, NY, USA), except the hot wire anemometer used for measuring wind speed (Modern Device, Brooklyn, NY, USA). The thermocouples inside the nesting box and at the opening of the cavities (face temperature) were type-K thermocouples attached to MAX31855 amplifier boards, while the ambient temperature sensor was built into the SHT-30 mesh-protected sensor that also recorded humidity. We 3D-printed a Stevenson screen to protect sensors from solar radiation and precipitation. The Stevenson screen housed one temperature and air pressure sensor and the humidity sensor and was secured to the wooden shelter (Fig. 12). The second thermocouple was placed in the center of the nest box inside a nesting cavity that was sealed to preventing nesting, while the third was positioned on the front of the box to measure temperature with the influence of solar radiation. A previous study using 3D printed nest boxes showed that internal cavity temperatures can vary 1.2°C across the box when faced southeast like the boxes in this study (Wilson, et al., 2021). Data were recorded every minute to an SD card.

Three identical activity boxes were placed in an alfalfa field from June 29th-September 5th, 2019. Twenty, individually-marked females and ten, unmarked males were released at each nest box twice during the season. The first cohort (early season) was released on June 29th and observed until July 15th, and the second cohort (late season) was released on August 18th and observed until September 5th. Data from SD and microSD cards were collected every week and transferred to external hard drives (Drobo, Inc., Sunnyvale, California, USA) for long-term storage.

To determine an individual's first flight, videos were played back at 2x speed until a flight event was observed. The time of first flight and bee color (ID) was recorded for each flight. Data on environmental variables were downloaded as .csv files and stored on an external hard drive (Drobo, Inc., Sunnyvale, California, USA).

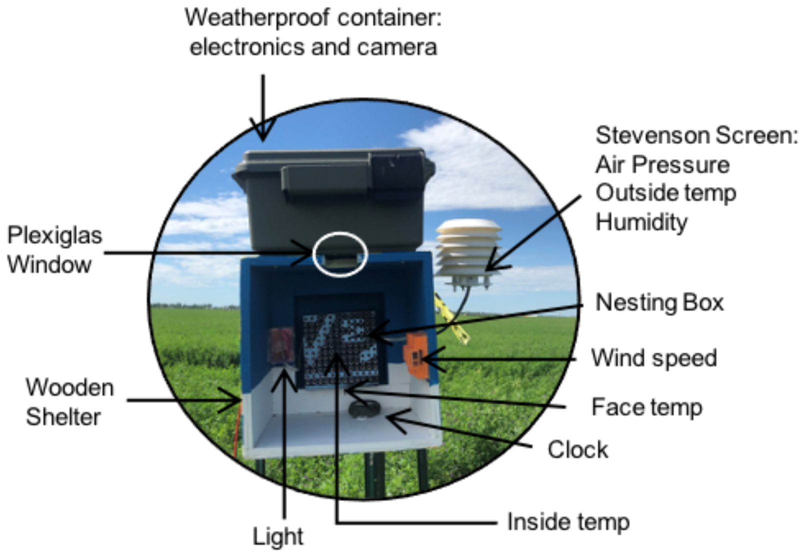

Figure 12. Diagram of the weather station activity monitor. Arrows indicate the sensor placement and the environmental variable recorded. Electronic components were located in the weatherproof container with the camera.

Heated Microclimate

To determine if increased temperatures would encourage earlier flight in lower ambient temperatures, we used a similar activity monitor as described above with several modifications. Instead of using purchased Styrofoam, nest blocks were made using conductive aluminum tubes and plates to allow for heat transfer. The nest box had 15 nesting cavities (3 holes x 5 holes; Fig.

13) made of aluminum that were anchored to an aluminum plate. The aluminum panel was heated by a heating pad (Adafruit Industries, New York, NY, USA). The metal tubing and heating pad were enclosed in wood panels and painted to look like a nest box. Hollow spaces were filled with spray foam to insulate the tubes and prevent heat transfer within the nest. The heating pad and thermocouples were controlled by Arduino Uno boards and programmed in Arduino. An additional motor shield (Adafruit Industries; New York, NY, USA) was added to accommodate the higher power requirements to heat the metal. The heating pad was programmed to heat the nest box to 30°C at 6 am so that the nest temperature would be higher than ambient temperature after sunrise. Each heated nest box was paired with a control nest box made out of the same materials but not heated. The two nest boxes were housed in an open-faced, wooden shelter as described above. Additional foam insulation was placed between the heated and control nest boxes to prevent heat transfer. GoPro cameras were placed above the nest box pairs and controlled as described above. Thermocouples recorded temperatures at the center of the nest box and the ambient temperature every minute. Three, replicated nest box pairs were placed in an alfalfa field from August 7th-26th, 2020. An excess of unmarked females (n = 40) and males (n = 20) were released at each pair of boxes to ensure nesting due to multiple attempts with no nesting establishment. Lower sample sizes (n = 20 females, n = 10 males) were used the previous year with little to no nesting.

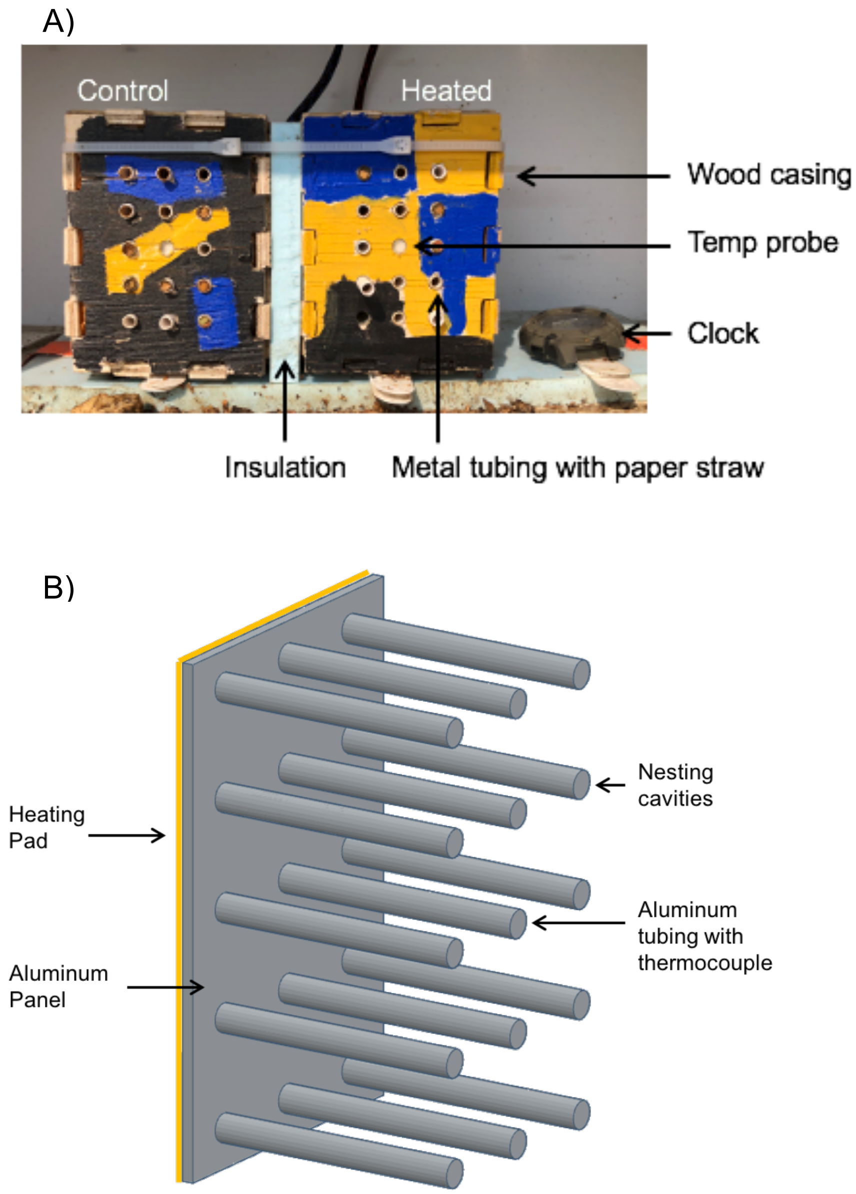

Figure 13. A) Diagram of the heated and control nest boxes. Arrows indicate differences in design compared to the weather station activity box (Figure 12). **B)** The internal aluminum structure within the wooden nest boxes. Aluminum tubes used for cavities were anchored to an aluminum panel in the back, which was heated using an Adafruit heating pad. The center tube had a thermocouple to measure internal temperature. The cavity was then closed to prevent nesting.

Statistical Analysis

Data were analyzed in R (Version 1.1.423; R Core Team, 2019) using the following packages: *car*, *dplyr*, *lmer4*, *plyr*. All data were assessed for normality and equal variance. All data are presented as mean ± standard error of the mean (S.E.M.), and significance was determined as $p < 0.05$. Six days in July were not included in the analysis because of a failed battery that resulted in no recorded data. A hierarchical cluster analysis was performed in JMP to determine the leading variables affecting flight. The leading variable was whether bees were measured in early or late season; therefore, variables were compared between early and late season using a nonparametric t-test, except temperature which was compared using an ANOVA. Tukey posthoc tests were performed to determine which treatments were significantly different.

The environmental variables at first flight were compared between the control and heated nesting boxes. To analyze the temperatures at first flight, an unpaired two-sample Wilcoxon test was used for both ambient and internal nest box temperature between the control and heated nesting boxes. An ANCOVA was performed to compare changes in temperature for both the ambient and internal temperatures across the four-hour window using three days of sampling. An additional Wilcoxon test was used to compare the time of first flight between bees from the control and heated boxes.

Results

Weather Station Activity Monitor

The hierarchical cluster analysis determined that season was the leading variable affecting flight conditions; therefore, environmental variables were compared between early and late season. Later in the season, light readings at first flight were higher ($W = 1554$, $p < 0.0001$; Fig. 15A), while humidity at first flight was lower ($W = 3572$, $p = 0.006$, Fig. 15B). Wind speed at first flight was also higher in late season ($W = 2066$, $p = 0.004$; Fig. 15C). Air pressure readings at first flight did not significantly differ between the early and late seasons ($W = 3217$, $p = 0.161$; Fig. 15D). There was a significant interaction between early and late season with the location of the temperature probe ($F_{2,2275} = 114$, $p < 0.0001$; Fig. 14). A Tukey posthoc test determined that there was no significant difference between inside and the face of the box early in the season.

Heated Microclimate

During the 4-hour recording, temperatures measured inside the heated nest box were higher than those of the control box ($F_{1,2863} = 753$, $p < 0.0001$; Fig. 16). Temperatures in both boxes increased linearly due to solar heating, with the control box heating faster than the heated box (Fig. 16). At first flight, the internal nesting box temperatures were higher in the heated box compared to the control box ($W = 5292$, $p < 0.0001$; Fig. 17A). Bees that were nesting in the heated boxes flew at lower ambient temperatures compared to bees nesting in the control boxes ($W = 11281$, $p = 0.0013$; Fig. 17B). Also, bees nesting in the heated nesting box flew earlier compared to control nesters ($W = 13450$, $p < 0.0001$; Fig. 18). Overall, there were more first flight observations of bees nesting in the heated box ($n = 167$) compared to the control ($n = 110$; Fig. 18).

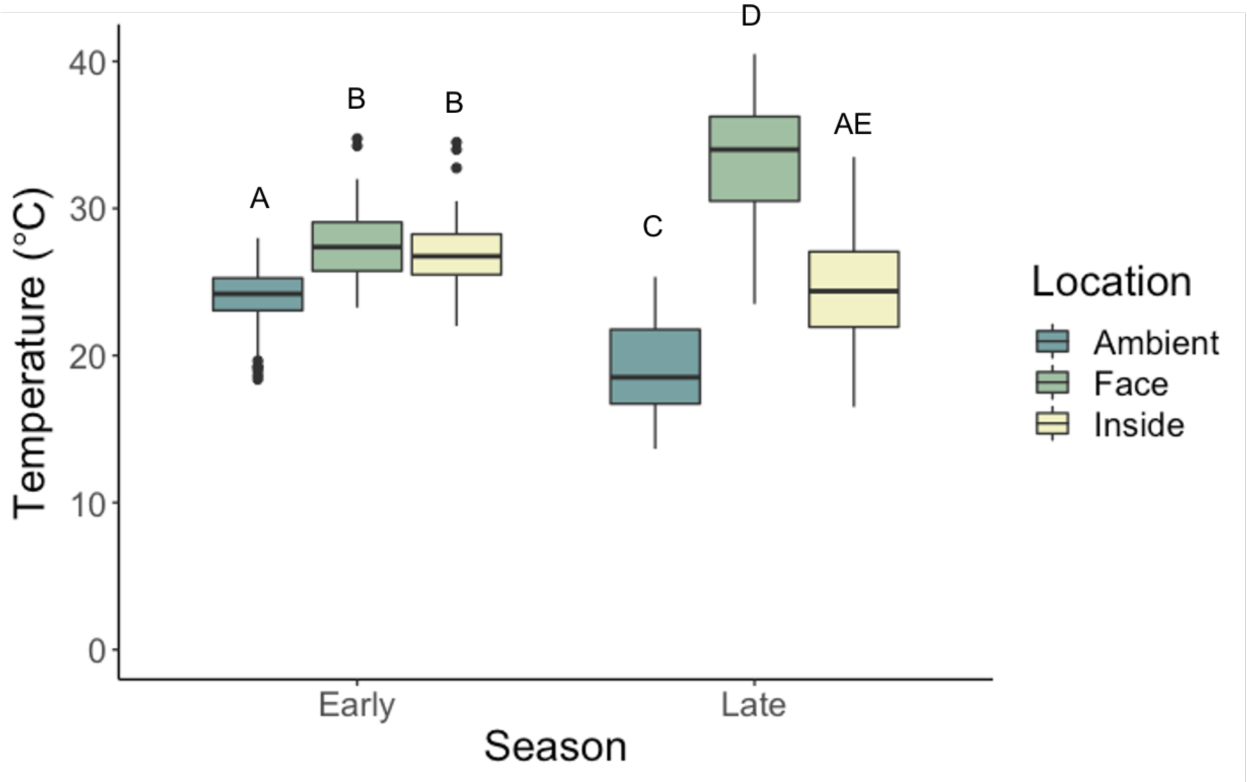

Figure 14. Temperature (°C) at first flight at the three thermocouple locations for early and late season bees. Boxes represent the 1st to 3rd quartile, while the lines extending from the box show the 25th and 75th percentiles of the data. The median is indicated by the line in the center of the box. Boxplots that share at least one letter above them are not significantly different by treatment.

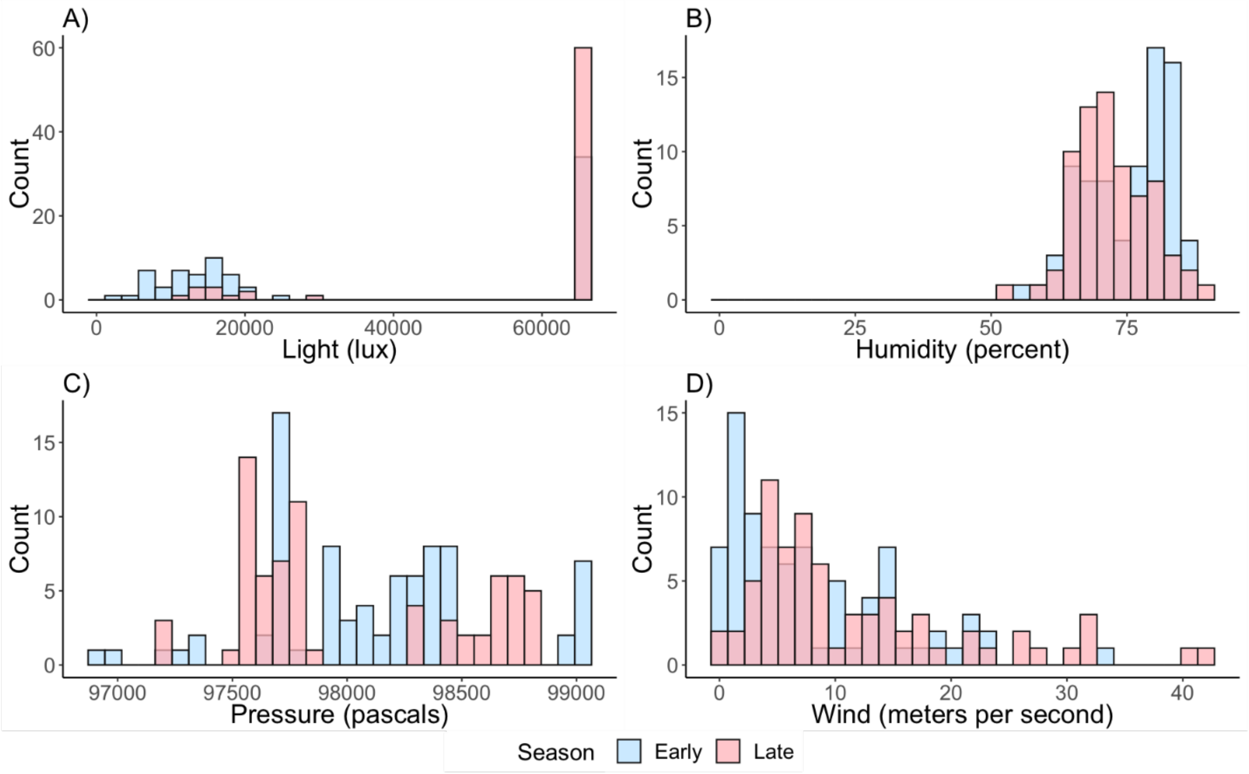

Figure 15. Distribution comparing first flights of M. rotundata in early and late seasons across the following environmental variables: **A)** light (lux), **B)** humidity (percent), **C)** pressure (pascals), and **D)** wind (meters per second).

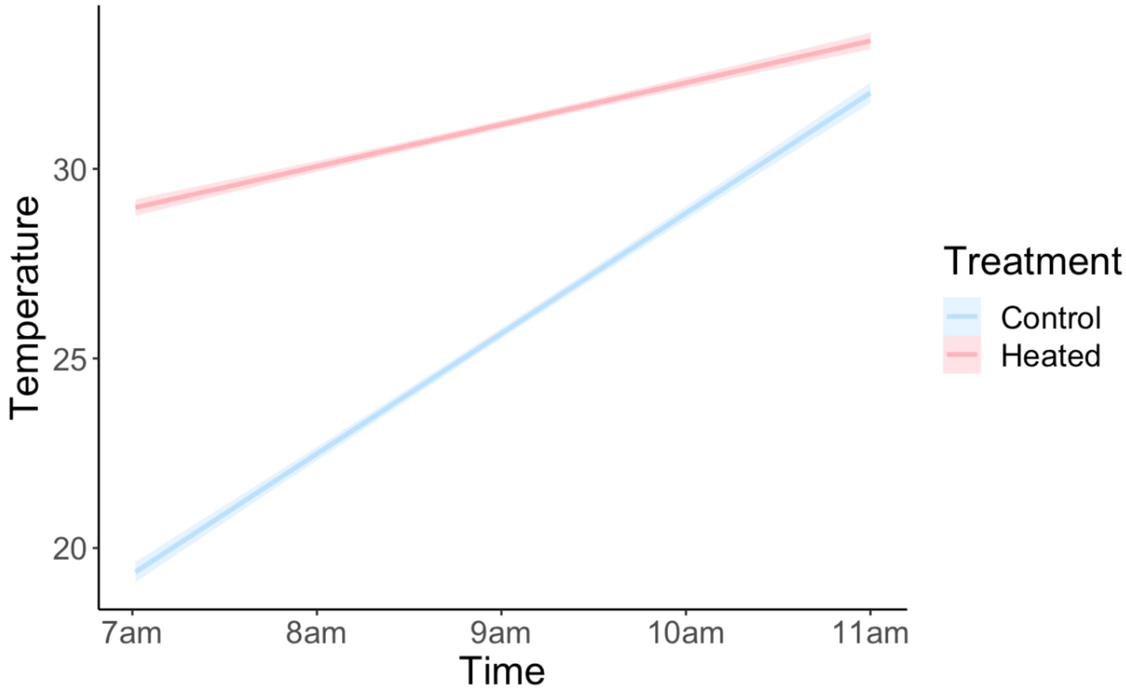

Figure 16. Regression lines showing the change in temperature measured by the internal thermocouples inside the control and heated nest boxes for 3 days. Shading represents the 95% confidence intervals.

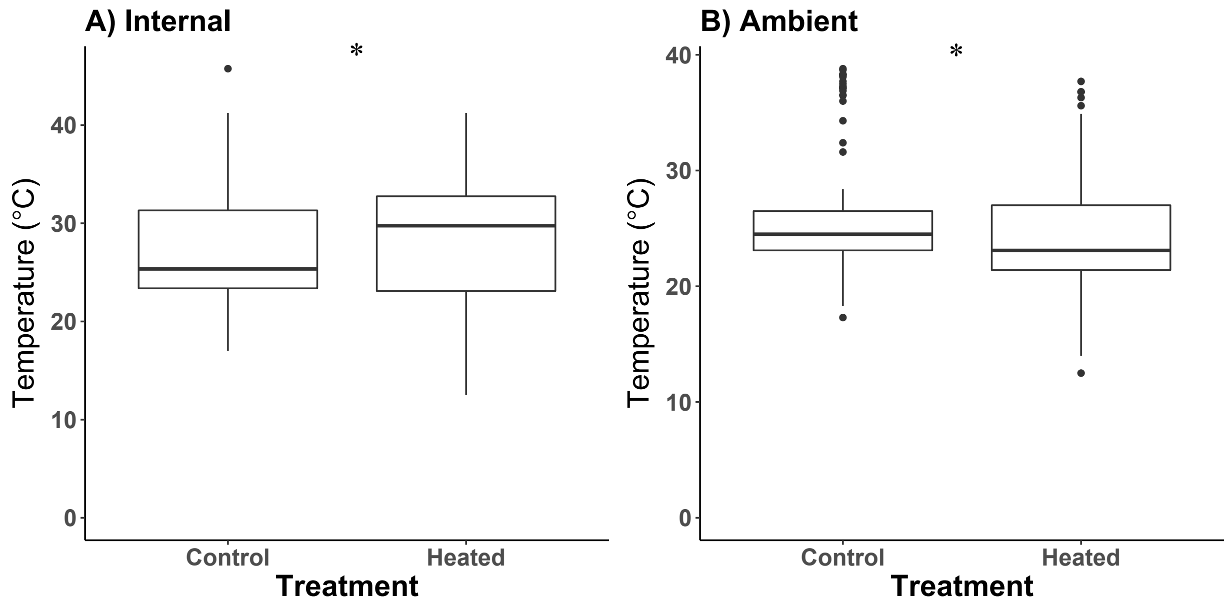

Figure 17. A) Internal nest box and **B)** ambient temperatures at which M. rotundata first flew. Bees nesting in the heated nest box flew when ambient temperatures were lower, but internal temperature was higher. There was a significant difference in both comparison of internal and ambient temperatures and is indicated by *. Boxes represent the 1st to 3rd quartile, while the lines extending from the box show the 25th and 75th percentiles of the data. The median is indicated by the line in the center of the box.

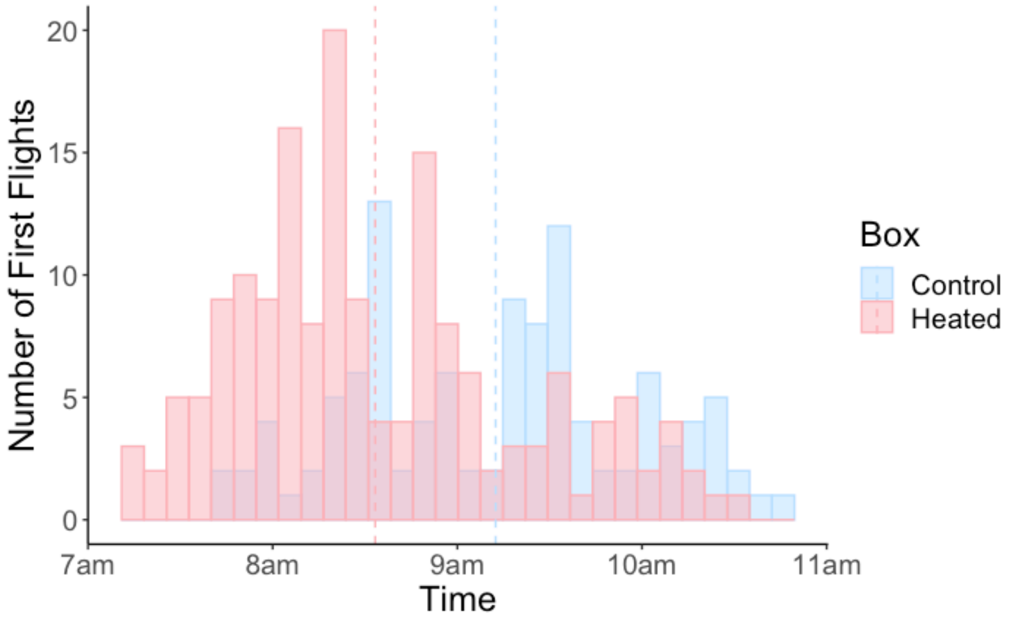

Figure 18. Distribution of first flight across time of bees nesting in control (blue) and heated (pink) boxes. Megachile rotundata nesting in the heated nest boxes flew earlier than bees in the unmanipulated control boxes. Dashed vertical lines indicate the mean for each treatment.

Discussion

Flight is a critical aspect of life history that influences an insect's ability to forage, nest, and find mates. Microclimate around nesting cavities has been found to be important for nest selection (Wilson et al., 2020). Our results show that environmental conditions at first flight differ depending on the time of the season. Later in the season, temperatures varied significantly based on the location of the thermocouple, even though they were close to each other. Later season had more days with higher light readings; therefore, solar radiation could be contributing to higher temperatures at the face of the nesting box. The only abiotic variable at first flight that did not differ between the early and late season was air pressure. Later in the summer, bees took their first flights when temperatures were lower, but it was sunnier, suggesting an important role for solar radiation in their heat budget during flight. Without enough heating prior to flight, bees could fall midflight (Herrera 1995a). The effects of various weather conditions on activity has been observed in other pollinating insect species, spanning many orders, including Hymenoptera, Diptera, Coleoptera, and Lepidoptera (Abrol, 2010; Vicens and Bosch, 2000). Some species measured were more closely related to *M. rotundata*, including *Osmia cornuta* and *Megachile lanata* (Hymenoptera: Megachilidae; Abrol and Kapil; 1986; Vicens and Bosch, 2000). Again, many of the studies collect weather data adjacent to the field site and not directly inside and around the nest or nesting cavity.

Light and temperature were previously shown to be important factors influencing flight in *M. rotundata* (Lerer et al., 1982; Szabo and Smith, 1972). However, in those studies, flight activity was measured by the presence of bees foraging and/or using macroclimate data, such as ambient temperature, light, and vapor pressure. Until now, studies have not investigated the effects of other abiotic conditions that could influence flight, including additional temperature measurements inside and around the nesting cavity, while measuring bees as they leave their nest. Nest boxes used for agriculture have a plethora of cavities, which makes measuring activity with sensors difficult, especially because bees and other insects often rest on or around cavity entrances (Klostermeyer and Geber, 1969; personal observation). Being able to measure bee activity in a less intrusive way while capturing various weather data provides a better understanding the conditions that cavity-nesting insects face daily.

Environmental conditions, such as low temperature, limit pollination services of *M. rotundata* in northeastern North America (Javorek, 1996; Javorek et al., 2002). Manipulating the internal temperature of nesting cavities could potentially promote flight when temperatures may be unfavorable. Our hypothesis that increased nesting temperature promotes earlier flight when ambient temperatures are lower was supported. *Megachile rotundata* that nested in artificial nesting boxes designed to heat before sunrise promoted bee flight 39 minutes earlier than that of bees nesting in the control box. Bees in the heated box flew when ambient temperatures were lower (24.4°C ± 0.4) than when control bees flew (26.2°C ± 0.5; Fig. 16). Internal temperatures of the heated box were 3.7°C higher (32.4°C ± 0.22) than controls (28.7°C ± 0.56) at first flight. The control box was 2.5°C warmer (28.7°C ± 0.56) than the ambient temperature (26.2°C ± 0.52) when bees first flew. Temperatures inside both nesting boxes increased linearly over time. The temperature inside the heated box exceeded the set temperature of 30°C because of additional solar heating. While this study only heated to one temperature, future studies could investigate additional temperatures to see how flight is impacted. Further, long-term effects on offspring developing in heated cavities should be investigated to determine if there are lasting effects of warming nesting temperatures. Ideally, an internal temperature should be below the threshold before a stress response occurs and heating may be more beneficial during early season or in locations where ambient temperatures could not contribute to heat stress to adults and offspring. The heated nest box could also provide close insight into insect response to climate change.

References

Abrol, D. P. (2010) Foraging behaviour of *Apis florea* F., an important pollinator of *Allium cepa* L. *J. Apic. Res.* **49**(4), 318-325.

Abrol, D. P., Kapil, R. P. (1986) Factors affecting pollination activity of *Megachile lanata* Lepel. *Proc. Indian Acad. Sci. (Anim. Sci.)* **95**(6), 757-769.

Arroyo, M. T. K., Armesto, J. J., Primack, R. B. (1985) Community studies in pollination ecology in the high temperate Andes of Central Chile II. Effect of temperature on visitation rates and pollination possibilities. *Plant Syst. Evol.* **149**, 187-203.

Bradner, N. R., Frakes, R. V., Stephen, W. P. (1965) Effects of bee species and isolation distance on possible contamination in alfalfa. *Agron. J.* **57**, 247-248.

CaraDonna, P. J., Cunningham, J. L. and Iler, A. M. (2018) Experimental warming in the field delays phenology and reduces body mass, fat content, and survival: implications for the persistence of a pollinator under climate change. *Funct. Ecol.* **32**, 2345–2356.

Chen, Y., Seybold, S. J. (2014) Crepuscular flight activity of an invasive insect governed by interacting abiotic factors. *PLoS ONE* **9**(8), E105945.

Duffy, G. A., Coetzee, B. W. T., Janion-Scheepers, C., Chown, S. L. (2015) Microclimate-based macrophysiology: implications for insects in a warming world. *Curr. Opin. Insect Sci.* **11**, 84-89.

Dyer, A. G, Whitney, H. M., Arnold, S. E. J., Glover, B. J., Chittka, L. (2006) Bees associate warmth with floral colour. *Nature* **442**, 525-525

Fauria, K., Campan, R., Grimal, A. (2004) Visual marks learned by the solitary bee *Megachile rotundata* for localizing its nest. *Anim. Behav.* **67**(3): 523-530.

Gerber, H. S., Klostermeyer, E. C. (1972) Factors affecting the sex ratio and nesting behavior of the alfalfa leafcutter bee. *Wash. Agric. Exp. Stn. Tech. Bull.* **73**, 1-11.

Herrera, C. M. (1995a) Floral biology, microclimate, and pollination by ectothermic bees in an early-blooming herb. *Ecol.* **76**, 218-228.

Herrera, C. M. (1995b) Microclimate and individual variation in pollinators: Flowering plants are more than their flowers. *Ecol.* **76**, 1516-1524.

Javorek, S. K. (1996) The potential of the alfalfa leafcutting bee *Megachile rotundata* F. (Hymenoptera: Megachilidae) as a pollinator of lowbush blueberry (*Vaccinium angustifolium* Ait. *V. mytilloides* Michx.). M.Sc. thesis, Acadia University, Wolfville, Nova scotia.

Javorek, S. K., Mackenzie, K. E., Vander Kloet, S. P. (2002) Comparative pollination effectiveness among bees (Hymenoptera: Apoidae) on lowbush blueberry (Ericaceae: *Vaccinium angustifolium*). *Ann. Entomol. Soc. Am.* **95**(3), 345-351.

Kim, J. (1999) Influence of resource level on maternal investment in a leaf-cutter bee (Hymenoptera: Megachilidae). *Behav. Ecol.* **10**(5), 552-556.

Kingsolver, J. G., Woods, H. A., Buckley, L. B., Potter, K. A., MacLean, H. J., Higgins, J. K. (2011) Complex life cycles and responses of insects to climate change. *Integr. Comp. Biol.* **51**(5), 719-732.

Klostermeyer, E. C., Gerber, H. S. (1969) Nesting behavior of *Megachile rotundata* (Hymenoptera: Megachilidae) monitored with an Event Recorder. *Ann. Entomol. Soc. Am.* **62**(6), 1321-1325.

Lerer, H., Bailey, W. G., Mills, P. F., Pankiw, P. (1982) Pollination activity of *Megachile rotundata* (Hymenoptera: Apoidae). *Environ. Enomol.* **11**(5), 997-1000.

Maino, J. L., Kong, J. D., Hoffmann, A. A., Barton, M. G., Kearney, M. R. (2016) Mechanistic models for predicting insect responses to climate change. *Curr. Opin. Insect Sci.* **17**, 81-86.

McCall, C. Primack, R. B. (1992) Influence of flower characteristics, weather, time of day, and season on insect visitation rates in three plant communities. *Am. J. Botany* **79**(4), 434-442.

Mousseau, T. A. and Dingle, H. (1991) Maternal effects in insect life histories. *Annu. Rev. Entomol.* **36**, 511–534.

O'Neill, K. M., Pearce, A. M., O'Neill, R. P. and Miller, R. S. (2010) Offspring size and sex ratio variation in a feral population of alfalfa leafcutting bees (Hymenoptera: Megachilidae). *Ann. Entomol. Soc. Am.* **103**, 775–784.

Pankiw, P., Siemens, B. (1974) Management of *Megachile rotundata* in northwestern Canada for population increase. *Can. Entomol.* **87**, 922-927.

Peterson, J. H. and Roitberg, B. D. (2006a) Impact of flight distance on sex ratio and resource allocation to offspring in the leafcutter bee, *Megachile rotundata*. *Behav. Ecol. Sociobiol.* **59**, 589–596.

Peterson, J. H. and Roitberg, B. D. (2006b) Impact of resource levels on sex ratio and resource allocation in the solitary bee, *Megachile rotundata*. *Environ. Entomol.* **35**, 1404–1410.

Peterson, J. H., Roitberg, B. D. (2016) Variable flight distance to resource results in changing sex allocation decisions, *Megachile rotundata*. *Behav. Ecol. Socio.* **70**, 247-253.

Pincebourde, S., Dillon, M. E., Woods, H. A. (2021) Body size determines the thermal coupling between insects and plant surfaces. *Func. Ecol.* **35**, 1424-1436.

Pincebourde, S., Woods, H. A. (2020) There is plenty of room at the bottom: microclimates drive insect vulnerability to climate change. *Current Opinion in Insect Science* **41**, 63-70.

Pitts-Singer, T. L., Bosch, J. (2010) Nest establishment, pollination efficiency, and reproductive success of *Megachile rotundata* (Hymenoptera: Megachilidae) in relation to resource availability in field enclosures. *Environ. Entomol.* **39**, 149-158.

Pitts-Singer, T. L., Cane, J. H. (2011) The alfalfa leafcutting bee, *Megachile rotundata*: The world's most intensively managed solitary bee. *Annu. Rev. Entomol.* **56**, 221–237.

Pitts-Singer, T. L., James, R. R. (2008) Do weather conditions correlate with findings in failed, provision-filled nest cells of *Megachile rotundata* (Hymenoptera: Megachilidae) in Western North America? *J. Econ. Entomol.* **101**(3), 674-685.

Potter, K., Davidowitz, G., Woods, H. A. (2009) Insect eggs protected from high temperatures by limited homeothermy of plant leaves. *J. Exp. Biol.* **212**, 3448-3454.

Shrestha, M., Garcia, J. E., Bukovac, Z., Dorin, A., Dyer, A. G. (2018) Pollination in a new climate: assessing the potential influence of flower temperature variation on insect pollinator behaviour. *PLoS ONE* **13**(8), e0203153.

Smith, W. K. (1978) Temperature of desert plants: another perspective on the adaptability of leaf size. *Science* **201**, 614-616.

Szabo, T. I., Smith, M. V. (1972) The influence of light intensity and temperature on activity of alfalfa leaf-cutter bee *Megachile rotundata* under field conditions. *J. Apic. Res.* **(11**), 157-165.

Taylor, L. R. (1963) Analysis of the effect of temperature on insects in flight. *J. Anim. Behav.* **32**(1), 99-117.

Totland, O. (1994) Influence of climate, time of day and season, and flower density on insect flower visitation in alpine Norway. *Arctic Alpine Res.* **26**, 66-71.

Vicens, N., Bosch, J. (2000) Weather-dependent pollinator activity in an apple orchard, with special reference to *Osmia cornuta* and *Apis mellifer* (Hymenoptera: Megachilidae and Apidae). *Environ. Entomol.* **29**(3), 413-420.

Wilson, E. S., Murphy, C. E., Rinehart, J. R., Yocum, G. P., Bowsher, J. H. (2020) Microclimate temperatures impact nesting preference in *Megachile rotundata* (Hymenoptera: Megachilidae). *Environ. Entomol.* **49**(2), 296-303.

Woods, H. A. (2010) Water loss and gas exchange by eggs of *Manduca sexta*: trading off costs and benefits. *J. Insect Physiol.* **56**, 480-487.

Woods, H. A., Dillon, M. E., Pincebourde, S. (2015) The roles of microclimatic diversity and of behavior in mediating the response of ectotherms to climate change. *J. Therm. Biol.* **54**, 86-97.

Zurbuchen, A., Cheesman, S., Klaiber, J., Müller, A., Hein, S., Dorn, S. (2010) Long foraging distances impose high costs on offspring production in solitary bees. *J. Anim. Ecol.* **79**, 674-681.

CHAPTER 5: CONCLUSIONS

Changes in the environment can affect an organism's physiology and life history. Environments vary greatly depending on the time of the year and location. To overcome small daily and longer dramatic changes in the environment, organisms have evolved adaptive mechanisms to survive through phenotypic plasticity (Bowler and Terblanche, 2008; Mallard et al., 2020; Schlichting, 1986). Ectotherms, such as insects, are especially vulnerable to drastic changes in environmental conditions (Deutsch et al., 2008; Kingsolver et al., 2011). The life stage during which an insect experiences an environmental stressor may also affect how severe the physiological and ecological consequences are. For instance, holometabolous insects may be immobile while undergoing metamorphosis and unable to seek more optimal conditions (Kingsolver et al., 2011), thus rendering that stage more vulnerable to stressors as they are undergoing extensive physiological and morphological changes. As adults, unfavorable conditions may limit the ability to forage for food (Arroyo et al., 1985, McCall and Primack, 1992; Totland, 1994). Effects of environmental stress can even extend beyond the organism by affecting its subsequent offspring (Mousseau and Dingle, 1991). The purpose of this dissertation was to show how environmental variation, such as changes in temperature, affects short- and long-term physiological mechanisms and life history in a solitary, cavity-nesting bee, *Megachile rotundata.*

Sudden bouts of low temperature are one environmental stressor than insects may experience while developing in the spring. In Chapter 2, my results show that experiencing one week of low temperatures as pupae has both long- and short-term effects on *M. rotundata*. Previous research has shown that while a week of the same low temperatures slows development, survival was not affected (Rinehart et al., 2011); however, my results show that despite being alive, exposed pupae often develop morphological deformities that prohibit flight. Deformities were separated into two main categories depending on if the deformity was visible or not. Some bees did not have any visible deformities but were still unable to fly. However, it is unclear whether the cause of bees without deformities' inability to fly is due to neurological or muscular injury. This also strengthens previous arguments that survival is not be the best metric for determining insect quality.

Megachile rotundata undergo facultative diapause, meaning that some bees bypass diapause to continue development, depending on the timing and environmental conditions of the season (Hobbs and Richards, 1976; Krunic, 1972; Tasei and Masure, 1978, Tepedino and Parker, 1986). However, the exact conditions that contribute to that "decision" are not well described. In this study, offspring produced by parents that were exposed to fluctuating low temperatures were more likely to enter diapause. This result indicates that diapause may be maternally regulated and that temperatures experienced by a mother before she reaches the adult stage could be an important factor. Entering diapause at an unideal time not only disrupts mutualisms with flowering plants but also could expose individuals to additional high temperature stress (CaraDonna et al., 2018; Tougeron et al., 2020; Tuljapurkar and Istock, 1993). During the summer, cavities in which offspring are oviposited often experience frequent high temperatures that decrease survival (Wilson et al., 2021). The effects of entering diapause at a suboptimal time during the season are still unknown in *M. rotundata* and should be investigated in the future.

Exposure to low temperatures during development may also affect offspring investment. Parental investment in offspring may be affected by various conditions, such as resource availability and foraging distances (Peterson and Roitberg, 2006a,b). Females sometimes alter offspring investment by altering sex ratios and the amount of provisions given to each offspring, which in turn affects body size (Trostle and Torchio, 1994). Results from Chapter 2 show that females that received a constant low temperature stress during metamorphosis had fewer offspring with higher body mass, indicating that females altered offspring investment. This result also supports the terminal investment hypothesis, which states that individuals that experience stress will increase their reproductive investment in preparation for future stress (Clutton-Brock, 1984). Females that were exposed to constant low temperatures as pupae were less likely to be able to fly and the ones that could, altered their offspring investment.

Chapter 2 focused on the effects of two different low temperature types (constant versus fluctuating); however, *M. rotundata* pupae could be exposed to a wide range of temperatures during the development. In Chapter 3, pupae were exposed to a wide range of temperatures (6-48°C), and oxygen consumption was measured. The response to changes in temperatures mostly followed a typical thermal performance curve with the exception of the predicted negative slope as supraoptimal temperatures are reached. Duration of the exposure may have been too short to see the negative slope, as duration can be an important factor in response to temperature stress (Marshall and Sinclair, 2015). Temperatures above 40°C have been found to increase heat shock protein expression and decrease survival (Undurraga & Stephen, 1980, Yocum et al., 2005). Future experiments could include looking at longer exposure durations. Results from this experiment would be helpful in measuring other performance matrices in *M. rotundata*, including reproductive fitness and prevalence of deformities. Additionally, Chapter 3 validates measuring oxygen consumption in a small terrestrial insect using a closed-system respirometer designed to measure aquatic organisms. This opens up endless possibilities to measure oxygen consumption in hard-to-measure insects.

Changes in environmental conditions also may affect adults by limiting flight, which is essential for mating, foraging, and offspring provisioning. Two different nesting boxes were created to measure several environmental conditions and to manipulate the temperature inside the nesting cavity. Chapter 4 shows that multiple factors were important for flight determination in *M. rotundata* by using the weather station nest box. The nest box could be easily adapted depending on the life history and nesting patterns of other organisms to measure activity and behavior. The use of GoPro cameras also provides the ability to record behavior without invasive methods. Heating the nest cavities promoted earlier flight in colder ambient temperatures. Both nest box designs can be used to investigate the effects of climate change on bee activity. Additional experiments could include adding a cooling nest box to determine nesting preference and activity when bees have the choice between a heated, cooled, and control nest boxes. Results from Chapter 4 show that the microclimate in and around nesting cavities is important for flight determination in a variable environment. The conditions around the nest box are more accurate to what a bee experiences than macroclimate measurements.

Additionally, *Megachile rotundata* is important in agriculture for alfalfa pollination. Farmers will place developing *M. rotundata* at lower temperatures to time adult emergence with alfalfa bloom (Rinehart et al., 2011; Pitts-Singer and Cane, 2011; Yocum et al., 2010). Results from Chapters 2 and 3 suggest that low temperatures affect morphology, metabolic rate, reproduction, and offspring characteristics. The prevalence of deformities affecting flight and feeding would result in decreased alfalfa pollination and return rates. Pollination rates would also decline if offspring entered diapause too early in the season or could be exploited to conserve bees for the following season. The thermal performance curves generated in Chapter 3 are a starting point to understanding stress response to temperature in *M. rotundata* pupae by using oxygen consumption. The heated nesting boxes used in Chapter 4 could be adapted to promote flight in unfavorable conditions early in the blooming season.

Overall, this dissertation has many implications for improving bee management practices and also promotes further research to enhance the understanding of thermal stress physiology in insects. I have demonstrated that the life stage which an insect receives a stress can affect their offspring by altering life history trajectory and triggering trade-offs on quantity vs body size. Also, I have shown that oxygen consumption can be reliably measured for pupae inside their brood cells using equipment originally designed for aquatic organisms. Lastly, I have demonstrated that measuring environmental conditions in and around nesting cavities is important for flight determination and that altering the internal temperature can promote flight when temperature is lower.

References

Arroyo, M. T. K., Armesto, J. J., Primack, R. B. (1985) Community studies in pollination ecology in the high temperate Andes of Central Chile II. Effect of temperature on visitation rates and pollination possibilities. *Plant Syst. Evol.* **149**, 187-203.

Bowler, K., Terblanche, J. S. (2008) Insect thermal tolerance: what is the role of ontogeny, ageing and senescence? *Biol. Rev.* **83**, 339-355.

CaraDonna, P. J., Cunningham, J. L. and Iler, A. M. (2018) Experimental warming in the field delays phenology and reduces body mass, fat content, and survival: implications for the persistence of a pollinator under climate change. *Funct. Ecol.* **32**, 2345–2356.

Clutton-Brock, T. H. (1984) Reproductive effort and terminal investment in iteroparous animals. *Am. Nat.* **123**, 212-229.

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C., Martin, P. R. (2008) Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci.* **105**(18), 6668-6672.

Hobbs, G. A. and Richards, K. W. (1976) Selection for a univoltine strain of *Megachile* (Eutricharaea) *pacifica* (Hymenoptera: Megachilidae). *Can. Entomol.* **108**, 165-167.

Kingsolver, J. G., Woods, H. A., Buckley, L. B., Potter, K. A., Maclean, H. J. and Higgins, J. K. (2011) Complex life cycles and the responses of insects to climate change. *Integr. Comp. Biol.* **51**, 719–732.

Krunic, M. D. (1972) Voltinism in *Megachile rotundata* (Megachilidae: Hymenoptera) in southern Alberta. *Can. Entomol.* **104**, 185-188.

Mallard, F., Nolte, V., Schlötterer, C. (2020) The evolution of phenotypic plasticity in response to temperature stress. *Genome Biol. Evol.* **12**(12), 2429-2440.

Marshall, K. E. and Sinclair, B. J. (2015) The relative importance of number, duration and intensity of cold stress events in determining survival and energetics of an overwintering insect. *Funct. Ecol.* **29**, 357–366.

McCall, C. Primack, R. B. (1992) Influence of flower characteristics, weather, time of day, and season on insect visitation rates in three plant communities. *Am. J. Botany* **79**(4), 434-442.

Peterson, J. H. and Roitberg, B. D. (2006a) Impact of flight distance on sex ratio and resource allocation to offspring in the leafcutter bee, *Megachile rotundata*. *Behav. Ecol. Sociobiol.* **59**, 589–596.

Peterson, J. H. and Roitberg, B. D. (2006b) Impact of resource levels on sex ratio and resource allocation in the solitary bee, *Megachile rotundata*. *Environ. Entomol.* **35**, 1404–1410.

Pitts-Singer, T. L. and Cane, J. H. (2011) The alfalfa leafcutting bee, *Megachile rotundata*: The world's most intensively managed solitary bee. *Annu. Rev. Entomol.* **56**, 221–237.

Rinehart, J. P., Yocum, G. D., West, M. and Kemp, W. P. (2011) A fluctuating thermal regime improves survival of cold-mediated delayed emergence in developing *Megachile rotundata* (Hymenoptera: Megachilidae). *J. Econ. Entomol.* **104**, 1162–1166.

Schlichting, C. D. (1986) The evolution of phenotypic plasticity in plants. *Ann. Rev. Ecol. Syst.* **17**, 667-693.

Tasei, J. N., Masure, M. M. (1978) Sur quelques facteurs influencant le développement de *Megachile pacifica* Panz. (Megachilidae). *Apidologie* **9**, 273-290.

Tepedino, V. J., Parker, F. D. (1986) Effect of rearing temperature on mortality, second-generation emergence, and size of adult in *Megachile rotundata* (Hymenoptera: Megachilidae). *J. Econ. Entomol.* **79**(4), 974-977.

Totland, O. (1994) Influence of climate, time of day and season, and flower density on insect flower visitation in alpine Norway. *Arctic and Alpine Research* **26**, 66-71.

Tougeron, K., Brodeur, J., Le Lann, C., Van Baaren, J. (2020) How climate change affects the seasonal ecology of insect parasitoids. *Ecol. Entomol.* **45**, 167-181.

Tuljapurkar, S. and Istock, C. (1993) Environmental uncertainty and variable diapause. *Theor. Popul. Biol.* **43**, 251-280.

Undurraga, J. M., Stephen, W. P. (1980) Effect of temperature on development and survival in post-diapausing alfalfa leafcutting bee prepupae and pupae (*Megachile rotundata* (F.): Hymenoptera: Megachilidae). I. High temperatures. *J. Kansas Entomol. Soc.* **53**(3), 669-676.

Wilson, E. S., Murphy, C. E., Rinehart, J. P., Yocum, G. D., Bowsher, J. H. (2021) Microclimate temperatures impact nesting preference in *Megachile rotundata* (Hymenoptera: Megachilidae). *Behav. Ecol.* **49**(2), 296-303.

Yocum, G. D., Kemp, W. P., Bosch, J., Knoblett, J. N. (2005) Temporal variation in overwintering gene expression and respiration in the solitary bee *Megachile rotundata*. *J. Insect Physiol.* **51**(6), 621-629.

Yocum, G. D., Rinehart, J. P., West, M. and Kemp, W. P. (2010) Interrupted incubation and short-term storage of the alfalfa pollinator *Megachile rotundata* (Hymenoptera: Megachilidae): A potential tool for synchronizing bees with bloom. *J. Econ. Entomol.* **103**, 234–241.