

NESTING CAVITY TEMPERATURE IMPACTS NESTING CHOICE AND DEVELOPMENT
OF THE ALFALFA LEAFCUTTING BEE (*MEGACHILE ROTUNDATA*)

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

By

Elisabeth Susan Wilson

In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Department:
Biological Sciences

March 2019

Fargo, North Dakota

North Dakota State University
Graduate School

Title

NESTING CAVITY TEMPERATURE IMPACTS NESTING CHOICE
AND DEVELOPMENT OF THE ALFALFA LEAFCUTTING BEE
(*MEGACHILE ROTUNDATA*)

By

Elisabeth Susan Wilson

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

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Julia Bowsher

Chair

Joe Rinehart

Jason Harmon

Approved:

4/1/19

Date

Kendra Greenlee

Department Chair

ABSTRACT

Megachile rotundata is an agriculturally important pollinator of alfalfa and is not doing well in the U.S due to a 50% return rate (Pitts-Singer and Bosch, 2011). Nesting boxes can reach temperatures between 35°C-48°C (see Chapter 2). These temperatures can cause damage to multiple stages of development, including death (Barthel et al. 2002, Rossie et al. 2010). To date, it is unclear how cavity temperature varies across the box. Traditionally, nesting cavity temperatures have been monitored using 1-3 temperature probes to measure thousands of cavities (CaraDonna et al. 2018). These methods do not account for the accuracy of the temperature probe to depict temperatures several cm away from the probe. To assess this variation I have designed a 3D printed nesting box that holds one temperature probe for every four cavities. I found that cavity temperature impacts nesting preference, and survival, of the alfalfa leafcutting bee.

ACKNOWLEDGEMENTS

Thank you to Nyle Jonason, Courtney Grula, and Korie Debardlabon for continued field support. Thank you to George Yocum for hours spent discussing experimental design, statistics and finding every missed comma in my R script. Thank you to my committee Julia, Joe, and Jason for continually pushing my projects to be the best they could be. This project was supported by the Shockey Scoby Fellowship from the Biological Sciences Department. Thank you to the USDA, NDSU Biological Sciences Department, and The College of Science and Mathematics for funding this project. This project was supported by NSF IOS 1557940 and NSF EPSCoR 1826834.

DEDICATION

This thesis is dedicated to my family Laura Wilson, Keith Wilson, Jacob Wilson, and Jake Rhodes, without your love and continued support this document would not exist.

TABLE OF CONTENTS

ABSTRACT.....	iii
ACKNOWLEDGEMENTS.....	iv
DEDICATION.....	v
LIST OF FIGURES.....	viii
INTRODUCTION.....	1
Aims.....	9
Aim 1: Nesting preference and temperature.....	9
Aim 2: Temperature, development, and fitness.....	9
Aim 3: Temperature and body size.....	9
MICROCLIMATE TEMPERATURES IMPACT NESTING PREFERENCE AND FITNESS IN <i>MEGACHILE ROTUNDATA</i>	10
Abstract.....	10
Introduction.....	11
Methods.....	14
Field site set up.....	14
Monitoring nesting behavior and nest size.....	15
Cavity nest preference.....	16
Data analysis.....	16
Results.....	17
Nest cavity temperatures during foraging.....	17
Rate of nest completion by direction.....	19
Nest cavity preference by temperature.....	20
Nest size.....	21
Discussion.....	22

MICROCLIMATE TEMPERATURES ON DIAPAUSE, BODY SIZE, AND SURVIVAL OF <i>MEGACHILE ROTUNDATA</i>	27
Abstract	27
Introduction	28
Methods	31
Field site set up	31
Monitoring nesting behavior and nest size	32
Data analysis	34
Results	35
Nesting box temperatures and microclimates	35
Incidence of diapause and nondiapause	37
Body size by temperature	39
Survival	41
Discussion	42
Non-diapausers by temperature	43
Body size by temperature	44
Survival	45
CONCLUSION	47
REFERENCES	53

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1. How Temperature Impacts Performance in Insects. Figure adapted from (Colinet et al. 2015).	2
2. Hypothesized impacts of temperature on diapause. A spike in temperature in the later season could cause offspring to avert diapause and emerge in the same season as the F1 generation.	7
3. Field Design. A) Replicate 1, at alfalfa field site with wooden base. B) Animated rendering of 3D printed design, with places for iButtons along the back of each block, and terminology.	14
4. Average Field Temperatures. A) Average temperature (C) per day for by direction. B) Average temperature (C) by direction for entire nesting season. C) Average foraging temperature (C) by each row for entire nesting season, color by direction. D) Average foraging temperature (C) by each column for entire nesting season, color by direction.	18
5. Nest Completion by Direction. Kaplan-Mier of box fill over nesting season (p<0.0001). NE and NW cavities filled 7 days earlier than SE and SW cavities.	20
6. The Probability to Predict Nesting. A) Probability of temperature to predict nesting. B) Probability of direction to predict nesting. C) Probability of row to predict nesting.	21
7. Nest Size by Direction. Nest size significantly differed by direction One-Way ANOVA (p<0.001). Tukeys HSD denoted by letter.....	22
8. Methods Figure. How nests were continually monitored throughout the season.	33
9. Experienced Field Temperatures. A) Average temperature by direction B) Amount of time spent above 40°C by direction C) Amount of time spent above 35°C by direction D) Average temperature by block E) Amount of time spent above 40°C by block F) Amount of time spent above 35°C by block.....	36
10. Probability of Diapause Incidence. A) Probability to predict diapause by average temperature B) Probability to predict diapause by date of capped nest.	39
11. Body Size by Temperature and Development Stage. A) Average temperature on body weight B) Time spent above 40°C by body weight C) Time spent above 35°C by body weight D) Development stage on body weight.....	40
12. Factors impacting Offspring Survival. A) Probability of predicting survival by amount of time spent above 40°C B) Probability of predicting survival by date of capped nest C) Probability of predicting survival by development status.....	42

INTRODUCTION

This thesis looks explores how nesting cavity temperature impacts the prominent pollinator *Megachile rotundata* (Hymenoptera, Megachilidae), the alfalfa leafcutting bee. The nesting cavity may represent a microclimate that exposes both adult nesting bees and the offspring to fluctuating temperatures throughout the nesting season. For my study I define microclimate as the true experienced temperature of an organism that varies from the ambient temperature. Chapter two, aim one explores if the nesting box represents a microclimate, and how variations in temperature impact nesting cavity choice and fitness. Chapter three, aim two and aim three explores how microclimate impacts the offspring within the cavity, through studying body size, diapause aversion, and survival. High temperature has been shown to have detrimental effects on this species (Pitts-Singer and Cane 2011, Kemp and Bosch 2001, Fischmann et al. 2017) and understanding how cavity temperature may impact fitness could improve management practices.

Insects, as ectotherms, are intimately linked to temperature. However, in insects, the relationship between temperature and many biological variables is non-linear. Jensen's inequality describes the relationship between increasing temperature and performance in insects (Fig 1). As temperature increases, performance increases rapidly until the thermal performance optimum in which small increments of temperature cause decreases in performance (Colinet et al., 2015). This concave and convex relationship makes temperature extremely important in studying insect survival, reproduction, and physiology in fluctuating environments, because small temperature increases past the thermal optimum can cause drastic changes in performance. Due to the inequality around the optimum, a 1°C increase past the optimum could lead to decreased fitness and survival. However, a 1°C decrease from the optimum will not cause as

much damage and the insect will still operate around peak performance values (Fig 1). This inequality explains why insect internal body temperature is often right below the optima and are “sub-optimal” in order to mitigate consequences from fluctuating temperatures past the optimum (Martin and Huey, 2008).

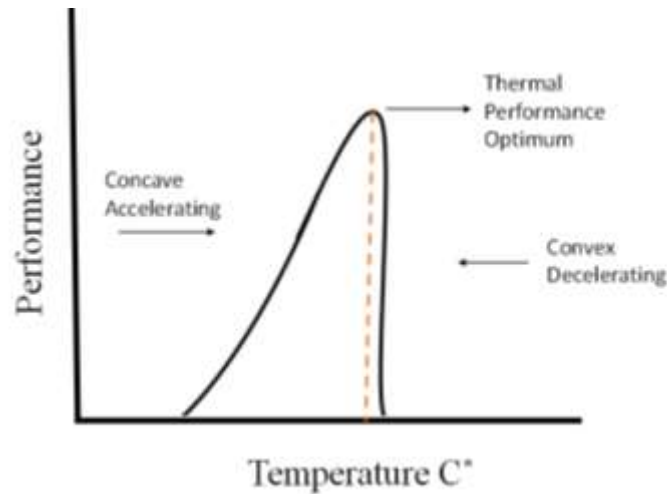


Figure 1. How Temperature Impacts Performance in Insects. Figure adapted from (Colinet et al. 2015).

This relationship between temperature, performance, and the thermal performance optimum becomes even more critical when considering the environmental crisis of climate change. Under current climate predictions, by the year 2071 the temperature could increase by 3-5°C (National Climate Assessment 2014). While these incremental changes do not seem to be vast, a 2-5°C increment in temperature can cause a decrease in insect performance due to the inequality around the optimum (Fig 1). A decrease in insect performance can come at a high cost to food systems, due to the reliance on pollination services. Today, the U.S. agricultural industry uses multiple bee species to pollinate crops. One of these, the alfalfa bee *Megachile rotunda* (Fab.) (Hymenoptera: Megachilidea), is responsible for pollinating alfalfa for seed. As temperature increases and these species show poor performance, we must begin to understand how fluctuating temperatures in the fields are impacting the survival, reproduction, and

physiology of our prominent pollinators. *Chapter two of my thesis explores how natural fluctuations in temperature impact the development, reproduction, and physiology of M. rotundata.*

M. rotundata is the primary pollinator of alfalfa in the U.S. and is likely to experience heat stress both through climate change and industrial rearing practices. *M. rotundata* is a cavity nester that uses natural cavities in wood and manmade cavities in the agricultural industry (Pitts-Singer and Bosch 2011, Richards 1987). Currently, the U.S. loses 50% of the managed alfalfa bee population each season to unknown factors (Pitts-Singer and Bosch 2011). This population decline is only experienced in the United States whereas Canada often produces a surplus (Pitts-Singer and Cane 2011). It has been observed that the temperatures between the United States and Canada significantly vary (Pitts-Singer and James, 2005), but how this difference correlates with reproductive output has not been explicitly explored.

In 1990 the U.S. spent over 11 million dollars to import this primary pollinator to help pollinate alfalfa (Peterson et al. 1992). This cost will only have increased due to inflation and increase in farm land. Even with a vast amount of money spent on these pollinators, the U.S. is unsuccessful in rearing populations (Pitts-Singer and Bosch 2011). The agricultural industry uses commercial bee boxes made of polyurethane and corrugated cardboard to rear alfalfa bees and suggests placing these large nesting shelters facing southeast (Stephen 1981). By facing the southeast the sun can strike the shelters early in the morning and allow the bees to start pollination services as early as possible. *However, preference for direction has not been tested in this species. In Chapter two, aim one explores how preference for direction correlates with temperature.*

Potential correlations between nesting preferences and temperature are important as several field and lab-based studies have indicated that solitary bees are impacted by increasing temperatures (Richards 1996, Kemp Bosch 2001, Rossi and Pitts-Singer 2009). A recent paper on *Osmia lignaria*, a cavity nesting solitary bee, significantly manipulated field nesting temperatures by painting nesting boxes with black, clear, and neutral enamel and found that warmer boxes experienced 30-70% mortality compared to cooler boxes (CaraDonna et al. 2018). This study demonstrated that nesting boxes can vary in temperature and have reproductive consequences for solitary bees. However, these studies used ambient temperatures, or very few temperature probes. It is imperative that we examine the effects of heat stress by cavity under field conditions, because field nesting cavities mimic both industrial rearing practices, and biologically relevant conditions.

While chapter two studies the effects of nesting cavity temperatures on the adult nesting preference and fitness, chapter three studies the effects of cavity temperature on offspring. Nesting box temperatures have the potential to impact developing offspring at both the larval and pupal stage. Adult female alfalfa leafcutting bees build linear nest structures, lay eggs in cavities, and reside in the cavity overnight. Thus, both the offspring and adults will be exposed to nest box temperatures. Temperatures ranging from 35°C-47°C have been shown to have multiple detrimental effects at various stages of development of the alfalfa leafcutting bee (Barthell et. al. 2002).

In the prepupa stage, the alfalfa leafcutting bee experiences several detrimental effects at high temperatures. Alfalfa leafcutting bee prepupa produce Heat Shock Proteins (HSP70) starting at 35°C and these proteins peak at 42.5°C (Barthell et. al. 20002). Heat Shock Proteins are one possible indicator of heat stress. Not only do prepupa indicate heat stress through HSP70

proteins, but they also show 0% survival at 52.2°C (Barthell et. al. 2002). Considering nest box temperatures can easily reach these values, it is likely *M. rotundata* is experiencing detrimental effects of heat stress in early developmental stages.

In the adult stage, *M. rotundata* are also vulnerable to high temperatures. *M. rotundata* show behavioral and physiological damage in response to heat stress. As mean temperatures increased, *M. rotundata* males increased harassment of females, with temperatures reaching up to 44°C, and decreased overall nest production (Rossi and Pitts-Singer, 2009). However, a population of *M. rotundata* in Alberta, Canada had increased nest and offspring production under warmer temperatures, but these temperatures were only analyzed above 30°C, with the highest production of capped nests at 25°C (Richards, 1996). A recent survey found that the number of viable *M. rotundata* brood cells was greater in cooler regions (James and Pitts-Singer 2013). Considering the nesting cavities may reach temperatures exceeding 40°C for compounding hours, it is imperative to explore how these nesting temperatures impact reproduction. *Chapter three, aim two, explores how nesting cavity temperatures impact the number of viable brood cells for M. rotundata.*

While overall survival of *M. rotundata* is important for population level effects, body size may also be impacted by high temperatures and can give insight to individual level consequences of nesting cavity temperature. In solitary bees, body size is primarily determined by provision size (Klostermeyer et al. 1973). However, temperature has been described as the “most influential abiotic ecological factor in offspring body size” (Radmacher and Strohm, 2010). The “temperature size rule” for ectotherms suggests that lower temperatures during development will lead to larger adult body size (Radmacher and Strohm, 2010). Adult females laying offspring into various cavities expose the offspring to a microclimate within the cavity. Considering

nesting cavity temperature significantly varies by position and direction, the nesting box may impact body size. *Chapter three, aim three, will explore how cavity nesting temperature influences body size in solitary bees.*

While body size and survival may be impacted by exposure to nest box temperature, diapause incidence can also be impacted by warming temperatures. The alfalfa leafcutting bee undergoes facultative development, suspended development, in early fall to emerge in the following spring to warmer temperatures. However, there are a few bees that skip diapause and develop straight to adulthood. These offspring emerge in the same summer as the parental generation and are referred to as 2nd Generation or non-diapause individuals (Kemp and Bosch 2000).

The mechanism behind the outcome of diapause or non-diapause is unknown. However, there are two hypothesized mechanisms that could cause an offspring to go straight through development; maternal control, and stress (Fig 2). It is hypothesized that the decision to enter diapause or 2nd generation is primarily under maternal control. It is proposed that the adult female will lay eggs that will either go through diapause or become a 2nd Generation. However, a pattern has been shown in the literature of when a female will lay each type of egg. Kemp and Bosch found a higher incidence of direct development, 2nd Generation, in eggs laid earlier in the season than late laid eggs (Kemp and Bosch 2001). In 1983, 55% of offspring laid before July 29th were 2nd Generation, with only 3.9% 2nd Generation by Aug. 19th (Parker and Tepedino, 1988). In 1981, the switch between laying more 2nd Generation to more diapause eggs happened on Aug. 10th (Parker and Tepedino, 1988). Kemp and Bosch 2001 found 0% of non-diapausing cells by July 21st, and then saw an increase to 30% of non-diapausing cells on July 30th. While

these dates do not hint at the mechanism to maternal decision making, they indicate a pattern in which you are likely to see diapause vs. non-diapausing eggs.

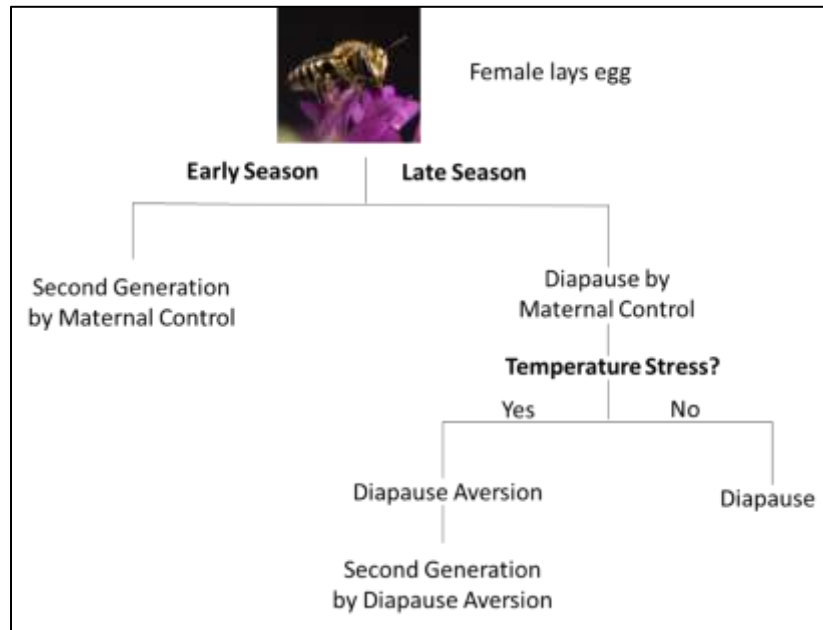


Figure 2. Hypothesized impacts of temperature on diapause. A spike in temperature in the later season could cause offspring to avert diapause and emerge in the same season as the F1 generation.

Stress is hypothesized as a second mechanism that could cause the production of a 2nd Generation. Stress has been hypothesized to cause an offspring to “change its mind” after the mother has determined a diapause route, and this observation would be called diapause aversion (Kemp and Bosch 2001). Diapause aversion is when an organism was on a route to enter a diapause state and then “changes its mind” during the latency stage and directly develops into an adult (Fig 2). One possible stress that could induce diapause aversion in *M. rotundata* is temperature stress. Kemp and Bosch 2001 found that *M. rotundata* prepupae were more likely to diapause avert, as constant temperatures increased. At 18°C only 6.8% of prepupa averted diapause compared to 45.5% of prepupa exposed to a constant 32°C (Kemp and Bosch 2001). However, this observation has not been specifically observed in the field under natural

fluctuating temperatures. Considering nest box temperatures can be highly variable and extremely high, initiation of diapause may be impacted by nesting temperature.

In Chapter three, aim two explores how high temperatures lead to a higher incidence of diapause aversion. By following nests for the entirety of the season, I will be able to track if a temperature spike correlates with increase in diapause aversion. Although we will be unable to separate completely the maternal effect vs. the temperature effect, using the dates of nest capped will be an indicator of maternal preference. The literature suggests that the majority of non-diapause eggs will be laid before the last week in July, suggesting that temperature may be a stronger cue later in the season for both the mother and offspring. Offspring may be especially sensitive to temperature changes, due to being in a light restricting environment. Bennett et al. 2018 found that while both photoperiod and temperature synced emergence of diapausing *M. rotundata*, temperature was a stronger cue. This result suggests that prepupa would be responsive to temperature increases.

A 2nd generation bee that develops faster than its siblings and becomes a 2nd generation is a threat to both the agricultural industry and the population. A 2nd Generation bee must chew through its brood cell and the brood cells of its siblings to emerge from the nest. Not only could this emergence kill other offspring, but the movement of an individual through the nest could spread pathogens and disease, further decreasing fitness (James and Pitts-Singer, 2013). *Through monitoring natural temperature variation over the course of the nesting season chapter three will explore if a high temperature period experienced during the later season correlates with increased diapause aversion.*

Heat stress experienced during nesting is likely to affect the alfalfa leafcutting bee both physiologically and reproductively. This thesis studies the impacts of nesting temperatures on

the alfalfa leafcutting bee using 3D printed nesting boxes that contain individual temperature probes. Individually printed boxes allow the accuracy of the iButton to fully account for the temperatures of each cavity. Previously, studies of nesting box temperatures have used 1-3 temperature probes to account for 1,000s of nesting cavities (CaraDonna et al. 2018). With a 3D printed design, I will be using 108 temperature probes to account for 432 nesting cavities. By 3D printing my own boxes and running multiple pre-trials I have ensured that each temperature probe accurately depicts the exact temperature of 4 cavities. *This thesis studies how naturally occurring nest box temperatures impact the preference, body size, diapause incidence, and survival of the alfalfa leafcutting bee.* This experiment will have both management and biological significance through studying the productivity of an agriculturally important pollinator as well as furthering the literature on the biological consequences of heat stress under natural conditions.

Aims

Aim 1: Nesting preference and temperature

- 1) How do cavity temperatures vary based on direction and position within nesting box?
- 2) How does nesting preference of *M. rotundata* vary based on temperature and direction?

Aim 2: Temperature, development, and fitness

- 1) How does cavity temperatures impact diapause incidence in *M. rotundata*?
- 2) How does cavity temperatures impact survival of *M. rotundata*?

Aim 3: Temperature and body size

- 1) How does cavity temperatures impact body size of *M. rotundata*?

MICROCLIMATE TEMPERATURES IMPACT NESTING PREFERENCE AND FITNESS IN *MEGACHILE ROTUNDATA*

Abstract

Nest micro-climates can have significant impacts on offspring development, size, and fitness of cavity nesting bees. Therefore, females may choose nest cavities that mitigate their offspring's exposure to potentially stressful temperatures. This study aims to understand how cavity temperature impacts the nesting preference and fitness of the solitary bee *M. rotundata* under field conditions. We designed a 3D printed nesting box that accurately measured the temperatures of 432 cavities. The nest boxes had sides that faced northeast, northwest, southeast and southwest. Boxes were observed daily for completed nests. Our study found that temperature significantly varies by direction of the nesting box, and the position of the cavity within the nesting box. The Southwest side recorded the highest temperatures and the Northeast side recorded the lowest temperatures. Cavities facing the Northeast and Northwest sides filled with nests faster than cavities facing the Southern sides, and nest preference correlated with lower average temperatures during foraging hours. Direction of cavity impacted number of off-spring per nest. The Southwest facing nest box had fewer offspring than nests laid on the cooler Northeast side. Our study indicates that the nesting box acts as a micro-climate and indicates that females may alter their behavior in response to aspects of nest box microclimate.

Key Words: Direction, Climate Change, Nesting Choice, Pollinators, Behavior, Thermal Performance Curve

Introduction

Insects are susceptible to fluctuations in temperature due to their ability to derive heat from their environment and the close relationship between external environmental temperature and internal body temperature (Martin and Huey, 2008, Jensen, 1906). Insect thermotolerance is susceptible to increases in temperature because insect performance is non-linear (Potter 2009, Colinet et al., 2015, Sinclair et al. 2016). Jensen's inequality models this non-linear response (Jensen, 1906, Colinet et al., 2015) in which insects experiencing temperatures past the optimum peak will have a steep decline in performance, while small decreases in temperature before the peak do not significantly change performance. If temperatures continue to rise past the optimal temperature, insect performance rapidly declines and can result in sub-lethal effects and eventually death (Colinet et al., 2015).

Thermal performance curves are context dependent and change with life stage and trait measured. In *Manduca sexta*, the pupal stage can withstand higher temperature than the adult stage (Potter et al 2011), and the first instar has a higher growth rate response to increasing temperature than the fourth instar (Kingsolver et al. 2011). In other ectotherms, the performance curve depends upon the trait being measured, with different optimum temperatures for movement, oxygen consumption, and digestion (Sinclair et al. 2016). Performance measures are chosen in order to represent insect fitness under the exposure temperature and it is imperative that the measurement is an accurate measure of fitness and that more than one performance measure is used in order to get a true estimate of the thermal performance curve. Multiple measurements, and temperature exposures are truly needed in order to accurately model insect response under thermal stress (Sinclair et al. 2016).

While atmospheric temperatures provide overall trends of increasing temperatures, microclimates often differ in terms of many abiotic factors, including temperature. A microclimate represents the specific temperature of a small area that differs from the ambient temperature, or macroclimate. Microclimate temperatures better predict insect performance than macroclimate temperature because microclimates are a more accurate measurement of the environment for insects (Richards, 1996). For example, A study done on the apple maggot *Rhagoletis pomonella* found that ambient temperatures reached peaks of 33°C, while apple temperatures peaked at 45°C (Lopez-Martinez and Denlinger, 2008). Leaf surfaces buffer temperatures experienced by insect eggs, producing a microclimate that increases hatching success (Potter et al. 2009). Nesting cavities of solitary bees have also been shown to increase mortality at higher temperatures (CaraDonna et al., 2018). Micro-climates have the potential to both protect from stressful temperatures or expose insects to heat stress, so an insect's ability to choose a micro-climate can have a significant impact on fitness.

Insects may be able to mitigate exposure to high temperatures by choosing to nest or reside in cooler micro-climates. Many organisms from a variety of taxa have been shown to mitigate exposure to high temperatures through moving to cooler microclimates. Bird communities of *Boscia albitrunca* spend more time in densely shaded trees on days above 35°C (Martin et al. 2015). Atlantic salmon, *Salmo salar* swim to cooler waters to avoid warmer temperatures (Breau et al. 2011). Several insect species have also been found to utilize mobility to decrease exposure to stressful temperatures (Huey et. al 2002, Kearney et al, 2009). Micro-climates can also dictate movement patterns of insects, with insects actively moving to cooler areas to avoid increased temperatures (Woods et al. 2014). However, eggs and larvae have limited ability to move from stressful micro-climates and must survive in the micro-climate

chosen by the parent. Females of the silver-spotted skipper, *Hesperia comma*, will lay eggs on warmer host plants during low temperatures and will lay eggs on cooler host plants during high temperatures resulting in increased fitness and habitat (Davies et al. 2006). Thus, not only can individuals mitigate temperature exposure for themselves, parent choice may mitigate offspring exposure through choosing cooler habitats.

Megachile rotundata, the alfalfa leafcutting bee, offers a great model for exploring how insects respond to microclimate and how insects may avoid exposure to sub-optimal temperatures. *M. rotundata* is a solitary, cavity nesting bee that builds linear nests out of leaves, making individual brood cells for each offspring (Pitts-Singer and Cane, 2009). *M. rotundata* is used by the agricultural industry and will nest in man-made boxes that include thousands of linear cavities (Stephen, 1981). Nesting cavities have different temperatures due to nest box material (Richards, 1996), and color (CaraDonna et al., 2018), and represent a dynamic microclimate. At increased temperatures, solitary bees have been shown to decrease in size (Fischman et al. 2017), lay fewer offspring (Pitts-Singer and James 2008), have a faster development rate (Kemp and Bosch, 2000; Fischman et al. 2017) and have increased pathogen susceptibility (Xu and James 2012). *M. rotundata* fitness is also impacted by higher temperatures, with increased larval death in warmer temperatures (Pitts-Singer and James 2008). These studies suggest that nest microclimates can impact fitness in *M. rotundata*, and that mothers could have the potential to influence offspring fitness by choosing nesting cavities with favorable micro-climates.

The goal of this study was to determine how micro-climate corresponds to nest choice and fitness in *M. rotundata* by tracking individual cavity temperature and nesting behavior. In order to expose *M. rotundata* to naturally occurring temperature fluctuations and micro-climates,

we established nesting boxes facing the four ordinal directions to act as temperature treatments. We found that females had a higher probability of nesting in cavities with cooler temperatures, and that females nesting on the SW side laid fewer offspring.

Methods

Field site set up

Three nest boxes were placed along the side of an alfalfa field in Fargo, North Dakota (46°55'15" N, 96°51'17" W). A drainage ditch containing multiple forbes and weeds ran along the side of the field. The replicates were placed 200m apart, which is a distance that minimizes adult migration between replicates (Bradner et al. 1965). Each nest box consisted of 36 smaller boxes that contained four nest cavities and were 3D printed using purple PLA plastic (#eSUN, ABS175Z1) in dimensions 60mm x 60mm x 82mm (Fig 3).

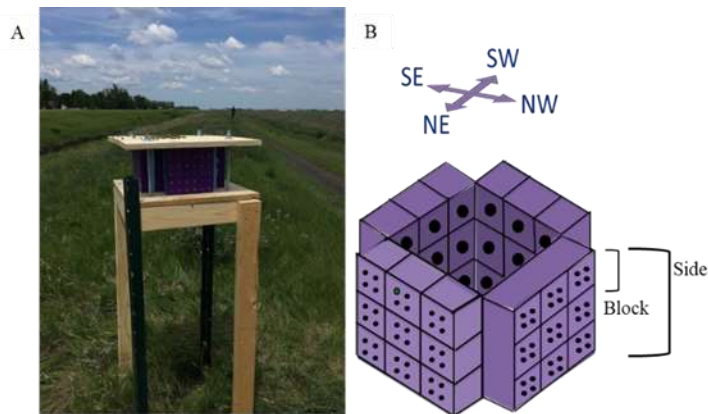


Figure 3. Field Design. A) Replicate 1, at alfalfa field site with wooden base. B) Animated rendering of 3D printed design, with places for iButtons along the back of each block, and terminology.

Boxes were printed on a Taz 5 and Taz 6 3D printer with a 20% infill. The four nest cavities were spaced equal distance apart. The diameter of the cavity was 7mm in diameter and length of cavity was 78mm. (Fig 3). Nest boxes were made by stacking blocks in a three by three pattern, resulting in 36 nesting cavities per side, and a total of 144 cavities per nest box. A hole

was added to the back middle of each four-cavity block to accommodate a Thermocron 5 ibutton (DS1921G-F5#-ND) measuring 10.25mm x 41mm. To ensure that one ibutton was able to accurately measure the temperatures of each of the four cavities within one block, an incubator pre-trial was run. Four blocks were placed in an incubator with a HOBO temperature probe in each cavity (ONSET, U12-006) and an ibutton placed in the back. The incubator was set to ramp from 10°C to 30°C then back down to 10°C over the course of 4 days. This trial showed no significant difference between the HOBO probes and each of the four iButtons (ANOVA, $p=0.981$, $p=0.941$, $p=0.948$, and $p=0.978$). This pre-trial confirmed that one ibutton would be able to accurately measure the temperature of the four surrounding cavities.

Each nest box was oriented in the field so that the sides faced northwest (NW), northeast (NE), southwest (SW), southeast (SE) (Fig 3). Traditionally farmers place their boxes SE, but preference for this direction has not been previously tested (Stephen 1981). Nest boxes were placed on a wooden base approx. 4ft above the ground and topped with a wooden board with 7.62 cm of overhang to provide shade (Fig 3A), similar to the agricultural set up. Straws measuring 7.5cm were placed in each cavity to allow for nest removal and analysis. The back of each block contained an ibutton that recorded the block temperature to the nearest 0.5°C every 15 min. Ibuttons were downloaded and reprogrammed approximately every 20 days. iButtons were initially deployed on June 21st, 2018 and the final temperature reading was September 22, 2018. Out of 540 iButtons three had failures that resulted in data loss.

Monitoring nesting behavior and nest size

Megachile rotundata purchased from JWM Leafcutters (Nampa, Idaho) were released on top of the nest block and allowed to nest in any of the available cavities. 500 bees were released at each nest box replicate on June 20th, 2018 and 1,068 bees were released at each replicate on

June 26th, 2018. Starting on June 25th, 2018 boxes were checked every day for capped nests. Once a nest was capped, the date and location was recorded.

Capped nests were X-rayed every Monday and Thursday from July 6th- Aug. 2nd. Nests were X-rayed on a (Faxitron 43855A) four 4 seconds, at 28 KVM, machine. Once the nesting activity has stopped, capped nests were X-rayed on Aug 15, Sept. 3, and Sept. 22nd. X-rays were recorded and saved by date and nest ID. Number of brood cells was measured through x-rays.

Cavity nest preference

Nest boxes were checked each day for completed nesting. After nests were completed the nests were x-rayed twice a week until Aug. 15th 2019. The first time each nest was x-rayed was used to determine the number of brood cells in each nest. *M. rotundata* build about one brood cell per day (Maeta and Kitamura 2005). Assuming a rate of one brood cell per day, the start date of each nest was calculated using the date of capped nest subtracted by the number of brood cells. This analysis gave the start date for each nest. We hypothesized that temperature on the first day of nesting would be the most important temperature in determining nest choice. Rstudio (1.1.419) was used to calculate the average temperature for each cavity for all of the dates of completed nests. We used the first 30 days of capped nests for this analysis. To calculate choice nests were either labeled with 0 for nest unfilled by calendar date or 1 for filled on that calendar date. Cavities that were already chosen from a previous day were labeled with a “removed” and removed from analysis. Thus, each female’s preference was only calculated based on the number of cavities available on the first day of nesting.

Data analysis

IButtons were downloaded individually and then combined using Rstudio (1.1.419) and R (3.5.2) with packages (lubridate, tidyr, and stringr). *M. rotundata* are only actively foraging

during the daylight hours (Lerer et al. 1982 and Szabo and Smith 1972), thus average foraging temperature was determined by temperatures collected between 9 am and 9 p.m. using the package `dplyr` and subset from R studio. Date capping was used to track nest box fill over the course of the experiment. Survival curves were generated using JMP Pro version 14. All other analyzes were done in Rstudio. Ambient temperatures were obtained from the closest weather station to the field site, Hector International Airport, and downloaded from the National Climatic Data Center.

Results

Nest cavity temperatures during foraging

M. rotundata will only actively build a nest during the daylight hours, thus, we wanted to understand how average cavity temperature changed during the foraging hours over the course of the experiment, and whether there were patterns in cavity temperature by the cardinal direction the nest box was facing and the position of the cavity. The temperature data was subset using R studio, from 9 am to 9 pm. The average temperature from June 22nd until August 15th by direction, block in the nest box, and replicate was calculated using the `plyr` package in R studio (Fig 4A).

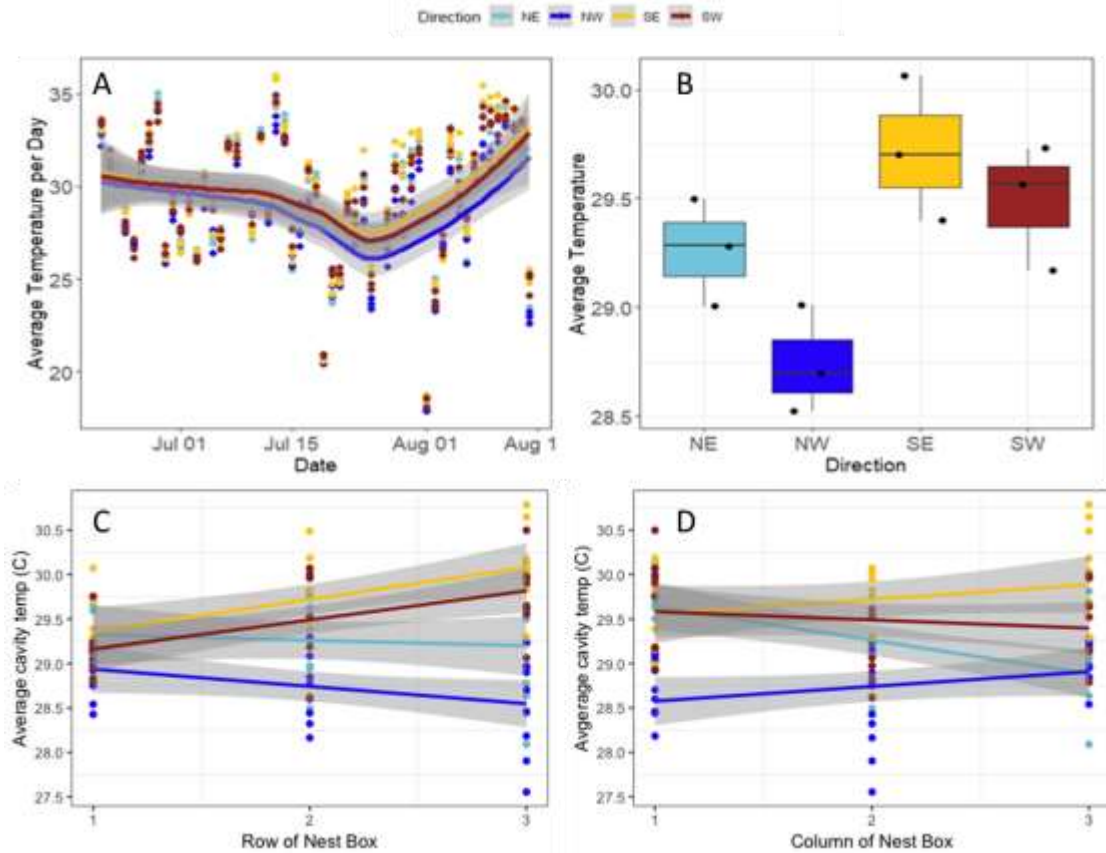


Figure 4. Average Field Temperatures. A) Average temperature (C) per day for by direction. B) Average temperature (C) by direction for entire nesting season. C) Average foraging temperature (C) by each row for entire nesting season, color by direction. D) Average foraging temperature (C) by each column for entire nesting season, color by direction.

The minimum nest cavity temperature during foraging hours was 9.5°C, and nest temperatures were recorded up to 46.5°C. All sides experienced similar minimum temperatures, but the maximum temperature was much more variable and ranged from 42°C on the NE and 46.5°C on the SW side. Interestingly, the minimum temperatures were all reached on the same date of Aug. 2nd, however the maximum temperatures for each direction were reached on different days. The SW side reached its maximum temperature on July 13. The NW side reached the max on June 21st, SE August 8th and NE on June 28th. Average ambient temperatures did not reach temperatures this high and varied from 12°C-28°C with a maximum temperature of 33°C (National Climatic Data Center 2018).

Nest boxes were constructed by stacking three rows with three columns of individual blocks. In order to test the influence of block position and cardinal direction on nest cavity temperature, we tested linear models that incorporated cardinal direction as a categorical variable and row and column as continuous variable. Replicate was analyzed as a random effect. For example, we were interested in whether the first row of cavities was cooler due to the shaded overhang on top of the box. There were significant interaction terms between row and direction ($F(3,100)=8.0721$, $p<0.0001$) and column and direction ($F(3,100)=6.9834$, $p=0.0003$). Direction was a significant predictor of cavity temperature ($F(3,100)=19.411$, $p<0.0001$, $R^2=0.3504$). When nest box row and column was added, row was significant ($F(1,100)=4.8565$, $p=0.0299$, Fig. 4C), but column was not ($F(1,100)=0.5058$, $p=0.4787$, Fig 4D). The combination of row, column and direction explained 53.19% of the variation in average cavity temperature. The shading on the top of the boxes made the first row have similar temperatures across directions, with the SE and SW directions increasing in warmth in cavities in rows two and three (Fig 4C). The NE and NW directions decreased in average temperature in rows two and three. These results support the idea that microclimate exist across the nest box, allowing the possibility of cavity choice influencing maternal decisions and offspring environment.

Rate of nest completion by direction

The agricultural industry places shelters facing southeast, however preference has not been tested in this species. Thus, we wanted to understand if *M. rotundata* favored a particular direction when nesting. Over the course of the nesting season all boxes filled to full capacity. However, rate of fill was significant by direction (chi-squared (3) =1627.065, $P<0.0001$, Fig 5). The Northwest and Northeast cavities were preferred by nesting females and capped first, followed by the Southeast and Southwest facing cavities. The Northwest and Northeast sides

reached 50% capacity and full capacity approximately five days before the Southwest and Southeast sides (Fig 5). A Tukey's Post hoc showed that females preferred NE to SW ($p < 0.0001$) and SE ($p = 0.0004$). The NW side was also significantly different from the SW ($p < 0.0001$) and SE ($p < 0.0001$). SE and SW preference were not significant ($p = 0.871$), and NE and NW preference was not significant ($p = 0.7096$).

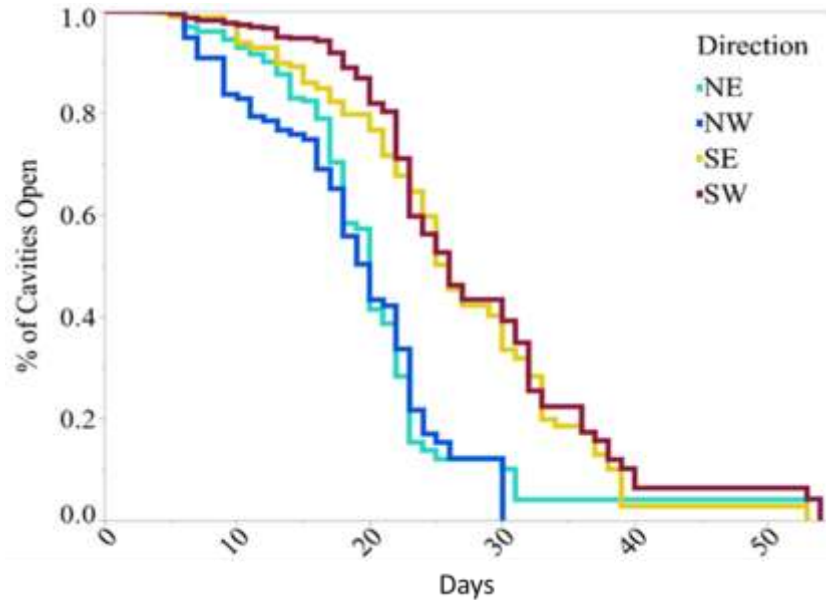


Figure 5. Nest Completion by Direction. Kaplan-Meier of box fill over nesting season ($p < 0.0001$). NE and NW cavities filled 7 days earlier than SE and SW cavities.

Nest cavity preference by temperature

To determine if the difference in nesting patterns was dependent on temperature a binomial, general linearized model was run. The response variable for our model was filled (1) or unfilled (0) for each date and accounts for the variation in number of cavities throughout the nesting season. The fixed effects were average foraging temperature of each day, cardinal direction, row and column of nest box, and replicate was included as a random effect. Column was not significant ($F(1,6007) = 0.0004$, $p = 0.9840$), so it was dropped. None of the interaction terms were significant. The final model included the average temperature during foraging hours

($F(1,6015)=7.9369$, $p=0.0049$), cardinal direction ($F(3,6015)=12.8016$, $p=0.0003$) and row ($F(1,6015)=22.8035$, $p<0.0001$). The probability that a female would nest in a cavity decreased with increasing temperatures (Fig 6A). As had been found in the Kaplan Meier analysis, females favored the NE and NW cardinal directions (Fig 6B), and females preferred the top rows of the nest box (Fig 6C). The variance inflation factors were all below three demonstrating that the preferences for north-facing sides of the nest box, and top rows of the nest box was independent of their temperature and thus, temperature, direction, and row contributed to nest choice independently of one another. However, these three factors together only explained 6.08% of the variation in nest choice, and the predictive power of the factors is low. For example, the average probability that a female would choose a 20°C cavity was only 8.91%. Females showed a preference for cooler, north-facing cavities at the top of the nest box, but this preference was weak (Fig 6).

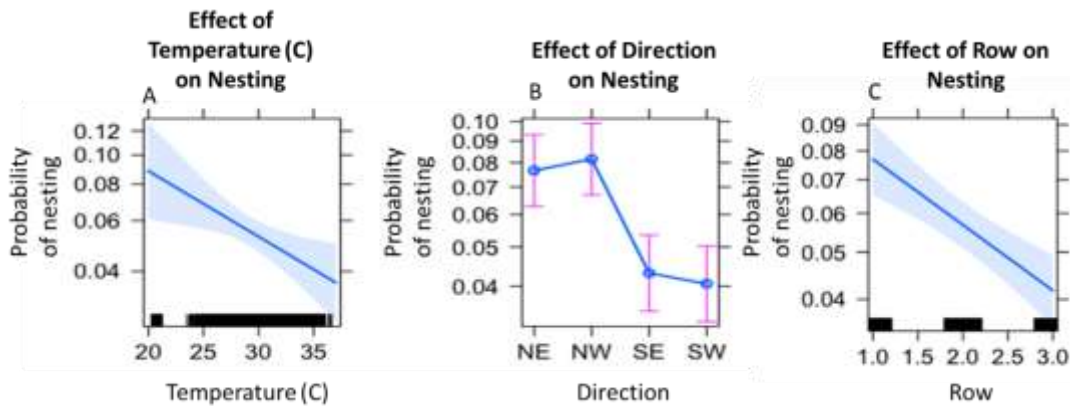


Figure 6 The Probability to Predict Nesting. A) Probability of temperature to predict nesting. B) Probability of direction to predict nesting. C) Probability of row to predict nesting.

Nest size

Females can choose how many brood cells to build per cavity and we hypothesize that they would lower their reproductive investment in nest cavities with poor microclimates. We tested a linear model that used average temperature of the cavity, cardinal direction, row and

column of the nest block as fixed effects and replicate as a random effect. The temperature of the cavity had no effect on the number of brood cells a female built ($F(1,6006)=0.3099$, $p=0.5778$), nor did the column of the nest box ($F(1,6006)=0.2045$, $p=0.6511$), so these were dropped from the model. The cardinal direction ($F(3,6007)=142.08$, $p<0.0001$: Fig 7) and the row of the nest box ($F(1,6007)=579.2$, $p<0.0001$) did have a significant influence on brood cell construction. Females built more brood cells in NE cavities, and did not favor the SW (Fig 7), and nesting females preferred rows in the top of the nest box (Fig 6C). The combined effect of cardinal direction and row explained 14% of the variation in the number of brood cells.

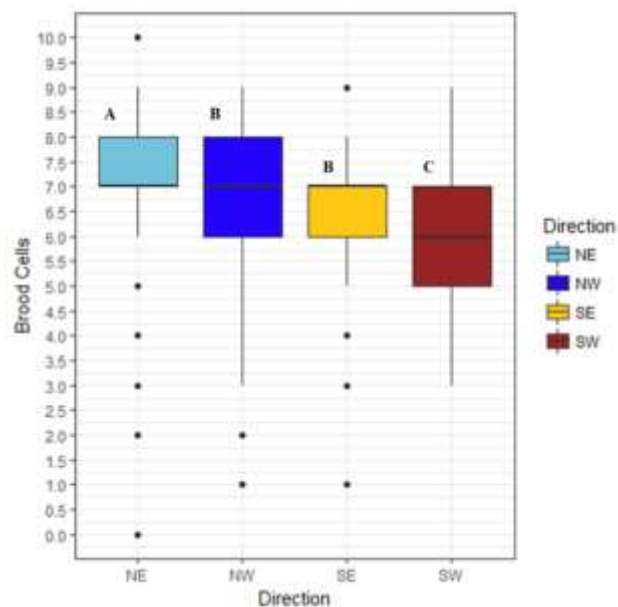


Figure 7. Nest Size by Direction. Nest size significantly differed by direction One-Way ANOVA ($p<0.001$). Tukeys HSD denoted by letter.

Discussion

Insect performance has a non-linear relationship to temperature. Fluctuating temperatures have continually been shown to influence insect performance (Colinet et al. 2015, Sinclair et al. 2016). Increasing temperature can improve insect performance up until a thermal optimum after which further increases cause a steep decrease in performance (Jensen 1906). However, insects

can behaviorally regulate their exposure to harmful temperatures by choosing favorable microclimates. Some insects have juvenile stages that are immobile and are not able to behaviorally regulate their exposure to temperatures. Hymenopterans tend to have immobile larva, whose mothers choose the location for juvenile development. For cavities nesting bees like the alfalfa leafcutting bee, *M. rotundata*, females have the potential to limit the exposure of their offspring to harmful temperatures by choosing favorable cavities for their nests. Our goal was to test whether nest cavities are microclimates with respect to temperature and whether female bees make reproductive decisions based on cavity temperature.

We found that nesting cavities can act as a microclimate, with some cavities warming to high temperatures. We found nesting cavities are highly variable in average temperature and maximum temperature, while the minimum temperature was shared across cavities. Cavity average temperature between the SE and NE side varied by 1°C. Maximum temperatures are especially important in the relationship between performance and temperature, because increases could potentially exceed the thermal optimum where one degree past the optimum causes disproportionate decreases in performance (Jenson 1906). We found that the SW side reached 46.5°C, 1.5°C above the 45°C that has been shown to cause a decrease in survival to developing pupae (Undurraga and Stephen 1980). However, the cavities facing the NE never reached this threshold for decrease survival. Nesting box cavities increases by row from top to bottom within the nesting box on the southern facing sides (Fig 4A-D), but cavities on the northern sides did not have this pattern (Fig 4C-D). Our maximum temperatures are similar to other reported field studies in the US, where tents containing nesting boxes reached temperatures of 44°C (Rossie et al. 2010). Nesting boxes placed in Arizona reached temperatures exceeding 45°C (CaraDonna et al. 2018). While previous studies have reported similar overall temperatures, our temperatures

were more variable and indicated that one measure of temperature for multiple cavities is not enough to accurately capture the range of temperatures across cavities. These temperature ranges indicate that not all nest cavities are favorable microclimates for offspring development, and that females have the potential to influence offspring fitness through choosing cavities that will not reach high temperatures.

This study also further indicates that ambient temperature is not a reliable predictor of cavity temperatures experienced and resembled a microclimate. The average ambient temperatures throughout the course of the experiment varied from 12°C-28°C and reached a maximum temperature of 34°C (National Weather Service 2018), while nesting boxes reached much higher temperatures above 40°C. These variations in temperature determine that the nesting box is a microclimate that varies from ambient temperature. *M. rotundata* nesting success has been tied to mean actual temperature, heat units, and average temperature rather than area projections (Richards 1996). *Rhagoletis pomonella*, that reside in apples, experienced temperatures reaching upwards of 45°C in the apple, while ambient temperatures rose only to 34°C (Lopez-Marinez and Denliner, 2008). We found the cavities in *M. rotundata* nesting box heat up disproportionately throughout the day and there is significant variability in temperatures even between cavities facing the same cardinal direction (Fig 4). These temperature variations indicate that ambient temperature would not be able to predict the true variability of all cavities and are not an accurate predictor of temperature exposure.

Due to the variability in temperature, our data suggest that female bees may have more of a choice in mitigating exposure to high temperature than previously considered. Movement away from warm temperature as an avoidance strategy has been demonstrated in multiple insects (Woods et al. 2014, Huey et. al 2002, and Kearney et al., 2009). We find that *M. rotundata*

females nested in cooler cavities on the northern sides faster than the warmer cavities on the southern sides (Fig 5), suggesting female preference to cooler cavities. Further analysis on temperatures chosen by nesting females indicated that up to 8.9% of cavity choice can be explained by temperature and as temperatures increase, the probability of nesting in that cavity decreases (Fig. 6A).

However, direction as its own independent factor was also a predictor of cavity choice, while a smaller predictor than temperature, it is possible that other abiotic factors such as wind, may also influence this choice and explain further predictability (Fig 6B). Bumblebees have been shown to prefer the nesting boxes facing the north by relying more heavily on light distribution in the absence of wind, however, in the presence of wind bumblebees equally nested on the north and south (Hemple de Ibarra et al. 2009). Yet, trap nesting for bees and wasps has been found to be most successful when turned away from prevailing winds (Martins et al. 2012). Nevertheless, we found that direction influences the temperatures experienced in the cavities as seen by the variation in foraging temperature (Fig 4) and max temperatures reached, therefore this link may also explain why direction alone, was a significant predictor. Further testing will need to be done to decouple the effects of temperature vs. direction on nesting choice.

Microclimates will become increasingly more important under climate change. Temperatures are predicted to rise, and experience higher fluctuations (Vasseur et al. 2014), and while ambient air temperature are not as strong of predictor as experienced temperature, fluctuating and higher ambient temperatures will impact microclimates through solar irradiation. We found significant variation across 36 cavities, and these variations may be exacerbated by increasing temperatures making the warmest cavities even warmer than they are and further pushing organisms past their thermal optimums. An increase in climate modeling has occurred in

order to predict the effects of climate change across a wide range of landscapes (Colinet et al. 2015, Sinclair et al. 2016). Microclimate provides a more reliable measurement of temperature and the true amplitude of fluctuations cavity nesters may experience in the field. These temperatures will only improve the ability to explore how climate change may impact insects and predict how performance varies with climate change.

The ability to predict insect response will be even more important when considering economically viable pollinators, like *M. rotundata*. In a managed setting *M. rotundata* nest in large polystyrene bee boxes (Richards 1996). However, in the U.S. *M. rotundata* does not reproduce well and has a 50% return rate (Pitts-Singer and Bosch, 2011). *M. rotundata* is used throughout the US in multiple environments including Central Valley California where temperatures can consistently rise above 40°C (Barthell et al., 2002). Currently, the agricultural industry faces their shelters facing the Southeast with the polystyrene boards slid into the wooden shelter (Stephen, 1981). While these studies often make connections to temperatures and reproduction, our studies explore the full variations of temperatures experienced by these bees and makes a link between temperatures and decreases in fitness. Our study found that through facing the box facing a more suitable microclimate a female bee will lay one additional offspring. For growers this could be an 15% increase of offspring laid and additional pollinators for the nest season. Our study demonstrates that growers may be able to manipulate the microclimate experienced by *M. rotundata*, and lead to increased nesting rates and offspring laid.

MICROCLIMATE TEMPERATURES ON DIAPAUSE, BODY SIZE, AND SURVIVAL OF *MEGACHILE ROTUNDATA*

Abstract

Offspring of *M. rotundata* will spend their entire developmental period in the nesting cavity without the ability to move or change their environment. The direction and position the cavity faces influences temperature experienced (see Chapter 2). The nesting cavity can reach temperatures that have been shown to decrease survival and influence development rate under constant lab conditions (Kemp and Bosch 2000). However, the nesting box fluctuates in temperature throughout the day and it has not been shown how natural fluctuating temperatures impact developing offspring. Often the nest box is measured as a constant unit of temperature, but this neglects the variation of temperatures offspring can experience. To estimate the true impact of experienced cavity temperature on offspring, we designed a 3D printed box that measures individual cavity temperature. We monitored nesting temperature from June 21st -Sept. 22nd and followed offspring through development to measure mortality, development, and body size. We find that nesting cavity temperatures reach lethal temperatures that decrease survival of developing offspring. However, these stressful temperatures have no impact on adult body weight. We find that nesting cavity temperature does not influence diapause aversion, and that photoperiod is a strong cue for diapause incidence. In conclusion, we demonstrate that nesting cavity temperature impacts survival of offspring, and through changing direction or position of the nesting box, growers can decrease mortality associated with temperature. Future studies must consider the nesting box not as a cohesive environment for solitary bees, but a microclimate and continually measure multiple positions throughout the nesting box to account for variation.

Key words: Diapause incidence, pollinators, preference, climate change, fitness

Introduction

Megachile rotundata, the alfalfa leafcutting bee, has a facultative bivoltinism diapause that is permanent (Tepedino and Parker, 1986). Typically, *M. rotundata* adults emerge in the spring and begin to build a linear nest made of individual cells of leaves and provisioned with pollen and nectar (Pitts-Singer and Cane, 2011). Once a brood cell is complete the female will lay an egg on top of a provision, and then begin the second cell. Once the nest is complete the female will seal off the nest with a cap. Eggs will complete embryogenesis in approximately 2-3 days and continue to develop through 5 instars (Trostle and Torchio, 1994). Individuals enter diapause in the pre-pupa stage. However, some offspring will skip diapause and emerge in the same summer as the parent generation. These individuals are referred to as 2nd Generation or nondiapausing individuals. Due to the facultative diapause structure, *M. rotundata* are a prime model to explore diapause incidence.

In the United States, 2nd Generation rates can reach up to 90% of individuals laid for the nests made in the earlier season, and 0% by late August (Johansen and Eves, 1973). However, Canadian populations only reach up to 5% of summer adult emergence (Kronic, 1972). Currently, it is unclear the exact mechanism causing increased nondiapausing individuals, but the leading hypothesis is that the decision to enter diapause is under maternal control (Tepedino and Parker 1986). However, several studies have noted environmental factors that may influence diapause incidence or aversion including; amount of food (Rank and Rank, 1989), length of photoperiod (Pitts-Singer and Cane 2011) and warm temperatures (Tepedino and Parker 1986, Kemp and Bosch 2000 and Kemp and Bosch 2001).

Diapause initiation is often energetically costly, and other insect species are able to recognize when enough energy is available to initiate diapause (Hahn and Denlinger 2011). *M.*

rotundata females provision each offspring independently, finishing one brood cell before another is started (Pitts-Singer and Cane 2011) and the provisioning strategy of this species is impacted by sex, position within the nest, and environmental factors. The amount of provision depends on the sex of the offspring, with females typically receiving higher amounts of provision (Pitts-Singer and Cane 2011). Sex of the offspring is under maternal control, with the majority of female offspring laid in the back of the nest (Yocum et al. 2006). Not only does sex play a role in provision amount but the probability of entering diapause was influenced by provision size as well and scaled according to sex (Fischmann et al. 2017), suggesting that larval provision may be a factor in initiation of diapause. Thus, an adult female may be able to control sex, amount of provision, and diapause incidence in developing offspring.

However, even though the adult female may provision a nest with a certain amount of provision this does not guarantee that provision will be eaten. While the majority of offspring eat all of the provision provided (Trostle and Torchio 1994), environmental factors can influence consumption. Temperature can impact the amount of provision consumed regardless of amount provisioned. Increasing temperatures up to 30°C, increase the amount of provisions left uneaten in the cocoon and contribute the decrease in body size (Radmacher and Strohm, 2009). Provision size is highly correlated with adult body weight in *M. rotundata* (Klostermeyer et al. 1973), and heritability for body size in solitary bees is found to be low (Tepedino et al., 1984), indicating that collecting data on provision size or adult weight provides insight to either variable. It has been found that diapause individuals usually weigh more than non-diapausing individuals (Tepedino and Parker 1988, Pitts-Singer and Bosch 2010). However, provision size is under maternal control while amount eaten is controlled through offspring and impacted by abiotic temperatures. It is important that studies of diapause incidence take into account provision size

as well, as body weight in order to separate the parental from offspring effects. Thus, if diapause incidence is controlled through the provision it is currently unclear, whether provision provided has a direct effect on diapause or is an effect through other means, such as temperature, sex, or offspring consumption.

Several studies have shown that 2nd Generation individuals often emerge during the first half of the summer and are often offspring from the first nests laid. In 1972 2nd Generation bees peaked at 37.9% of brood cells and were laid on July 7th and by July 30th 2nd Generation bees fell to 1.4% (Kronic 1972). In 1988 2nd Generation reached 34% by median date laid July 15, and fell to 7% by median date laid, July 23 (Kemp and Bosch 2000 and Kemp and Bosch 2001). Nondiapausing individuals reached 54% by July 14th and fell to 0% by August 5th (Tepedino and Parker 1988). All of these studies indicate that earlier laid nests contain a higher percentage of non-diapausers and that the percentage falls to 0% of non-diapause individuals by the end of July early August. Adult females could be responding to amount of daylight early in the season and laying 2nd Generation eggs. Through monitoring nesting completion rates and emergence dates we can explore how time of year influences diapause incidence in *M. rotundata* under natural field conditions.

M. rotundata are likely to be impacted by heat stress through the nesting box with temperatures reaching above 40°C (see Chapter 2). Heat stress has been shown to decrease development time and lead to higher rates of 2nd Generation (Pankiw, Lieveise, and Siemens 1980, Kemp and Bosch 2000, Kemp and Bosch, 2001). The majority of nondiapause individuals come from nest laid earlier in the summer and the mechanism behind 2nd Generation is under maternal control (Tepedino and Parker 1986). However, several studies have noted that stress, specifically temperature stress, may also lead to more diapause averting individuals (Kemp and

Bosch 2000, Kemp and Bosch 2001). Specific development stages of *M. rotundata* are more sensitive to temperature stress than others (Barthell et al. 2002, Undurraga and Stephen 1980), and could potentially indicate that *M. rotundata* sensitivity to temperature could impact decision to diapause.

It is difficult to separate photoperiod, food provision, and temperature as separate study systems, because all of these factors happen simultaneously in the *M. rotundata* system. However, we have designed a nesting box that records exact cavity temperature in the field (see Chapter 2). By using these boxes, we will measure the effect of temperature on diapause incidence, and the use of these boxes throughout the nesting season will lead to key insights on how photoperiod impacts diapause decision rates. Our study aims to understand how nesting temperature and photoperiod interacts to influence diapause incidence in *M. rotundata*.

Methods

Field site set up

Three nest boxes were placed along the side of an alfalfa field in Fargo, North Dakota (46°55'15" N, 96°51'17" W). A drainage ditch containing multiple forbes and weeds ran along the side of the field. The replicates were placed 200m apart, which is a distance that minimizes adult migration between replicates (Bradner et al. 1965). Each nest box consisted of 36 smaller boxes that contained four nest cavities and were 3D printed using purple PLA plastic (#eSUN, ABS175Z1) in dimensions 60mm x 60mm x 82mm (Fig 3). 108 boxes were printed on a Taz 5 and Taz 6 3D printer. The four nest cavities were spaced equal distance apart and were 3.5mm x 78mm (Fig 3). Nest boxes were made by stacking blocks in a three by three pattern, resulting in 36 nesting cavities per side, and a total of 144 cavities per nest box. A hole was added to the back middle of each four-cavity block to accommodate a Thermocron 5 iButton (DS1921G-F5#-

ND) measuring 10.25mm x 41mm. To ensure that one iButton was able to accurately measure the temperatures of the four cavities on the front of the box, an incubator pre-trial was run. Four boxes were placed in an incubator with an HOBO temperature probe in each cavity (ONSET, U12-006) and an iButton placed in the back. The incubator was set to ramp from 10°C to 30°C then back down to 10°C over the course of 4 days. This trial showed no significant difference between the HOBO probes and each of the four iButtons (ANOVA, $p=0.981$, $p=0.941$, $p=0.948$, and $p=0.978$). This pre-trial confirmed that one iButton would be able to accurately measure the temperature of the four surrounding cavities.

Each nest box was oriented in the field so that the sides faced Northwest (NW), Northeast (NE), Southwest (SW), and Southeast (SE) (Fig 3B). Nest boxes were placed on a wooden base approx. 4ft above the ground and topped with a wooden board with 7.62 cm of overhang to provide shade (Fig 3A), similar to the agricultural set up. Straws measuring 7.5cm were placed in each cavity to allow for nest removal and analysis. The back of each block contained an iButton that recorded the block temperature to the nearest 0.5°C every 15 min. IButtons were downloaded and reprogrammed approximately every 20 days. iButtons were initially deployed on June 21st, 2018 and the final temperature reading was September 22, 2018. Out of 540 iButtons three had failures that resulted in data loss.

Monitoring nesting behavior and nest size

Megachile rotundata purchased from JVM Leafcutters (Nampa, Idaho) were released on top of the nest block and allowed to nest in any of the available cavities. 500 bees were released at each nest box replicate on June 20th, 2018 and 1,068 bees were released at each replicate on June 26th, 2018. Starting on June 25th, 2018 boxes were checked every day for capped nests. Once a nest was capped, the date and location within the nesting box was recorded.

Capped nests were X-rayed every Monday and Thursday from June 6th- Aug. 2nd. Once the nesting activity has stopped, capped nests were X-rayed on Aug 15, Sept. 3, and Sept. 22nd. X-rays were recorded and saved by date and nest ID. Number of brood cells was measured through x-rays and through nest dissection at the end of the experiment. Nest parameters measured through x-rays were brood cell position, diapause vs non-diapause, and parasitism. Offspring that reached the adult stage and were non-diapausing, 2nd Generation, were dissected from the nest with their position and date recorded (Fig 8)

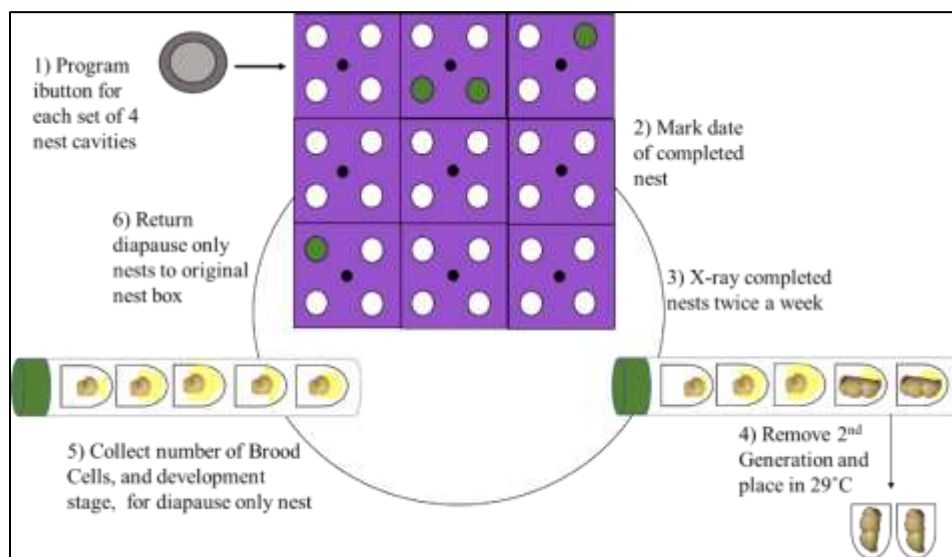


Figure 8. Methods Figure. How nests were continually monitored throughout the season.

The nondiapause individuals were placed in 24 well plates in a 29°C incubator and allowed to emerge. Emergent date, and sex was recorded. Offspring that ceased development in preparation for diapause were dissected from the nest on Sept. 22nd and placed in 24 well plates. Plates were put in a 6°C incubator for the remainder of the diapause period. Plates were then placed in 29°C on Dec. 11th and allowed to emerge (Yocum et al. 2006). Emergence date and sex was recorded for each offspring. Once individuals emerged, they were dried in individual well capsules with ID number, sex, and nest position recorded. Dry weight, IT Span, and Head Width

were measured following (Cane, 1997). Images were taken on a microscope (SteREO, Discovery.V8, Zeiss) with a Canon camera (EOS Rebel T3 EOS 1100D) and analysis was done on ImageJ (Windows, 64-bit, 1.8.0_112).

Data analysis

Buttons were downloaded individually and then combined using Rstudio (1.1.419) and R (3.5.2) with packages (lubridate, tidyr, and stringr). Time spent above 35°C and 40°C by block, replicate, and direction was calculated using subset and the dplyr package. Time spent above 35°C and 40°C was calculated into minutes and hours. Hours spent above 40°C was used for survival analysis in order to decrease variation on the axis. At 35°C *M. rotundata* start to produce HSP70 proteins and at 42°C HSP70 proteins peak (Barthell et al. 2002) and provided the rationale for our chosen temperature thresholds. Average temperature by block, replicate, and direction was calculated using the dplyr package. Offspring were separated into three categories, emerged, dead, and removed. Emerged individuals successfully emerged from their brood cells. Dead individuals failed to exit their brood cells. Removed individuals were offspring that were in the same nest as a nondiapauser individual, that were diapauser individuals. These individuals were sacrificed, because the nest was destroyed in collecting the non-diapauser individuals and unable to be placed back in the field. These individuals in the removed category were assumed to be diapausing individuals because they did not transition to the adult stage at the same time as the 2nd Generation individuals (Fig 8). For survival analysis emerged individuals were assigned a 1 and dead individuals were assigned 0. Date of capped nest was converted to a continuous numeric with 1 equaling the date of the first nest completed and 52 equaling the date of the last nest completed.

Results

Nesting box temperatures and microclimates

Offspring are exposed to a highly variable range of temperatures in the nesting box throughout the season. The nesting boxes reached a minimum temperature of 4.5°C and a maximum temperature of 48.5°C. The NE reached a maximum temperature of 42°C. The NW reached a maximum temperature of 45.5°C. The SE reached a maximum temperature of 44°C and the SW reached a maximum temperature of 48.5°C.

We wanted to understand if direction and block position influenced, influenced average temperature experienced, time spent above 40°C, and time spent above 35°C. We used parametric and non-parametric ANOVAs to analyze variation in temperature by cardinal direction. Direction significantly impacted temperatures experienced (Fig 9A-C). Average temperature varied by less than 1°C and was significant by direction ($F(3)=414.3$, $p<0.0001$) (Fig 9A). All post-hoc comparisons by directions were significant from each other at ($p<0.0001$) and the SE reached the highest average temperature. Exposure to stressful temperatures also varied by direction (Fig 9B-C). Time spent above 40°C and 35°C were significant by direction ($\chi^2(3)=1335.7$, $p<0.0001$, and $f(3)=805.8$, $p<0.0001$) respectively. Time spent above 40C was analyzed using a non-parametric ANOVA to account for unequal variance, and time spent above 35C was analyzed using a One-way ANOVA. All post-hoc comparisons for time spent above 35°C and 40°C were significant ($p<0.0001$) (Fig 9B-C). The SW spent the greatest amount of time above both 35°C and 40°C while the NE spent the least amount of time above 35°C and 40°C (Fig 9B-C).

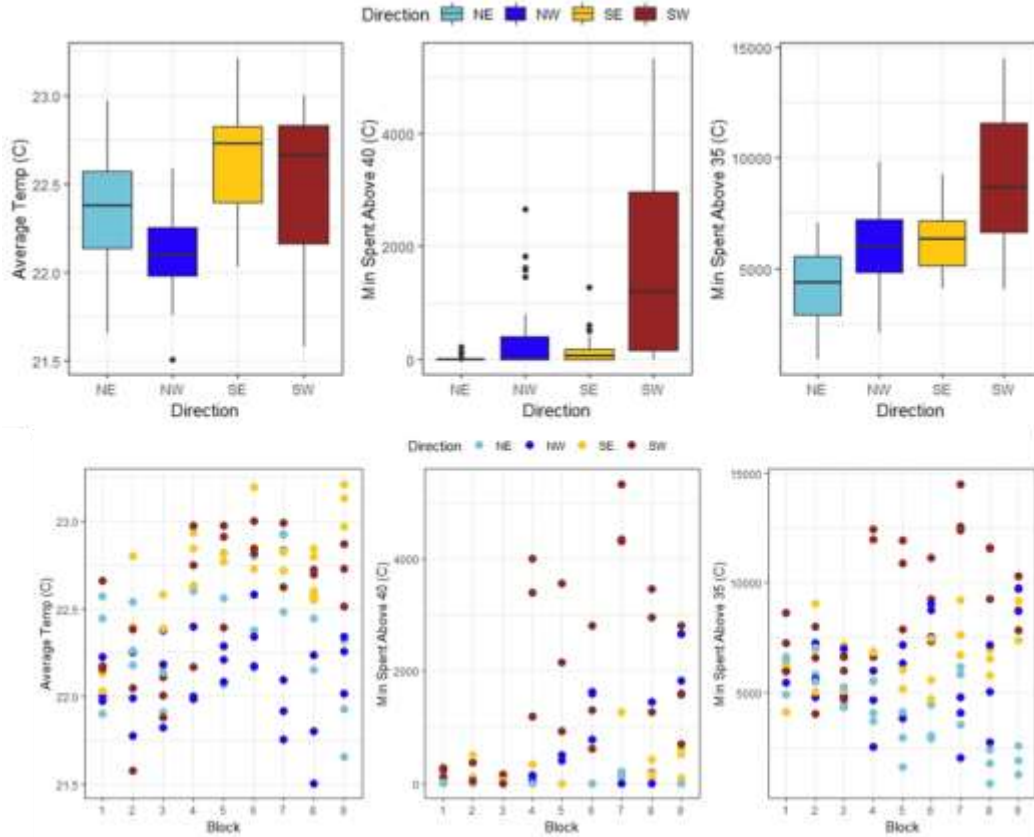


Figure 9. Experienced Field Temperatures. A) Average temperature by direction B) Amount of time spent above 40°C by direction C) Amount of time spent above 35°C by direction D) Average temperature by block E) Amount of time spent above 40°C by block F) Amount of time spent above 35°C by block.

To analyze if temperatures were significantly different by block, we tested linear models that incorporated temperature treatment as a continuous response variable, cardinal direction as a categorical variable and block as a categorical variable and replicate as a random effect. We ran three separate models with each response variable represented as either average temperature, time spent above 40°C or time spent above 35°C. Not only were temperatures impacted by direction, but cavity temperature varied by block as well (Fig 9D-F). The interaction between block and direction was also significant ($F(24)=119.54$, $p<0.0001$). Average temperature by block and direction were significant. Block and Direction were significant by themselves ($F(8)=357.49$, $p<0.0$, $F(3)=1074.49$, $p<0.0$). The model explained 72.75% of the variance in average

temperature when all variables were included. Time spent above 40°C was also significant by block and direction. The full model explained 84.78% of the variance in time spent above 40°C. The interaction between block and direction was also significant ($F(24)=326.15$, $p<0.0001$). Block and direction by themselves were significant ($F(8)=396.16$, $p<0.0001$, $F(3)=2548.99$, $p<0.0001$). Time spent above 35°C was significant by both block and direction. The interaction between block and direction was also significant ($F(24)=249.78$, $p<0.0001$). The full model explained 81.08% of the variance in time spent above 35°C. Block and direction were significant by themselves ($F(8)=32.801$, $p<0.0$, $F(3)=2462.13$, $p<0.0001$). These findings follow the pattern established in the first chapter of the manuscript and further support the idea that the nest box is a microclimate. Average temperature, time spent above 40°C and time spent above 35°C are significantly impacted by the block in which the cavity faces and the direction the cavity is facing. Due to the significant interaction effect the amount of variation explained directly by direction or block is unable to be determined.

Incidence of diapause and nondiapause

The total number of individuals that reached adulthood during the summer were calculated as nondiapause individuals. Nondiapause individuals by direction were, NE=144, NW=65, SE=61, and SW=42. The percentage of dead non-diapause individuals were, NE=38%, NW=49%, SE=34%, and SW=33%. All directions had nests that contained both nondiapause and diapause individuals, the number of individuals removed from the nest by direction were, NE=123, NW=84, SE=47, and SW=42. Removed individuals are diapause individuals that were in the same nest as a non-diapause individual.

M. rotundata experience a facultative bivoltinism and we wanted to understand if increased time spent under high temperatures influenced diapause aversion. We tested non-

diapause incidence against three temperature parameters; average temperature, time spent above 40°C and time spent above 35°C. We used linear models with temperature as a continuous variable, diapause incidence as a binomial factor of 0 or 1, and date completed as a continuous variable. Error structure for our model was calculated with replicate as a random effect, and nested within replicate was nest in order to account for individuals within the same nest. For analysis, diapausing individuals were assigned the number 0 and nondiapause individuals were assigned number 1 and treated as the response variable. Date capped was changed from a date into a continuous number for analysis. Average temperature was significant ($z=-2.247$, $p=0.0246$) and date completed was significant ($z=-6.678$, $p<0.0001$ Fig 10). Each factor was then analyzed for strength of predictability diapause/nondiapause and we found that date of capped nest predicts 29% of nondiapause incidence, while average temperature only predicts 13% of nondiapause incidence (Fig 10). Due to very few non-diapausing individuals spending time above 40°C and 35C a linear model could not be run. The majority of non-diapausing individuals were laid in the NE facing cavities. These cavities spent the least amount of time above 35°C and 40°C (Fig 9A-F) and thus a model cannot be run because there are so few non-diapausing individuals spending significant time above these temperatures. (Fig 10).

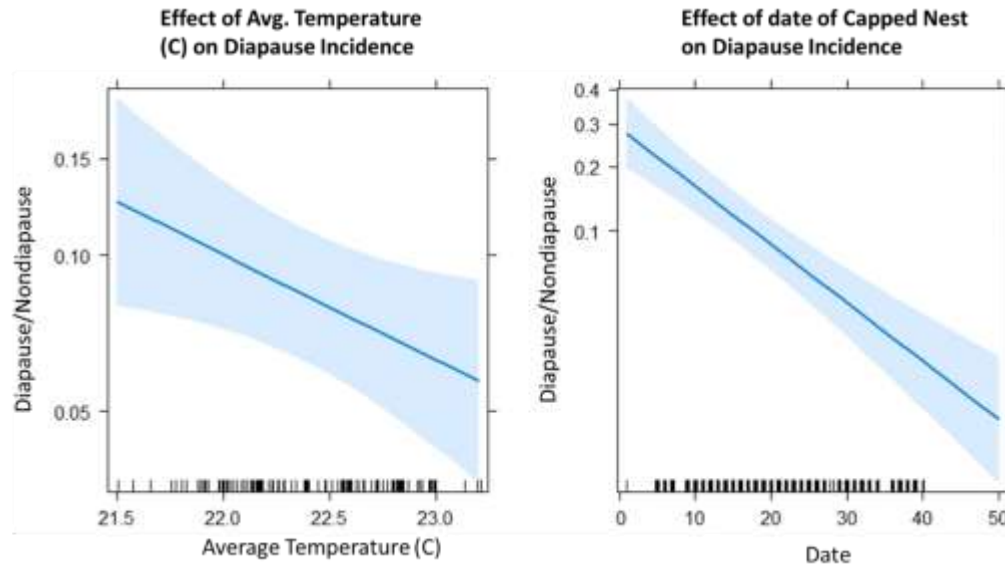


Figure 10. Probability of Diapause Incidence. A) Probability to predict diapause by average temperature B) Probability to predict diapause by date of capped nest.

Body size by temperature

Lab studies have shown that body size decreases with increasing temperature in the solitary bee *Osmia bicornis* (Radmacher and Strohm 2010). We wanted to see if increased exposure to high temperatures in the nesting box decreased body size in *M. rotundata*. We utilized linear models with body weight as a continuous variable, temperature parameter as a continuous variable, development, non-diapause or diapause, as a categorical variable, sex as a categorical variable, and date completed as a continuous variable. Error for our model was included as replicate as a random effect, and nested within replicate was nest in order to account for individual brood cells within the nest. Temperature over the course of the entire experiment was utilized in the models. We ran three linear models with each temperature parameter; average temperature, time spent above 35°C, and time spent above 40°C. Body size was not impacted by any measure of temperature in our study (Fig 11A-D). The factors with the highest impact on weight were development, date capped and sex. The first analysis modeled weight as a function of average temperature, development, date completed and sex. The full model explained 48.28%

of the variance in dry weight. Development is significant ($F(1)=101.1387$, $p<0.0001$). Sex is significant ($F(1)=1355.9190$, $p<0.0001$). Average temperature was not significant ($F(1)=0.0425$, $p=0.8366$, Fig 11A). Date completed was significant ($F(35)=2.6324$, $p<0.0001$). The second model included weight compared to time spent above 40°C, development, sex, and date completed. This model explained 48.30% of the variance in dry weight. Sex is significant ($F(1)=1354.3881$, $p<0.0001$). Development is significant ($F(1)=101.7930$, $p<0.0001$). Time spent above 40°C is not significant ($F(1)=0.5316$, $p=0.466$, Fig 11B). Date completed was significant ($F(1)=2.6476$, $p<0.0001$). Our third model tested the impact of time spent above 35°C on weight and included development, sex, and date completed. This model explained 48.36% of the variance in dry weight. Development is significant ($F(1)=103.1654$, $p<0.0001$). Sex is significant ($F(1)=1353.8752$, $p<0.0001$). Time spent above 35°C was not significant ($F(1)=2.2097$, $p=0.1373$, Fig 11C). Date completed was significant ($F(1)=2.6857$, $p<0.0001$). There was no significant interaction effect between development and sex in any of the models.

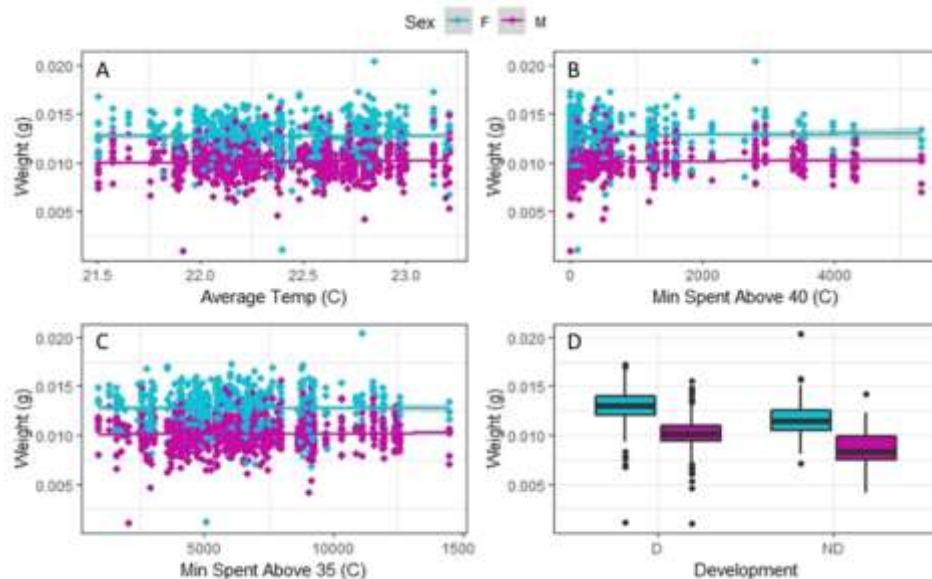


Figure 11. Body Size by Temperature and Development Stage. A) Average temperature on body weight B) Time spent above 40°C by body weight C) Time spent above 35°C by body weight D) Development stage on body weight.

Survival

Increased exposure to high temperatures could decrease survival in *M. rotundata*, we used linear models that utilized temperature treatment as a continuous variable, survival as a binomial factor of 0 or 1, and replicate as a random effect. Time spent above 40°C was significant in decreasing survival of offspring (Fig 12). Intercept was significant (z value=14.300, $p < 0.0001$). Time spent above 40 was significant (z=-3.026, $p=0.00248$). Correlation of fixed effects = -0.277. Neither average temperature nor time spent above 35°C was significant in impacting offspring survival with ($p=0.0675$, and $p=0.392$). We then wanted to see if date of completed nest and development also had an impact on survival. The further into the nesting season laid the greater chance of survival, general linearized model with survival as a binomial factor of 0 or 1, date completed as a continuous variable, time spent above 40°C as a continuous variable, development as a factor, and replicate as a random effect. Intercept was significant (z=2.284, $p=0.022381$). Time spent above 40°C was also significant (z=-3.799, $p=0.000145$). Development was also significant (z=-6.143, $p < 0.0001$). Predictability factors of survival were then run on time spent above 40°C, date capped, and development (Fig 12). We find that time spent above 40°C and developmental stage has the widest range of predictability on survival, ranging from 78%-61% (Fig 12).

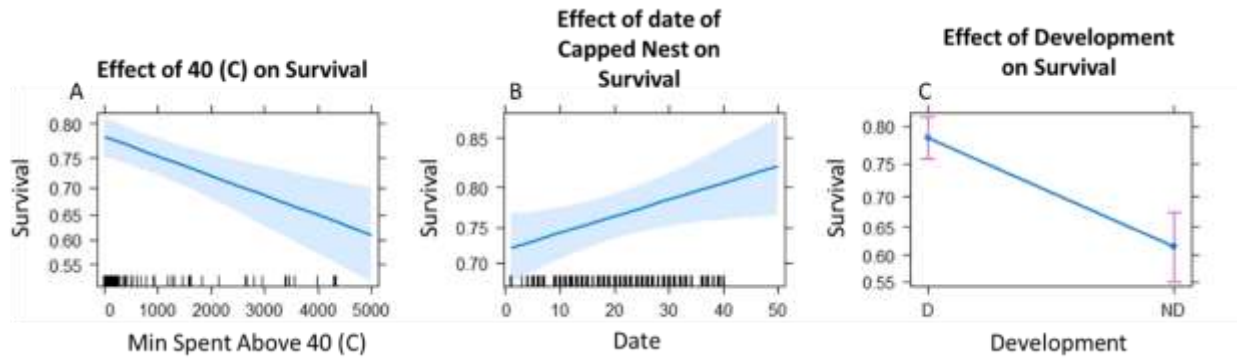


Figure 12. Factors impacting Offspring Survival. A) Probability of predicting survival by amount of time spent above 40°C B) Probability of predicting survival by date of capped nest C) Probability of predicting survival by development status

Discussion

Diapause allows insects to avoid harsh environments and time access to optimal resources such as flower availability (Denlinger 2002). Diapause incidence cues vary across species (Bale and Hayward 2009). Tiger moth *Cymbalophora pudica* relies on photoperiod as a cue for diapause with long photophases shortening diapause and short photophases lengthening diapause (Kostal and Hodek 1997). All insects will have a critical day length (CDL) that can program diapause (Bale and Hayward 2009) and in a temperate zone most insects rely on diapause to overwinter (Denlinger 2002). Diapause is also regulated through temperature, with *C. vicina* produce fewer diapausing offspring at 20°C than at 15°C (McWatters and Saunders 1988). Determining the cause of diapause incidence could not only aid in understanding a biological mechanism, but aid in industry. Triggers of non-diapause incidence in *M. rotundata* have been shown to be influenced by photoperiod (Pitts-Singer and Cane 2011), temperature (Tepedino and Parker 1986, Kemp and Bosch 2001), and provision size (Fischmann et al. 2017). Through utilizing a 3D printed nesting box that can record exact temperature, and monitoring throughout the entire nesting season, this study can look at the individual impacts on diapause of these environmental factors.

Non-diapausers by temperature

Several studies have indicated that development speed from egg to adulthood increases with temperature, with temperatures of 29°C showing the fastest completion to adulthood in both diapausing and nondiapausing individuals (Kemp and Bosch 2000). Temperatures between 29°C and 32°C have been reported to increase incidence of diapause aversion (Tepedino and Parker 1986, and Kemp and Bosch 2001). We predicted that the warmest cavities would have the highest number of nondiapause individuals. The warmest cavities in our study were the SW and SE facing cavities which spent the most time above 35°C and 40°C (Fig 9A-F). We predicted that these cavities would have the highest number of nondiapause individuals. However, we found that the NE had the highest number of non-diapause individuals and contained the coolest cavities. Surprisingly, our results also suggested that as average temperature increases, diapause incidence increases. This is opposite of what previous work on temperature has reported (Kemp and Bosch 2000 and Kemp and Bosch 2001, CaraDonna et al. 2018). However, it is important to note, that diapause aversion in *M. rotundata* was only tested under a temperature range from 18-32°C (Kemp and Bosch 2001) and nest box temperatures exceed this range. However, all nest cavities reached these temperature thresholds, and if these temperatures were the true trigger of diapause aversion, we would have expected an equal representation of nondiapause individuals facing each direction.

While it is possible that we found the opposite result of diapause and temperature under field conditions, then what is previously established in the literature, we suggest that the NE side and coolest cavities contained the highest number of nondiapause individuals, because of the time in which the majority of nests were laid. Our models indicated that date of completed nest was significant in determining diapause/nondiapause incidence and had a higher predictability

than average temperature (Fig 10). All of the NE facing cavities were filled by July 22nd and was the first direction to be filled. Tepedino and Parker, found that the majority of 2nd Generation individuals are laid by July 22nd and argue that time of year/photoperiod is also a trigger for females to lay non-diapausing individuals (1988). The majority of non-diapausing individuals in our study were laid by July 23rd, which follows the pattern seen in several other studies across various latitudinal ranges (Krunic 1972, Tepedino and Parker 1986, Tepedino and Parker 1988, and Kemp and Bosch 2000). Only five nondiapause individuals were laid after July 23rd. We suggest that our increased number of nondiapause individuals on the coolest side is not due to the decreased temperature, but instead is more heavily influenced by time of year the eggs were laid. However, our models do suggest that average temperature may impact diapause incidence in some capacity that our models were unable to fully determine.

Body size by temperature

Fischmann et al. 2017 found that offspring that were nondiapausing individuals were provisioned less and therefore resulted in a smaller body weight. Our results found that development was a significant factor in in dry body weight (Fig 11A-D). Nondiapausers were smaller than diapausing offspring, following the results of Fischmann et al. 2017. *M. rotundata* have a body weight sexual dimorphism, with the females being larger than the males. We find that in both diapausing and nondiapausing populations that sex was a significant impact on body weight, with females being larger (Fig 11A-D) and scale proportionally with development status.

However, body weight can also be influenced by temperature. As temperature increases body size decreases in multiple insect species, including solitary bees (CaraDonna et al. 2018, Radmacher and Strohm 2010). In *Osmia bicornis* body size decreases with temperature and provision amount eaten decreases with increased temperature (Radmacher and Strohm 2010)

which can lead to smaller body size. In our study we find no impact of increased temperature on body weight (Fig 11A-C). In another cavity nesting, solitary bee, *Osmia lignaria*, researchers found that increased cavity temperatures up to 40°C did decrease adult body size (CaraDonna et al. 2018). It is unclear why we did not see an effect on body size in *M. rotundata*. The nesting box reached high temperatures that have previously been reported to decrease body size. However, it may be that the natural fluctuations in temperature allowed a “recovery period” that shielded offspring from consequences in weight reduction.

Survival

Previous work has indicated that nesting females lay fewer brood cells when nesting in warmer cavities (see Chapter 2). However, the cost to offspring has not been explicitly explored. Temperatures exceeding 35°C induce the production of HSP70 proteins, an indicator of stress (Barthell et al. 2002). We also see decreased number of viable brood cells with increased temperatures with some environments reaching 44°C (Rossi et al. 2010). Nest boxes consistently reach temperatures above these thresholds (see Chapter 2). We found that survival of offspring decreases with time spent above 40°C (Fig 12). Offspring that spent increased time above 40°C failed to emerge from the brood cell. Time spent above 35°C nor average temperature impacted survival. Thus, calculating the time spent above 40°C could act as a threshold to indicate good nesting position and increase offspring survival. Interestingly, we find that temperatures reach a critical threshold for survival but had no effect on body weight. Thus, we can conclude that the nesting box reaches a stressful temperature, but this stressful temperature does not impact overall dry weight.

Nondiapause bees can be devastating to farmers. To emerge these individuals must chew through their brothers and sisters to exit the nest, and cause death to diapausing offspring. We

found that non-diapausing individuals resided in the same nest as diapausing individuals, which further argues against the hypothesis that the entire nest will either be diapause or nondiapause individuals. Partial bivoltinism within the same nest can also spread disease throughout the nest and nesting box further impacting the population (Vandenberg and Stephen, 1982). Our study found that the largest predictor of nondiapause incidence is date the nest was completed (Fig 10). Date of nest completed is impacted by nesting preference (see Chapter 2). These interactions represent a multitude of factors that growers should consider in placing their boxes in open fields, and our research suggests that a constant suggestion of SE may not be optimal for a particular geographic area or population success.

CONCLUSION

Insect response to temperature has been well studied (Jensens 1906, Colinet et al. 2015, Sinclair et al. 2016). As temperature increases, insect performance will respond positively, until a tipping point is reached. At this peak, increases in temperature will decrease performance values until ultimately reaching death (Jensens 1906). Due to this close relationship with increases in temperatures insects are highly susceptible to increases in temperature under climate change (Bale and Hayward 2009). This connection between climate change and insect decline has sparked increasing news coverage of insect decline and has earned the media nickname “Insect Armageddon” (Kover 2017). Several new studies have come out attempting to quantify and warn of the devastating consequences to lose massive amounts of our insect population (Hallmann et al. 2017, Sánchez-Bayo and Wyckhuys 2019). Scientists have increasingly been attempting to model how insect populations will respond to climate change (Colinet et al. 2015 and Sinclair et al 2016, Hallmann et al. 2017).

However, when we talk about climate change, we often talk about the overall atmospheric increases of 3-5°F (National Climate Assessment 2014) or macroclimate, but this is not a guarantee on how individual environments, niches, or even geographical areas will change. A better representation of experienced temperature are microclimates. Microclimates more accurately depict the exact temperatures experienced by individual organisms. Microclimates scale down to particular environmental niches. Individual leaves can buffer ambient air temperature experienced by eggs of *Manduca sexta* and provide protection from high temperatures (Potter et al. 2009). Floral resources where caterpillars feed exhibit a gradient in temperature as well and differ from 20°C-36°C (Woods et al. 2014). Even differences in temperature can be experienced in the sun and the shade where organisms can take advantage.

The disparity between macroclimate and microclimate can be beneficial for organisms, because it leaves room for choice. If macroclimate temperatures increase 3-5°F (National Climate Assessment 2014), organisms with the ability to move, or adaptations to respond to increasing temperatures may be able to avoid detrimental increases. Thus, if microclimates may function as a refuge from macroclimate warming, where organisms can compensate for increasing temperatures by choosing specific microclimates.

Traditionally, the nesting box of solitary bees is looked as a macroclimate, with all cavities reaching the same temperature, or using ambient air temperature to indicate cavity temperature (Richards 1996, Rossi et al. 2010, Pitts-Singer and James 2008). However, ambient air temperature does not correlate as well as experienced temperature at the nesting box (Richards 1996). My first aim was to determine if the cavities in the nesting box resemble a microclimate. I found that cavity temperature across the nesting box are determined not only by position within the side of the box (Fig 9D-F), but the direction the box is facing (Fig 9A-C). These results influence two key ideas of how we study and understand *M. rotundata*. My research suggests that one temperature probe is not strong enough to catch the range of variation within even a small nesting box. My boxes contained 36 cavities, and commercial shelters often have space for thousands of cavities, if we are seeing variations across a small range of cavities, it is highly likely that large commercial shelters are experiencing a temperature range as well. Thus, in our continued study of how temperature is influencing *M. rotundata* populations, we must scale our experiments to looking at individual cavity temperature over large box macroclimate or ambient air temperature.

My results also find that direction has a significant effect on cavities, with the SW and SE directions reaching the warmest maximum temperatures and highest average temperatures (Fig 4

and Fig 9). Currently, farmers are suggested to place their boxes facing the SE (Stephen 1981), one of the warmest in my study (Fig. 4) and a side that had slower rates of nesting (Fig 5). For farmers in North Dakota, my results suggest that farmers should face their boxes facing the NE or NW. However, these bees are used from Central Valley CA to Logan, UT. While, the instinct is to suggest that the NE and NW directions are universal for lower temperatures, we have to remember that just as cavities within a nesting box represent a microclimate, geographic location may influence the temperatures experienced by each microclimate. Without evaluation of cavity temperatures experienced in CA or UT we are unable to suggest specific industry procedures across the country. However, my study does suggest that growers and industry could possibly make adjustments to nesting shelters in order to lessen exposure to high temperatures.

Not only do growers have possible choices in lessening exposure to high temperatures, our study indicates that the nesting females avoid nesting in warmer cavities (Fig 6A-C). In solitary bees, the mother has a large effect on the environmental experience of her offspring. The offspring of this species are unable to move or forage for themselves, thus, the cavity and amount of food left provisioned is all the offspring has access to. Both of these key environmental factors influence survival and fitness. Temperatures above 38°C have been shown to decrease fitness (Pitts-Singer and James 2008), and provision size is highly correlated to body size (Klostermeyer et al. 1973). Thus, females have a large effect on the microclimate and success of offspring. I find that females deter from choosing warmer cavities, which increases the number of eggs laid (Fig 5) and offspring survival (Fig 12). Females that nested on the SW side, which reached the highest maximum temperatures, laid one less offspring per nest (Fig 7) and offspring laid in the warmest cavities had decreased survival (Fig 12).

While the first aim of my study follows the consequences of microclimate temperatures on the parent generation, aim 2 and aim 3 focus on the consequences of microclimate temperatures on offspring. Offspring are unable to move from the cavity from the egg to the adult stage. Upon emergence the offspring gain full autonomy in caring for themselves. In *M. rotundata* there are two alternative life history strategies. A few individuals will emerge from the nest in the same season as the parental generation, these are referred to as nondiapausers or 2nd Generation (Pitts-Singer and Cane 2011). The remaining individuals will emerge the following spring as temperatures begin to warm. I explored if increasing high temperature exposure in the nesting cavity influences diapause incidence of *M. rotundata*. I found that our models predicted that high temperature decreases nondiapause incidence (Fig 10). However, this result does not fit with the previously known relationship between development and temperature. In *M. rotundata*, diapause aversion increases as temperatures increase (Kemp and Bosch 2000). Thus, I predicted that increased exposure to high temperature would increase the amount of non-diapausing individuals. Instead, we believe that this significance is due to the distribution of non-diapausing individuals that were not spread evenly throughout all of our temperature parameters.

In the second chapter I found that *M. rotundata* females preferred nesting on the NW and NE sides, and filled these cavities approximately 10 days faster than the SW and SE facing cavities (Fig 5). Nondiapause individuals have been shown to develop from the earliest laid nests with the highest percentage occurring in nests laid before the last week of July (Tepedino and Parker 1988, and Kronic 1972, Kemp and Bosch 2001). The majority of nondiapausing individuals in my study came from nests laid before or on July 23th, and because of adult nesting preference these nests were primarily on the NE side. Thus, we suggest that it is not the

temperature that impacted the lack of diapause incidence but instead the preference of nesting, and early completion of nests, this is supported in my strength of predictability (Fig 10).

While microclimate temperature does not seem to have a large effect on diapause incidence in offspring, it does impact offspring survival. I found that increased time spent above 40°C decreases survival of *M. rotundata* (Fig 12). Interestingly, I did not find that time spent above 35°C or average temperature impacted survival. I suggest that these results indicate a possible thermal threshold that can be used for future studies on nesting habitat. Currently, field studies implore a variety of high thermal thresholds, above 30°C (Richards 1996), above 38°C (Pitts-Singer and James 2008), or 44°C (Rossi et al. 2010) to measure success of *M. rotundata*. My results suggest that 40°C may be a strong initial marker to measure field temperature stress and provide a true marker of experienced temperatures within the nesting box.

Finally, I wanted to explore if increased exposure to high temperatures impacted body size of *M. rotundata*. High temperature has been shown to decrease body size in multiple insect species (CaraDonna et al 2018) and this pattern has been found in solitary bees (Radmacher and Strom 2010). However, the relationship between body size and temperature of *M. rotundata* is traditionally studied under lab conditions, with prolonged exposure to extremely high, constant, temperatures. My study provides information on if *M. rotundata* could be exposed to constant high temperatures in an ecologically relevant setting. Offspring residing in the nesting box will experience fluctuating temperatures throughout the day, thus, a critique of constant temperature studies in the lab, is that these temperatures may not be ecologically relevant. I find that body size was not impacted by any of the temperatures measured in our study (Fig 11A-D) even though temperatures were found to be lethal (Fig 12). This was quite a surprising result. The nesting boxes fluctuated rapidly throughout the day and over the course of the entire study saw a

range from 4.5°C to 48.5°C. It is possible that the nesting box fluctuations provided a “recovery time” and this hypothesis should be further explored.

It is clear that our study demonstrates that the nesting box of *M. rotundata* is not a homogenous unit when it comes to temperature. Temperature of nesting cavities is impacted by position within the nesting box (Fig 9A-C) as well as the direction the cavities are facing (Fig 9D-F).

These temperature differences within the position of the nesting box impact multiple traits of *M. rotundata*, nesting choice, number of offspring laid, and survival. Future studies must implore more through thermal measurements of these boxes in order to truly quantify the effects of temperature on this species. Studying the microclimates of organisms may lead to a greater understanding of how macroclimate temperature increases may impact individual populations and lead to better prediction of populations under climate change.

REFERENCES

- Atkinson, D. (1994). Temperature and organism size—a biological law for ectotherms? *Advanced Ecological Res.* 25: 1-58.
- Bale, J.S., and Hayward, S.A.L. (2009). Insect overwintering in a changing climate. *The Journal of Experimental Biology.* 213:980-994.
- Barthell, J.F., Hranitz, J.M., Thorp, R.W., and Shue, M.K. (2002). High Temperatures Responses in Two Exotic Leafcutting Bee Species: *Megachile apicalis* and *Megachile rotundata* (Hymenoptera: Megachilidae). *Pan-Pacific Entomologist.* 78(4): 235-246.
- Bradner, N.R., Frakes, R.V., and Stephen, W.P. (1965). Effects of bee species and isolation distance on possible varietal contamination in alfalfa. *Agron. F.* 57: 247-248.
- Breau, C., Cunjak, R.A., and Peake, S.J. (2011). Behavior during elevated water temperatures: can physiology explain movement of juvenile Atlantic salmon to cool water? *Journal of Animal Ecology.* 80:844-853
- Cane, J.H. (1987). Estimation of bee size using intertegular span (Apoidea). *Journal of the Kansas Entomological Society.* 60(1): 145-147.
- 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” *Functional Ecology.* 32(10): 2345-2356
- Colinet, H., Sinclair, B.J., Vernon, P., and Renault, D. (2015). Insects in Fluctuating Thermal Environments. *Annual Review of Entomology.* 60: 123-140.
- Davis, Z.G., Wilson, R.J., Coles, S., and Thomas, C.D. (2006). Changing habitat associations of a thermally constrained species, the silver-spotted skipper butterfly, in response to climate warming. *Journal of Animal Ecology.* (75): 247-256.

- Denlinger, D.L. (2002). Regulation of diapause. *Annual Review of Entomology*. 47:93-122
- Fischman, B.J., Pitts-Singer, T.L., and Robinson, G.E. (2017). Nutritional Regulation of Phenotypic Plasticity in a Solitary Bee (Hymenoptera: Megachilidae). *Environmental Entomology*. 46(5): 1070-1079.
- Hahn, D.A., and Denlinger, D.L. (2011). Energetics of Insect Diapause. *Annual Review of Entomology*. 56: 103-121.
- Hallmann, C.A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Muller, A., Sumser, H., Horren, T., Goulson, Kroon, H.D. (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. PLOS ONE. 12(10): e0185809. <https://doi.org/10.1371/journal.pone.0185809>.
- Hemple de Ibarra, N. Philippides, A., Riabinina, O., and Collett, T.S. (2009). Preferred viewing directions of bumblebees (*Bombus terrestris* L.) when learning and approaching their nest site. *The Journal of Experimental Biology*. 212:3193-3204.
- Huey, R.B., Carlson, M., Crozier, L., Frazier, M., Hamilton, H., Harley, C., Hoang, A., and Kingsolver, J.G. (2002). Plants Versus Animals: Do They Deal with Stress in Different Ways? *Integrative and Comparative Biology*. 42: 415-423.
- James, R.R., and Pitts-singer, T.L. (2013). Health Status of Alfalfa Leafcutting Bee Larvae (Hymenoptera: Megachilidae) in United States Alfalfa Seed Fields. *Environmental Entomology*. 42(6): 1166-1173.
- Jensen, J.L. (1906). Sur les fonctions convexes et les intelualites entre les valeurs moyenes. *Acta Main*. 30, 175-198.
- Johansen, C.A. and Eves, J. (1973). Effects of chilling, humidity and seasonal conditions on the emergence of the alfalfa leafcutting bee. *Environmental Entomology*. 2:23-26.

- Kearney, M., Shine, R., and Porter, W.P. (2009). The potential for behavioral thermoregulation to buffer “cold-blooded” animals against climate warming. *PNAS*. 106: 3835-3840.
- Kemp, W.P., and Bosch, J. (2000). Development and Emergence of the Alfalfa Pollinator *Megachile rotundata*. (Hymenoptera: Megachilidae). *Ann. Entmol. Soc. Am.* 93(4): 904-911.
- Kemp, W.P., and Bosch, J. (2001). Post-cocooning Temperatures and Diapause in the Alfalfa Pollinator *Megachile rotundata* (Hymenoptera: Megachilidae). *Physiology, Biochemistry, and Toxicology*. 94(2): 244-250.
- Kingsolver, J.G., and Huey, R.B. (2008). Size, temperature, and fitness: three rules. *Evolutionary Ecology Research*. 10:251-268.
- 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. *Integrative and Comparative Biology*. doi:10.1093/icb/icr015.
- Klostermeyer, E.C., Mech, S.J., and Rasmussen, W.B. (1973). Sex and weight of *Megachile rotundata* (Hymenoptera: Megachilidae) progeny associated with provisions weights, *Journal of Kansas Entomological Society*. 46:537-548.
- Kostal, V. and Hodek, I. (1997). Photoperiodism and the control of summer diapause in the Mediterranean tiger moth *Cymbalophora pudica*. *Journal of Insect Physiology*. 43(8): 767-777.
- Kover, P. (2017, October 30) Insect “armageddon”: 5 crucial questions answered. The Conservation. Retrieved from <http://www.scientificamerican.com/>
- Kronic, M.D. (1973). Voltinism in *Megachile rotundata* (Megachilidae: Hymenoptera) in Southern Alberta. *Canadian Entomologist*. 104:185-188.

- Lerer, H.I., Bailey, W.G., Mills, P.F., and Pankiw, P. (1982). Pollination activity of *Megachile rotundata* (Hymenoptera:Apoidea). *Environmental Entomology* 11: 997-1,000.
- Lopez-Martinez, G., and Denlinger, D.D. (2008). Regulation of heat shock proteins in the apple maggot *Rhagoletis pomonella* during hot summer days and overwintering diapause. *Physiological Entomology*. 33: 346-352.
- Maeta, Y., Kitamura, T. (2005). On the number of eggs laid by one individual of females in the alfalfa leaf-cutting bee, *Megachile (Eutricharaea) rotundata* (Fabricius) (Hymenoptera, Megachilidae). *Chugoku Kontyu* 19:39-43.
- Martin, R.O., Cunningham, S.J., and Hockey, P.A.R. (2015). Elevated temperatures drive fine-scale patterns of habitat use in a savanna bird community. *Journal of African Ornithology*. 86(1&2): 127-135
- Martin, T.L., and Huey, R.B. (2008). Why “suboptimal” is optimal: Jensen’s inequality and ectotherm thermal preferences. *American Naturalist*. 171(3): 102-118.
- Martins, C.F., Ferreira, R.P., and Carneiro, L.T. (2012). Influence of the orientation of nest entrance, shading, and substrate on sampling trap-nesting bees and wasps. *Neotropical Entomology*. 41(2):105-111.
- McWatters, H.G. and Saunders, D.S. (1998). Maternal temperature has different effects on the photoperiodic response and duration of larval diapause in blow fly (*Calliphora vicina*) strains collected at two latitudes. *Physiological Entomology*. 23: 369-375.
- Munyiri F., Shitani, Y., Ishikawa, Y. (2004). Evidence for the presence of a threshold weight for entering diapause in the yellow-spotted longicorn beetle, *Psacotheta hilaris*. *Journal of Insect Physiology*. 50:295-301.
- National Climate Assessment. (2014). Retrieved from <https://nca2014.globalchange.gov/report>.

- National Climatic Data Center (2018). WFO Monthly/Daily Climate Data [CXUS53 KFGF 011602] Retrieved from <http://www.ncdc.noaa.gov>.
- Pankiw, P., Lieverse, J.A.C., and Siemens, B. (1980). The relationship between latitude and the emergence of alfalfa leafcutter bees *Megachile rotundata* (Hymenoptera: Megachilidae). *Can. Entomol.* 112:555-558.
- Parker, F. D., and V. J. Tepedino. (1982). Maternal influence on diapause in the alfalfa leafcutting bee (Hymenoptera: Megachilidae). *Ann. Entomol. Soc. Am.* 75: 407D410.
- Peterson, J.H., and Roitberg, B.D. (2006). Impact of resource levels on sex ratio and resource allocation in the solitary bee, *Megachile rotundata*. *Environ. Entomol.* 35:1404-1410.
- Peterson, S. S., Baird, C.R., and Bitner, R.M. (1992). Current status of the alfalfa leafcutting bee, *Megachile rotundata*, as a pollinator of alfalfa seed. *Bee Science.* 2:135-142.
- 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 Bosch, J. (2010). Nest Establishment, Pollination Efficiency, and Reproductive Success of *Megachile rotundata* (Hymenoptera: Megachilidae) in relation to resource availability in field enclosures. *Environmental Entomology.* 39(1): 149-158.
- Pitts-singer, T.L., and Cane, J.H. (2011). The Alfalfa Leafcutting Bee, *Megachilie rotundata*: The World's Most Intensively Managed Solitary Bee. *Annual Review of Entomology.* 56: 221-237.
- 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" *Journal of Economic Entomology.* 98(6): 1785-1790.

- Pitts-Sinter, 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? *Apiculture and Social Insects*. 101(3): 674-685.
- Potter, K., Davidowitz, G., and Woods, H.A. (2009). Insect eggs protected from high temperatures by limited homeothermy of plant leaves. *The Journal of Experimental Biology*. 212: 3448-3454.
- Potter, K.A., Davidowitz, G., and Woods, H.A. (2011). Cross-stage consequences of egg temperature in the insect *Manduca sexta*. *Functional Ecology*. 25: 548-556.
- Radmacher, R., and Strohm, E. (2010). "Effects of constant and fluctuating temperatures on the development of the solitary bee *Osmia bicornis* (Hymenoptera: Megachilidae). *Apidologie*. 42: 711-720.
- Rank, G.H., and Rank, F.P. (1989). Diapause intensity in a French univoltine and a Saskatchewan commercial strain of *Megachile rotundata* (Fab.) *Canadian Entomologist*. 121:141-148.
- Rosenzweig, C., Iglesias, A., Yang, X.B., Epstein, P.R., and Chivian, E. (2001). Climate change and extreme weather events. *Global Change & Human Health*. 2(2):90-104.
- Richards, K.W., (1996). Effect of environment and equipment on productivity of alfalfa leafcutter bees (Hymenoptera: Megachilidae) in Southern Alberta, Canada. *Canadian Entomologist*. 128: 47-56.
- 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*. *Animal Behavior*. 79: 165-171.

- Sanchez-Bayo, F., and Wyckhuys, K.A.G. (2019). Worldwide decline of the entomofauna: A review of its drivers. *Biological conservation*. 232:8-27.
- Sinclair, B.J., Marshall, K.E., Sewell, M.A., Levesque, D.L., Willett, C.S., Slotsbo, S., Dong, Y., Harley, C.D.G., Marshall, D.J., Helmuth, B.S., and Huey, R.B. (2016). Can we predict ectotherm responses to climate change using thermal performance curves and body temperatures? *Ecology Letters*. 19:1372-1385.
- Stephen, W.P. (1981). The design and function of field domiciles and incubators for leafcutting bee management (*Megachile rotundata* (Fabricius)). *Oregon State College of Agriculture Exp. Stn. Bull.* 654:1-13.
- Stephen, W.P. and Undurraga, J.M. (1976). X-Radiography, and Analytical Tool in Population Studies of the Leafcutter Bee *Megachilie pacifica*. *Journal of Apicultural Research*. 15(2): 81-87.
- Szabo, T.I., Smith, M.V. (1972). The influence of light intensity and temperature on the activity of the alfalfa leaf-cutter bee *Megachile rotundata* under field conditions. *J. Apic Res.* 11: 157-65.
- Tepedino, V.J. (1982). Maternal influence on diapause in the alfalfa leafcutting bee *Megachile rotundata* (F) (Hymenoptera, Megachilidae). *Annals of the Entomological Society of America*. 75(4): 407-410.
- Tepedino, V.J., and 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.* 79:974-77.

- Tepedino, V.J., and Parker, F.D. (1988). Alternation of Sex Ratio in Partially Bivoltine Bee., *Megachile rotundata* (Hymenoptera: Megachilidae). *Annals of the Entomological Society of America*. 81(3): 467-476.
- Tepedino, V.J., and Torchio, P.F. (1982). Phenotypic variability in nesting success among *Osmia lignaria propinqua* females in a glasshouse environment (Hymenoptera: Megachilidae). *Ecological Entomology*. 7:453-462.
- Trostle, G., and Torchio, P.F. (1994). Comparative nesting behavior and immature development of *Megachile rotundata* (Fabricius) and *Megachile apicalis Spinola* (Hymenoptera, Megachilidae). *Journal of Kansas Entomological Society*. 67: 53-72.
- Undurraga, J.M., and 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. *Journal of Kansas Entomological Society*. 53(3): 669-676.
- Vandenberg, J.D., and Stephen, W.P. (1980). Spore load of *Ascospaera* spp. on emerging adults of the alfalfa leafcutting bee, *Megachile rotundata*. *Appl. Environ. Microbiol.* 39:650-655
- Vasseur, D.A., DeLong, J.P., Gilbert, B., Greig, H.S., Harley, C.D.G., McCann, K.S., Savage, V., Tunney, T.D., and O'Conner, M.I. (2014). Increased temperature variation poses a greater risk to species than climate warming. *Proc. R. Soc. B*. 281: <http://dx.doi.org/10.1098/rspb.2013.2612>.
- Woods, H.A., Dillion, M.E., and Pincebourde, S. (2014). The role of microclimatic diversity and of behavior in mediating the responses of ectotherms to climate change. *Journal of Thermal Biology*. 54: 86-97.

Xu, J., James, R.R. (2012). Temperature stress affects the expression of immune response genes in the alfalfa leafcutting bee (*Megachile rotundata*). *Insect Molecular Biology*. 21(2):269-280.