ENERGETICS OF INTERRUPTED DEVELOPMENT IN *MEGACHILE ROTUNDATA*

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ABSTRACT

Insects in temperate regions may experience temperature fluctuations during springtime development which can lead to low temperature stress. Previous research has shown that short artificial fluctuations in temperature during interrupted development are advantageous when compared to static temperatures, but it is unclear why. One idea is it allows insects to repair chill injury and maintain cellular membrane potential. My goal was to understand what macromolecules are maintaining ion balance through measuring the respiratory quotient (RQ), trehalose, glycogen, simple sugars, and lipids using biochemical assays. The development of *Megachile rotundata* was stimulated for two weeks before interrupting with either fluctuating or static temperature regimes. RQ was measured repeatedly over two weeks and subsets of bees from each treatment were frozen at the same time points for biochemical analyses. The RQ varied over time and lipids and trehalose had the biggest differences between static and fluctuating temperatures as well as over time.

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CHAPTER 1. LITERATURE REVIEW OF INSECTS IN FLUCTUATING TEMPERATURES AND *MEGACHILE ROTUNDATA*

1.1. Low Temperature Stress Tolerance

As ectotherms, insects are intimately connected to the temperatures of their physical environment. Exposure to low temperatures can be lethal or have sublethal effects on an insect's morphology and physiology through chill injury. Chill injury causes membranes to shift from a fluid to gel state which affects membrane permeability, leading to downstream problems such as protein denaturation and changes in cellular membrane potential (Denlinger & Lee, 1998; RamLøv, 2000).

As a result of low temperature exposure, insects evolved adaptative mechanisms that help them survive stressful thermal events. Known physiological mechanisms that aid in reducing chill injury include homeoviscous adaptation, metabolic depression, maintenance of ion balance, and cytoprotectant synthesis (Chown & Nicolson, 2004). Such adaptations allow insects to better withstand short- and long-term exposures to low temperatures.

In the wild, low temperature stress is more than just exposure to a single low temperature. However, experiments done in the laboratory to investigate the effects of low temperature stress often use a single low temperature exposure to simplify the experimental design (Colinet et al., 2015). Therefore, the results are not always applicable to what happens under natural conditions, because in nature, insects rarely are in environments with a single temperature exposure. Natural environmental temperatures can vary widely throughout weeks or even days, making experiments with single temperature exposures less useful in making predictions about what happens in natural conditions. Exposure to fluctuating low temperatures, even in a manner that seems artificial, can yield different results than exposure to constant temperatures (Meats, 1976;

Rinehart et al., 2011), making it a necessary research avenue to better understand the physiological responses to low temperature stress. In many cases, exposure to just one hour of a high temperature during low temperature stress improves survival in insects compared to those exposed to a constant low temperature (Rinehart et al., 2011; Yocum et al., 2010). However, the mechanisms promoting increased survival in fluctuating temperatures are unclear. Better understanding of the physiological changes that occur when insects are exposed to fluctuating temperatures can help discover the mechanisms that promote increased survival.

1.1.1. Metabolic Rate

Because metabolic rate in ectotherms is driven by the ambient temperature, exposure to low or high temperatures can alter energy demand and usage. As well as overall decreased survival, exposure to temperature extremes can cause detrimental changes to metabolic rates by slowing or stopping necessary metabolic processes (Storey & Storey, 1988, 2012). To survive extreme low temperature stress, insects evolved adaptive metabolic strategies to reduce damage caused by low temperature stress.

One such strategy is metabolic suppression. Across ectotherms, temperature and metabolic rate are positively correlated, meaning that in low temperatures, metabolic rates decrease accordingly (Gillooly et al., 2001). A decreased metabolic rate decreases the rate at which organisms transform energy, which in some cases may be advantageous. Such cases can be long non-feeding periods where a high metabolic rate would deplete energy stores (Koštál, 2006).

As insects experience fluctuating temperatures, the metabolic rate increases and decreases accordingly (Boardman et al., 2013; Lalouette et al., 2011; Yocum et al., 2011). Although fluctuating temperatures appear to suppress metabolism overall, metabolic rate still

increases during warming periods (Bozinovic et al., 2013; Yocum et al., 2011). An increased metabolic rate will use up energy reserves faster but could also allow for repair mechanisms to function where they would not have been able to in low temperatures (Colinet et al., 2015). Prior acclimation to fluctuating temperatures in *Pringleophaga marioni* resulted in a lower overall metabolic rate when exposed to low temperature stress compared to no acclimation which could decrease unnecessary energy usage (Chown et al., 2016). Metabolic rates during fluctuating temperatures could be species-dependent or based on other factors such as diet and length of exposure to high or low temperatures.

Metabolic rates can be determined by indirect measures of O_2 consumption and/or CO_2 production. Based on the stoichiometry of ATP production, O_2 consumption and CO_2 emission, the ratio of CO_2 production to O_2 consumption can be used to help determine what nutrients are being used for energy. This ratio is called the respiratory quotient (RQ) (Nunes et al., 1997). Although RQ is not the most accurate indicator of the nutrients being utilized for energy, the RQ can be helpful in understanding metabolism. When using carbohydrates for energy, the RQ is typically 1.0, catabolism of proteins/amino acids yields an RQ of 0.8-0.9, and burning lipids gives RQ measurements of 0.7 (Hall & Hall, 2020). Respiratory quotients above 1.0 may be associated with anaerobic metabolism or lipogenesis and below 0.7 may indicate ketogenesis or gluconeogenesis (Guenst & Nelson, 1994; Kerner & Hoppel, 2004; Kovacs & Westerterp-Plantenga, 2006).

In insects, research on the RQ and low temperature stress that are not related to the diapause state is limited. However, the relationship between energy substrate and RQ generally holds across taxa. In laboratory mice, heat stress caused a decrease in their overall RQ and an increase when exposed to low temperature stress (David et al., 2013), suggesting that mice

increase carbohydrate usage during low temperature stress. In contrast, human females had an overall downward shift of the RQ as low temperature stress increased, which would indicate lipid usage or ketogenesis/gluconeogenesis (Murray et al., 1986). In one of the few studies in non-diapausing insects, ghost moths (*Hepialus xiaojinensis*) had significantly lower RQ values as the temperatures decreased (Zhu et al., 2016). Another study, however, found that the RQ of the honey bee (*Apis mellifera*) was not related to temperature (Lighton & Lovegrove, 1990). While metabolic rate is positively correlated with temperature, the relationship between respiratory quotient and temperature is less definitive.

1.1.2. Energy Usage

Energy in the form of ATP is created from different substrates and used in various ways depending on the external environment, metabolic rate, and diet. Glycogen and trehalose are the main forms of stored energy in insects and can be quickly mobilized into glucose to generate ATP for energy-intensive activities, with trehalose usually being hydrolized first (Nation, 2015). For longer periods of high energy usage such as during flight, stressful events, and non-feeding periods, insects will rely on stored lipids in the fat body cells (Nation, 2015).

Such stressful events that would require mobilized energy include exposure to low temperatures. When exposed to low temperatures repeatedly, researchers found that *Drosophila melanogaster* had lower levels of overall glycogen, as well as a brief decrease in triglycerides when compared to single exposures or no exposure to low temperature (Marshall & Sinclair, 2012). Flies in this study were allowed to feed after each low temperature exposure, however, which could affect the amount of stored nutrients measured. This result suggests that flies were breaking down glycogen to increase overall amounts of glucose to be used for energy. In the false codling moth (*Thaumatotibia leucotreta),* body water and overall lipid contents were

unchanged during fluctuating temperatures, but heat shock protein 70, a molecular chaperone used to reduce or repair damage to proteins, was upregulated, suggesting that the ability to repair damage is a key component of fluctuating temperatures (Boardman et al., 2013).

Glucose produced during low temperature stress could also be used as a cytoprotectant. In amphibians, glucose transport proteins increase when exposed to low temperature temperatures to aid in movement of glucose across plasma membranes (King et al., 1993; Pessin & Bell, 1992). Glucose could also be converted into the potent cytoprotectant trehalose after exposure to low temperatures. Trehalose levels increased during low temperature exposure in insects and were positively correlated with high low temperature tolerance after previous low temperature exposures (Anduaga et al., 2018; Jagdale & Grewal, 2003; Lü et al., 2020), suggesting its importance to low temperature tolerance.

Ion pumps requiring ATP could be activated during warming periods due to the increased creation of ATP in warm temperatures. Previous research showed that when the flesh fly *Sarcophaga crassipalpis* were exposed to constant low temperatures, their muscular membrane potential was significantly altered (Kelty et al., 1996). Increased survival outcomes in fluctuating temperatures could be due to the ability of the insect's body to balance ions and water. This can then allow for repair functions such as antioxidant production (Lalouette et al., 2011; Torson et al., 2015) and protective measures like upregulation of heat shock proteins and cytoprotectant production (Boardman et al., 2013) to resume.

1.1.3. Homeoviscous Adaptation

Homeoviscous adaptation is a series of lipid membrane adjustments that promote cell membrane fluidity during exposure to low temperatures (Sinensky, 1974). Lipid membranes provide semi-permeable barriers that regulate the molecules that enter and exit cells. When

exposed to extreme temperatures, the fluidity of the membrane can change, disrupting ion homeostasis and water balance. Extreme high temperatures increase membrane fluidity, while extreme low temperatures decrease membrane fluidity. These changes in membrane fluidity negatively affect ion homeostasis, which could be detrimental to survival. Disturbance of ion homeostasis by the movement of positively charged sodium and potassium ions down their electrochemical gradients can alter membrane potential. Because of the highly important upstream role of ion homeostasis on physiological processes, the restoration of disrupted membrane potential could provide the ability to repair damage caused by low temperature stress. Preservation of correct electrochemical membrane potential is important for all physiological processes including movement (Djamgoz, 1987), antioxidant capacity (Tettamanti et al., 2008), signaling, and various others (Levin, 2014; Levin et al., 2017). To counteract these effects of temperatures stresses, cell membranes go through a series of changes to maintain membrane homeostasis. These changes are termed homeoviscous adaptation (Sinensky, 1974).

In low temperatures as membranes lose fluidity, the composition of fatty acids changes to include more shorter fatty acid chains to maintain membrane fluidity. Decreasing saturation in fatty acid chains also can increase fluidity by decreasing the weak hydrogen bonding between phospholipids (Bennett & Lee, 1997). Saturation changes are facilitated by elongases and desaturases that either extend hydrocarbon chains or insert double bonds respectively (Ernst et al., 2016). When experiencing fluctuating temperatures due to changes in the tide, oysters utilize homeoviscous adaptation to adjust membrane fluidity to an intermediate temperature, rather than changing as the temperature does (Pernet et al., 2007; Williams & Somero, 1996). A similar phenomenon could be occurring when insects are exposed to fluctuating temperatures, which

would optimize membrane fluidity for both low temperature and warm periods, as well as reducing potential costs associated with frequent fatty acid changes.

Insects exposed to fluctuating temperatures are less likely to have problems with ion balance than those exposed to constant low temperature stress. During exposure to constant low temperature, the European Firebug (*Pyrrhocoris apterus*) accumulated potassium ions in extracellular hemolymph (Koštál et al., 2007), which disrupts membrane potential and prevents many cellular functions. Ion levels in the European Firebug were more stable when exposed to fluctuating temperatures than when exposed to stable temperatures, suggesting that these insects could be using homeoviscous adaption. Exposure to fluctuating temperatures restores homeostasis through the use of energy to perform active ion pumping (Koštál et al., 2007).

However, other research showed that fatty acid composition did not change significantly when springtails (*Orchesella cincta)* were exposed to fluctuating temperatures (van Dooremalen et al., 2011). In fluctuating temperatures it would be expected to see an increase in the headgroup phosphatidylethanolamine (PE) due to the warm temperature pulses offering a prime opportunity for membrane reshuffling (Colinet et al., 2016). PE would be expected to occur at larger amounts in fluctuating temperatures because of its small, conical shape, which affects the whole fatty acid by increasing unsaturation in the sn-1 position (Logue et al., 2000). Instead, a study found that PE increased in response to constant low temperatures rather than in response to fluctuating temperatures which elicited no increase (Colinet et al., 2016). These results suggest that while ion homeostasis is being maintained, it may not be through homeoviscous adaptation.

1.2. Development and Low Temperature Stress

Insects show great variation from one another with regard to development. The three developmental paths an insect can follow are ametabolous, hemimetabolous, and holometabolous

(Truman & Riddiford, 1999). Adult ametabolous insects are largely the same as the juveniles, apart from size differences. Hemimetabolous insects follow an incomplete metamorphosis where juveniles are similar to adults except for wing and genitalia development. Holometabolous insects show large differences between larval, pupal, and adult developmental stages, undergoing complete metamorphosis.

As such, undergoing such massive changes from egg to adulthood could prove to be energetically complex. In insects, the energy in and energy out are usually equal between the insect and its environment, but during metamorphosis in holometabolous insects, the energy in is less than the energy out (Downer, 1981). This means that while no energy is entering the insect, large amounts of energy are being synthesized and used to power metamorphosis. The implication of this is that stored energy is being used to complete metamorphosis.

Because it is so complex, insect development is an energy intensive and sensitive period of an insect's life, especially in developmental pathways that include complete metamorphosis (Crompton & Birt, 1967). As such, exposure to low temperature stress amidst development could pose morbidity and mortality risks much more severe than when not in active development. Further research is needed to understand the physiological relationship between low temperature stress and active development.

Low temperature stress is usually studied in adult insects or during overwintering, which is a time of developmental arrest, so less is known about the effects of low temperature stress during active development and metamorphosis. This could be due to the fact that low temperature stress is most likely to occur during overwintering because it is a long period of low temperature exposure. Insect development is usually physiologically programmed to occur during optimal temperatures (Koštál, 2006), so historically it is less likely for insects to endure

unexpected low temperature stresses during development. With climate change, however, temperatures are predicted to change from historic temperatures, with large variability within a season which could cause new low temperature stresses during development (Easterling et al., 2000).

1.2.1. Energetics of Insect Development

To provide ATP for the energy-intensive process of metamorphosis, stored glycogen is mobilized in insects for use as glucose and other monomers for oxidative phosphorylation during the early stages of metamorphosis (Damos et al., 2011; Nestel et al., 2003; Tate & Wimer, 1971). Glycogen is also potentially a source for the creation of chitin during later developmental stages (Tate & Wimer, 1971). In the Medfly (*Ceratitis capitata),* glycogen sharply decreases within the first few hours of life, only to increase near adult eclosion (Nestel et al., 2003). In the blowfly (*Phormia regina),* a similar trend is observed early on in development, but glycogen levels never recover into adulthood (Tate & Wimer, 1971). During the larval to pupal transition of the blowfly (*Lucilia cuprina)*, glucose decreases greatly, only to accumulate again near adulthood (Crompton & Birt, 1967). Another study noted that in the blowfly (*Phormia regina)* that glucose was lowest at the transition from larva to pupa, and showed a sharp increase followed by a decrease later during pupation (Tate & Wimer, 1971). These findings could indicate usage of glucose throughout development or a conversion from glucose into trehalose.

Glucose derived from hydrolyzed glycogen can then be converted into trehalose. Trehalose is considered a necessary substrate for flight metabolsim in insects and increases before eclosion in blowflies (*Phormia regina*) (Tate & Wimer, 1971). In some cases, trehalose is completely undetectable during larval stages, indicating that it is most valuable later in development (Crompton & Birt, 1967). Another study in *Bombyx mori* showed that trehalose

was used largely during early stages of development, as well as right before adulthood (Hirano & Yamashita, 1980). These findings demonstrate the importance of trehalose for development.

Along with stored glycogen being utilized for energy during development, lipids are another large source of energy, especially in insects developing after long non-feeding periods. Lipid oxidation produces twice the metabolic water compared to carbohydrates, making it useful for maintaining water balance during non-feeding periods (Downer, 1981). In *Ceratitis capitata,* lipids increased during the larval to pupal transition, where afterwards they begin to be catabolized followed by a sharp decrease in lipid content right before adult emergence (Nestel et al., 2003). Similarly, this study also found that before adult eclosion glycogen also trends downward which suggests that the last stages of metamorphosis are exceptionally energetically demanding (Nestel et al., 2003).

1.2.2. Low Temperature Stress During Development

Quiescence is a direct physiological response to the low temperatures of the environment and occurs after diapause, which is a state of the developmental arrest (Koštál, 2006). In quiescence, warmer temperatures in spring can initiate development. Active development is a time when insects could be most susceptible to temperature fluctuations, such as during a springtime low temperature snap, because they may not have the protections against low temperatures that they had during diapause. Insects are energetically connected to their environments, with temperature changes having an effect on the type of macromolecules being utilized for energy (Acar et al., 2001). As such, temperature stress during metamorphosis could have a large impact on developmental success and adult performance. Because insects are so metabolically connected to the external environmental temperatures, optimal conditions are needed to maintain biochemical efficiency in insects (Acar et al., 2001). Understanding not only

the biochemistry behind development but also how insects cope with low temperature stress during that development is necessary for a comprehensive physiological understanding.

While some research exists on adult performance after enduring low temperature stress during development, less is known about the underlying physiological changes. Male alfalfa leafcutting bees (*Megachile rotundata*) had decreased flight performance when exposed to static low temperature stress compared to males exposed to fluctuating low temperature stress (Bennett et al., 2015). Malt fly larvae (*Chymomyza costata*), when not low temperature acclimated, showed increased chill injury in the fat body and Malpighian tubules when compared to those that were acclimated to low temperature temperatures (Des Marteaux et al., 2018). Low temperature stress during development is not insignificant, however the ability to acclimate to the low temperatures or repair damage done seems to be a feasible coping strategy for multiple insect species (Agrell & Lundquist, 1973). Still, less is known about the specifics of physiological repair mechanisms and acclimation during development.

1.3. The Alfalfa Leafcutting Bee, *Megachile rotundata*

Megachile rotundata is a solitary, gregarious, cavity-nesting bee. Depending on latitude, adult bees emerge throughout late spring, and female bees mate within a week of emergence (Richards, 1994). As solitary bees, *M. rotundata* do not live in hives, but rather females create nests in existing cavities. A nest consists of several brood cells, built by the female to house individual offspring. Mothers use their mandibles to cut leaf disks that they bring back to the nest for constructing brood cells. Brood cells are constructed sequentially in the cavity, each of which is provided its own pollen and nectar provision. Females lay one egg in each cell before sealing it and starting the next one. Offspring sex is determined by the female through fertilization, with

fertilized eggs becoming female and unfertilized eggs becoming male (Klostermeyer et al., 1973).

Once the egg hatches within its brood cell, the larva consumes the provision of nectar and pollen while progressing through five larval stages of development (Pitts-Singer & Cane, 2011). Larval development ends with the spinning of a cocoon inside of the brood cell as a pre-pupa. At this time, individuals will either develop through to adulthood in that same season or remain as pre-pupae throughout diapause development and emerge the following summer as adults. In *M. rotundata,* some evidence suggests that the photoperiod or thermal conditions experienced by the mother can impact whether an individual will undergo diapause development (Earls et al., 2021; Pitts-Singer, 2020).

As pollinators, *M. rotundata* have been widely used in agriculture for alfalfa pollination. Alfalfa production is a multibillion dollar industry in the United States with 64,000,000 pounds of alfalfa seed produced over 105,000 acres in 2017 (National Agricultural Statistics Service, 2017). Female *M. rotundata* are able to trip the staminal column on alfalfa flowers 78% of the time, making them much more effective pollinators for this plant when compared to honey bees, which trip the flowers only 22% of the time (Cane, 2002). When compared to the honey bee, *A. mellifera, M. rotundata* tripled alfalfa seed production when first introduced to the crop commercially (Pitts-Singer, 2008).

Unlike honey bees, *M. rotundata* can be more challenging to propagate because of low offspring production. American commercial farmers then need to purchase additional bees each spring because enough offspring are not produced to replace the previous year's bees. In a commercial setting, this can be expensive and inconvenient. More physiological information will

help to better inform rearing practices and be highly beneficial for commercial applications of *M. rotundata.*

Following overwintering in low temperature storage (6˚C), farmers attempt to time the peak alfalfa bloom with the development and emergence of adult bees. To initiate development, prepupal *M. rotundata* are placed in higher temperatures, (29 to 32°C), after which adult bees emerge in about three weeks. Sometimes, environmental factors, such as weather, may delay the bloom of alfalfa. Thus, farmers may seek to also delay bee development. To do this, farmers often place bees back into low temperature storage, referred to as interrupted development. Interrupted development results in mortality and sub-lethal, morphological defects (Rinehart et al., 2011). One method that has consistently abrogated the negative effects of interrupted development is the use of fluctuating temperatures (Colinet et al., 2016; Koštál et al., 2007). Instead of placing bees back into their overwintering temperature of constant 6˚C, bees are placed in a fluctuating temperature regime, 6˚C with 1 hour per day at 20˚C, which increases survival and eliminates some of the sublethal effects (Bennett et al., 2015; Rinehart et al., 2011). With this already established research on the benefits of fluctuating temperatures during development in *M. rotundata,* more information is needed to understand exactly why this occurs.

1.4. Conclusions and Significance

Because *Megachile rotundata* is a commercially managed species, increasing survival and fitness to increase crop yields and offspring production is highly beneficial. High alfalfa seed yields decrease the price of alfalfa which allows farmers to feed more livestock. Better understanding of bee physiology during interrupted development can play a part in designing low temperature-storage protocols and diet changes which will benefit agricultural growing of alfalfa and commercial rearing of the alfalfa leafcutting bee.

In *M. rotundata,* fluctuating temperatures have been used to increase long-term storage survival as well as to increase survival during interrupted development (Bennett et al., 2015; Rinehart et al., 2011; Yocum et al., 2010, 2019). Fluctuating temperatures are a natural part of living in the wild. Insects often experience low temperature stress and fluctuating temperatures during many parts of their lives, native and wild leafcutting bees included. Native species are important to local ecosystem pollination and are at risk of decreased population due to the effects of climate change. Understanding the physiology of a closely related leafcutting bee such as *M. rotundata* can help predict population dynamics or the range of species based on yearly temperatures.

Studying the effects of fluctuating temperatures on insect physiology is important because most insects are exposed to fluctuating temperatures throughout the course of their lifetime. Fluctuating temperatures occur in field studies and are increasingly being used in laboratory experiments because of the ability to control temperatures (Colinet et al., 2015). Fluctuating temperatures are a necessary part of experimental design because of the increasing body of information suggesting physiological differences from static temperatures. Understanding the physiological effects of fluctuating temperatures can better help us contextualize the results of experiments utilizing fluctuating temperatures. Physiological knowledge of an insect's reaction to fluctuating temperatures can also help rule out temperature variation as a confounding variable in experiments. It is often not possible to control the temperature in outdoor field experiments leaving temperature as a variable in itself when analyzing data. This can leave experiments conducted in the field unable to draw many conclusions because of the confounding variables.

In this study, I determined the differences between *M. rotundata* whose development was interrupted with different temperature regimes. Development was initiated at different times during their extended low temperature storage, and bees were interrupted for one or two weeks. I expected that bees treated with a fluctuating thermal regime would have higher metabolic rates as well as levels of trehalose and other free sugars and lower amounts of lipids and glycogen, indicating usage of energy reserves for repair mechanisms. I also expected to see bees that have been in low temperature storage for longer amounts of time to have lower amounts of stored glycogen and lipids, with bees in fluctuating thermal regimes having even less. Bees left in thermal treatments for longer periods of time would also be expected to show similar trends in stored macromolecules.

CHAPTER 2. ENERGETICS OF INCREASED SURVIVAL DURING INTERRUPTED DEVELOPMENT

2.1. Abstract

Insects inhabiting temperate regions often face large temperature stresses in the midst of development in the spring. When experiencing a spring low temperature snap, active development will be interrupted. This disruption in development can have deleterious effects on the rest of development and adulthood. Previous research has shown that interruption with fluctuations in temperature are beneficial during interrupted development when compared to static low temperatures. Maintenance of ion balance is a key hypothesis as to why fluctuations provide benefit over static temperatures. The warming periods allow for ion balance across cellular membranes to be maintained through active ion pumping. However, less is known about what macromolecules are being utilized for energy. I hypothesized that insects treated with fluctuating temperatures during development use stored energy, such as lipids and glycogen, as potential energy sources. To test this, I initiated development in overwintering *Megachile rotundata* by placing them in 29˚C for two weeks after which they were interrupted with either fluctuating or static temperature regimes. O_2 consumption and CO_2 production were measured on the last day of 29˚C, one day in treatment, one week in treatment, and two weeks in treatment. A subset of bees was frozen for biochemical analyses at the same time points. Trehalose, simple sugars, glycogen, and lipids were measures for each bee frozen at each time point. Metabolic rate was overall higher in bees treated with fluctuating temperatures, but there was no significant difference in respiratory quotient between temperature treatments. Notably, lipids increased in bees treated with fluctuating temperatures and decreased after two weeks, whereas bees treated with static temperatures showed the opposite trend.

2.2. Introduction

As ectotherms, insects are acutely tied to the temperatures of their external environment. Temperature thus affects many aspects of insect life, including behavior and physiological processes. As such, temperatures outside of the optimal range may negatively impact an insect's fitness. Insects in temperate environments often experience low temperature stress and have adapted to survive overwintering through diapause (Koštál, 2006). Diapause is a programmed state of developmental arrest (Koštál, 2006), often associated with various physiological changes, such as increased cryoprotectant production, decreased metabolic rate, and changes in membrane fluidity, all of which help insects survive low temperature stress (Hahn & Denlinger, 2011). Although there is a large body of research surrounding low temperature stress throughout overwintering, less is known about how low temperature stress affects insects during other seasons and life stages, such as during metamorphosis, when protections from diapause may no longer be present.

Many insects resume active development in the spring after overwintering. However, springtime is often associated with low temperature snaps, which are predicted to increase with climate change (Easterling et al., 2000). If development has already begun, insects could be vulnerable to low temperature stress during key developmental processes (Bennett et al., 2015). Low temperature stress in the wild is often experienced as large changes throughout the day or from week to week, but experimentally, low temperature stress is often studied using static low temperatures. This type of experimental design, while extremely practical, often cannot give an accurate representation of conditions experienced in the wild. These types of temperature changes could be stressful for an ectotherm and may result in negative or deleterious physiological changes, especially during juvenile development or metamorphosis.

Insects exposed to fluctuating temperatures as adults show increased survival and decreased morbidities when compared to static temperatures (Colinet et al., 2006, 2018; Davis et al., 2006; Dollo et al., 2010). The physiological mechanisms underlying this benefit, however, are largely unknown. The most widely accepted hypothesis is that the warmer periods in fluctuating temperatures allow repair mechanisms to start and for preventative cryoprotection, such as the creation of sugars that lower the freezing point, to occur (Baust & Miller, 1969; Torson et al., 2015). Research on the effects of fluctuating temperatures during low temperature stress in metamorphosis is largely lacking, despite fluctuating temperatures increasing survival over static temperatures (Rinehart et al., 2011; Yocum et al., 2010).

Previous research has shown that the warming periods of fluctuating thermal regimes allow for ion homeostasis (Koštál et al., 2007). During chill injury, positively charged sodium and potassium ions move down their electrochemical gradients, which can alter membrane potentials. The altered membrane potentials require a larger electrical stimulus to activate the action potential which will have large downstream physiological effects. ATPase ion pumping (specifically V-type) during warming periods is a likely explanation for balancing ions to maintain the electrochemical gradient potential (Koštál et al., 2007; Qian & Xue, 2016; Wieczorek et al., 1989). To fuel the ion pumps, breakdown of stored molecules, such as glycogen and lipids, may occur. Though, during development, devoting all of the stored energy to ion balance would leave little leftover to fuel development, indicating a potentially more complex balance.

Holometabolous insects undergo complete metamorphosis, which is physiologically complex and energetically expensive (Agrell & Lundquist, 1973). Over time, during metamorphosis, the metabolic rate follows a U-shape curve, with metabolic rate high at the

beginning, likely due to feeding periods for initial development, and with the increasing metabolic demand towards the end of development potentially indicating changes in oxygen demand for creation of adult reproductive organs (Damos et al., 2011; Schmolz et al., 2005). During these times, lipid was a primary energy source, and its usage increased when temperatures both increased or decreased from optimal (Damos et al., 2011). With this, at suboptimal temperatures, complete metamorphosis may not be able to occur because lipid stores would be depleted faster (Damos et al., 2011). There is not, however, an abundance of research on interruptions in metamorphosis with low temperature stress and how that affects energy storage and usage.

The type of substrate used to create energy can be estimated using the ratio of O_2 consumption and CO_2 production. This ratio is known as the respiratory quotient (RQ). When utilizing carbohydrates for energy, the RQ will be near 1.0, proteins will yield an RQ around 0.8-0.9, and lipids will yield an RQ of 0.7 (Nunes et al., 1997; Zhu et al., 2016). Respiratory quotients above 1.0 can be associated with anaerobic metabolism and lipogenesis while RQs below 0.7 can be ketogenesis or gluconeogenesis (Engelking, 2015; Kerner & Hoppel, 2004; Kovacs & Westerterp-Plantenga, 2006; Nunes et al., 1997). When exposed to low temperatures, the ghost moth (*Hepialus xiaojinensis*) had a significantly lower RQ compared to normal temperatures (Zhu et al., 2016). However, in the honey bee (*Apis mellifera*), the RQ was unaffected by temperature (Lighton & Lovegrove, 1990).

To understand the effects of fluctuating temperatures on low temperature stress during interrupted development, the alfalfa leafcutting bee (*Megachile rotundata*) was utilized. *Megachile rotundata* is a solitary, cavity nesting bee species that is widely used in commercial pollination of alfalfa seed and other cash crops, such as canola (Pitts-Singer & Cane, 2011). This

bee constructs brood cells for its offspring in cavities using cut leaf pieces and provisions of pollen and nectar (Klostermeyer et al., 1973). Each brood cell contains a single offspring, which hatches from an egg and develops through five larval instars and a pupal stage and then emerges as an adult (Pitts-Singer & Cane, 2011). In the pre-pupal stage, offspring commit to either direct development in which they continue into adulthood or overwintering in a state of suspended development known as diapause (Kemp & Bosch, 2000). After diapause, the bee remains developmentally halted in post-diapause quiescence, where the initiation of development may be determined by the environmental temperature. Farmers exploit this sensitivity to time adult emergence with alfalfa bloom by placing the bees into higher temperatures about 3 weeks before they are predicted to be needed for pollination. However, if the alfalfa bloom is delayed, the farmers may place the bees back into low temperature storage, referred to here as interrupted development (Yocum et al., 2012). The practice of interrupted development can have detrimental effects, including sublethal effects and mortality (Bennett et al., 2015). Previous research in this species indicates that exposure to fluctuating temperatures during interrupted development increases survival and decreases morbidities compared to interruption with a static low temperature (Bennett et al., 2015; Rinehart et al., 2011; Yocum et al., 2012).

Alfalfa is a widely utilized agriculture product, with alfalfa seed producing the alfalfa fed to commercially reared livestock. When compared to honey bees, *M. rotundata* initially increased alfalfa yields fivefold, (Pitts-Singer & Cane, 2011). Better understanding the physiological changes involved in fluctuating temperatures will help create better low temperature storage protocols and management practices in this species.

To test the hypothesis that low temperature storage procedures change the composition of macronutrients and metabolic rate in bees whose development has been interrupted, I used a

previously described interrupted development protocol. After two weeks of development at 29˚C, bees were randomLy assigned to either a fluctuating low temperature storage regime or a static low temperature storage regime for either one or two weeks. After that exposure, lipid, glycogen, simple sugar, and trehalose contents of each bee or the O_2 consumption and CO_2 emissions were measured and compared between thermal regimes. I predicted that there would be less lipids and glycogen in bees treated with fluctuating thermal regimes because the warming periods would allow for active repair mechanisms utilizing the lipids and glycogen for energy. I also predicted that the respiratory quotient of the bees would reflect primarily the usage of lipids for energy because of their long-term overwintering.

2.3. Methods

2.3.1. Collection and Treatment

Overwintering *Megachile rotundata* from the 2019 and 2020 field seasons were purchased as pre-pupae in 2020 and 2021 respectively (Mennie Bee Farms Inc., city, State). Bees in brood cells were stored in a low humidity (~30%) incubator at 6˚C (Percival, Perry, IA) until use. Bees from 2019 were used in September and November of 2020, and bees from 2020 were used in May of 2021. To compare how lipid, trehalose, glycogen, and simple sugars vary depending on the timing of interrupted development, prepupae were removed from the 6˚C incubator and placed into a 29˚C incubator in either September or November to stimulate development to the pupal stage. After two weeks of development, bees were removed from 29˚C and haphazardly assigned to one of three treatments. Control bees were frozen immediately. The other bees were placed into a fluctuating thermal regime (FTR; 21 hours at 6˚C with a daily 1-hour pulse of 20˚C, with a one hour ramp up and ramp down period) or a static thermal regime (STR; constant 6˚C) for low temperature storage (Figure 1. Originally appears in Torson et al.,

2017). After one week of treatment, bees were removed from their brood cells and placed in microcentrifuge tubes to be flash frozen in liquid N_2 and stored at -80˚C.

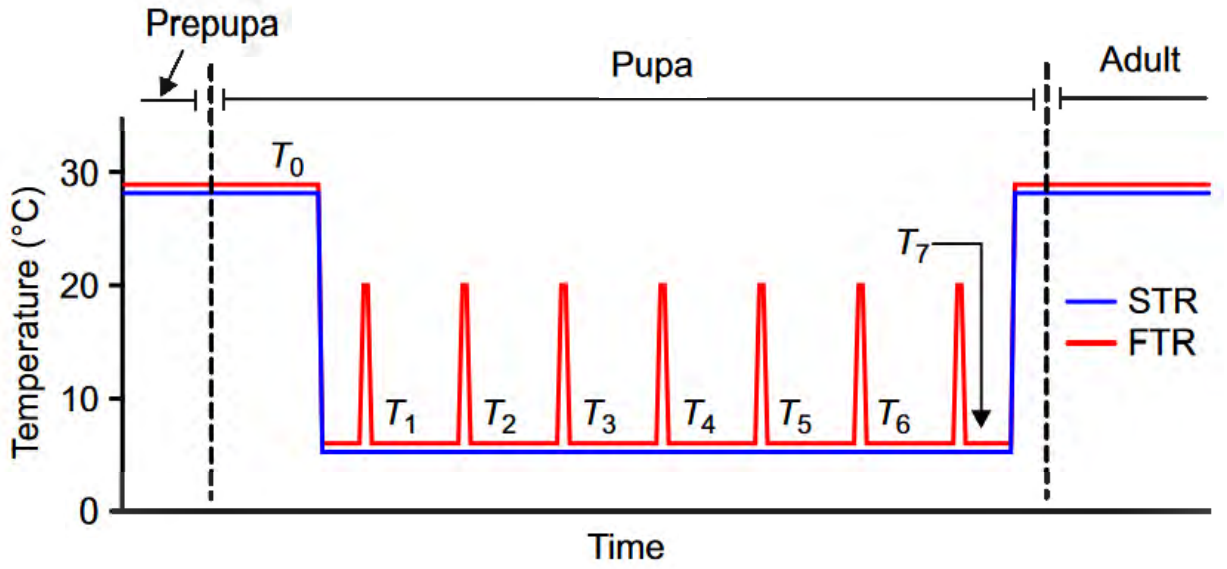

Figure 1. Experimental design. Bees were put in 29˚C for two weeks to initiate development into the pupal stage. Bees were then placed into either a static (STR, red) or fluctuating cold thermal temperature regime (FTR, blue) for either one or two weeks. Control bees (not shown) were allowed to develop without interruption at 29 ˚C (Torson et al., 2017).

To compare how trehalose, glycogen, and simple sugars vary depending on the amount of time in STR/FTR, bees from the 2020 field season were overwintered in 6˚C. To initiate development, bees were placed in 29˚C for two weeks. Bees were haphazardly assigned to both FTR or STR, and one week versus two weeks of treatment, with controls being bees immediately after being removed from 29˚C. After treatment bees were removed from their brood cells and placed in centrifuge tubes to be frozen and stored at -80˚C.

2.3.2. Biochemistry

After removal from -80˚C, bees were weighed using an analytical balance (Mettler Toledo, Columbus, OH) to obtain their wet weight. Bees were then transferred to new 1.5 mL screw-cap tubes (RINO® Next Advance Inc., Troy, NY) each containing 7 1 mm zirconium oxide beads (Next Advance Inc., city state) for homogenization. Total lipids were quantified

using a vanillin assay (Van Handel, 1985b). Trehalose, glycogen, and simple sugars were quantified using a modified anthrone assay for sugars (Skillman et al., 2018; Van Handel, 1965, 1985b, 1985a). Three bees from FTR, STR, and control group from both September and November were measured for each of the three days each assay was performed to ensure enough time to plate the samples since the assay color is time sensitive. Three bees from one day, one week, and two weeks of FTR or STR as well as the controls were measured each day the assay was performed totaling nine bees from each treatment at time point.

2.3.2.1. September versus November Treatment Assay Methods

To measure total lipids, a vanillin-based assay was used (Van Handel, 1985b). Each bee was placed in a 1.5 mL microcentrifuge tube, one mL of a 1:1 chloroform-methanol solution was added to each tube, and the tube was placed in a bead blender to homogenize the insect. The 18 tubes were then centrifuged for 3 minutes at 16,000 rpm to separate the lipids from the rest of the homogenate. A 100 µL aliquot of the supernatant was transferred into a glass tube. A soybean oil standard was created using 1mg of soybean oil per 1 mL of chloroform. The standard curve was generated using 0, 0.05, 0.1, 0.2, 0.25, 0.3 mg/mL of the soybean oil standard in glass tubes. All tubes were heated at 90˚C until the liquid evaporated. Additionally, 200 µL of 99% sulfuric acid was added to each of the tubes, and tubes were heated again at 100˚C for 10 minutes. After cooling until tubes were cool to the touch, 2.3 mL of the vanillin reagent (Van Handel, 1965) was added to each of the tubes before vortexing to combine. The color was left to develop for 5 minutes at room temperature before 125 µL was pipetted into wells in triplicate in a 96 well-plate. Plating took approximately 25 minutes. The absorbance was read with a spectrophotometer at a wavelength of 625 nm.

For the trehalose assay, the bees were homogenized using a bullet blender (Next Advance Inc., Troy, NY) in 4 µL/mg of bee mass of 2% sodium sulfate. This amount was based on the average mass across the 18 bees used that day. After homogenization, 1 mL of 100% methanol was added to each tube, and samples were centrifuged for 1 minute at 13,000 rpm. The supernatant was transferred to a glass tube, and 300 µL of reagent grade H_2O was added to the precipitate and vortexed. Then, 500 µL of 100% methanol was added to the dissolved precipitate and centrifuged at 13,000 rpm for 1 minute to allow for additional sugar extraction from the pellet into the supernatant. The supernatant was then combined with the previous supernatant in the same glass tube. The glass tubes were then heated at 90˚C until there was approximately 500 µL of liquid remaining. Additionally, 100 µL of the liquid from the glass tubes was added to a new glass tube. Then, 50 µL of 1M HCl was added and heated at 90˚C for 7 minutes. In addition, 150 µL of 1M NaOH was then added to the glass tubes and heated at 90˚C for 7 minutes. Finally, 2 mL of anthrone reagent was added to the tubes and heated at 90˚C for 17 minutes. The liquid was then allowed to cool before 125 µL of each sample was pipetted into each well in triplicate in a 96 well-plate and read at 625 nm.

Glycogen and simple sugars were extracted by adding 150 µL of 2% sodium sulfate to each beaded tube and homogenized as described above. Then, 950 µL of 1:2 chloroform-methanol was added to the tube. The tube was vortexed vigorously and centrifuged at 16,000 rpm for 3 minutes. The entire supernatant was added to a new glass tube and vortexed before 100 µL was aliquoted for use in the simple sugars assay. The multiplier for both glycogen and simple sugars was 10 since 10% of the initial extracted bee was used.

To quantify glycogen, 1 mL of 100% methanol was added to the remaining pellet in the homogenization tube. This was then homogenized and centrifuged as described above, and the

supernatant was discarded. Additionally, 975 µL of the anthrone reagent was added and homogenized. Then, 50 µL was aliquoted to a glass tube. Finally, 950 µL of additional anthrone reagent was added to the glass tube and vortexed. The tubes were heated at 90˚C for 10 minutes and then allowed to cool before 125 µL of each sample was pipetted in triplicate into a 96 well-plate and read at 625 nm.

Simple sugars were measured by concentrating the aliquoted 100 µL to 50 µL on a 90˚C hot plate to which 950 µL of the anthrone reagent was added. The solution was vortexed then heated at 90˚C for 10 minutes and allowed to cool before 125 µL of each sample was pipetted in triplicate into a 96 well-plate and read at 625 nm.

2.3.2.2. Length of Time in Treatment Assay Methods

For comparisons between the amount of time in treatment methods in this section are largely similar as the previous assay methods save for the fact that they were optimized for all macronutrient analyses to be performed on each bee. For nutrient extraction, frozen bees were weighed and placed in 1.5 mL centrifuge tubes (RINO® Next Advance Inc., Troy, NY) containing 7 1mm zirconium oxide beads and 150 µL 2% sodium sulfate. Bees were then crushed using a bullet mixer (Next Advance Inc., Troy, NY). Then, 950 µL of (1:2) chloroform-methanol was added and the tubes were vortexed. Tubes were centrifuged for 3 min at 16,000 x g. The entire supernatant was then removed and added to new centrifuge tubes. The new tubes were then vortexed and 50 µL was aliquotted into glass tubes for the lipid assay and 100 µL for each sugar assays. The pellet remained in the screw cap tubes for glycogen analysis.

For lipid analysis, a 1mg/mL of oil-chloroform solution was made to create a standard curve of 0, 0.05, 0.1, 0.2, 0.25, 0.3 mg/mL, and 50 µL was added to the glass tube. The glass tubes containing the standards and samples were heated at 90˚C until no liquid remained. Then,

40 µL of 99% sulfuric acid was added and heated at 90˚C for 2 min. The solution was then allowed to cool until the glass tubes were cool to the touch. Then, 960 µL of the vanillin reagent (Van Handel, 1965) was added to the glass tubes and vortexed. Finally, 125 µL of the solution was added in triplicate to a 96 well-plate and read at 625 nm.

For total carb analysis, the glass tube containing the total sugars fraction was heated at 90˚C and concentrated to 50 µL. Then, 950 µL of the anthrone reagent (Van Handel, 1965) was added to the tubes and vortexed. The tubes were then heated at 90˚C for 10 minutes and allowed to cool to the touch. Finally, 125 µL of the solution was added in triplicate to a 96 well-plate and read at 625 nm.

For glycogen analysis, the remaining pellet in screw-cap tubes was washed with methanol by adding 1 mL of methanol. The solution was mixed with a bullet mixer and centrifuged for 3 min at 16,000 x g. The remaining supernatant was discarded. Then, 975 µL of anthrone reagent was added and solids dissolved using the bullet mixer. A 50 µL aliquot of this solution was then added to a new glass tube. A glucose standard (50mg glucose /mL water; 0 ,0.5, 1, 1.5, 2 mg/mL) was then created which was utilized for both the glycogen and simple sugars assay. An additional 950 µL of anthrone reagent was added to both the standard and sample tubes and vortexed. The tubes were then heated at 90˚C for 10 minutes and allowed to cool to the touch. Finally, 125 µL of the solution was added in triplicate to a 96 well-plate and read at 625 nm.

2.3.3. Metabolic Rate and Respiratory Quotient Calculations

Closed-system respirometry was used to measure oxygen consumption and carbon dioxide emission rates to calculate the respiratory quotient (RQ) as an estimation of the energy source used to generate ATP. Post-diapause quiescent bees were removed from 6˚C and placed

in 29˚C incubators for two weeks, as we had done previously. Immediately following the two weeks at 29˚C, pupal bees within brood cells were placed into 5 mL syringes and flushed with CO_2 free air. The bees were then stored in a 29˚C incubator for approximately one hour, after which approximately 1 mL of air in the syringe was injected a stream of dry, CO_2-free, air at a flow rate of 200 mL min^{-1}, which was passed through an Ascarite® and Drierite® column, a LI-7000 CO_2 analyzer, and then an Oxzilla II oxygen analyzer (Sable Systems International, Las Vegas, NV, USA). Closed-system respirometry was repeated after the first day, one week, and two weeks after bees were placed into their treatments. Baselining was repeated between individual measurements by passing dry CO_2-free air directly through the analyzers.

2.3.4. Statistical Analysis

Statistics were conducted and figures were created using JMP Pro 15.1 (SAS, Cary, NC). Differences were considered significant if $p < 0.05$.

2.3.4.1. Timing of Interrupted Development Macromolecule Assays

Month of treatment (September or November) was treated as a fixed effect and not pooled when significantly different. Assay type (glycogen, simple sugars, trehalose, and lipids) was compared separately because each assay was run on separate bees. Month of treatment, treatment, and their interaction were compared together as predictor variables, with µg nutrient/mg body weight as the outcome variable. A generalized linear mixed model was used to compare variables. Post hoc comparisons were conducted using Tukey HSM.

2.3.4.2. Length of Interrupted Development Macromolecule Assays

Because these assays were all run on the same bee, a multivariate analysis of variance (MANOVA) was used to test the association between the four macronutrients (lipids, simple sugars, trehalose, and glycogen) and the treatment, time point, and treatment x time point

interaction utilizing Pillai's Trace as the test statistic. A discriminant analysis was used to determine significant differences between the treatments and time points and the contributing macronutrients. Control bees were randomly assigned to either FTR or STR for statistical analysis.

2.3.4.3. Respirometry

To determine if CO_2, O_2, and RQ varied among treatment and time point, repeated measures ANOVA was conducted with time point as the within-subjects factor and treatment as the between-subjects factor. Post hoc comparisons were conducted using Tukey HSM. Statistics were conducted both including and excluding the 29˚C control to first confirm the difference from the control and then assess nuanced interactions over time.

2.4. Results

2.4.1. Timing of Interrupted Development

There was no observed effect of month of treatment observed for glycogen, simple sugars, or trehalose (Figure 2; $p < 0.05$). However, lipids did show an effect of month of treatment (Figure 2; $F_{2,35} = 16.51$, $p < 0.001$) with December bees showing higher levels of lipids than September bees. Both FTR and STR treated bees, when compared with the control, had significantly higher levels of simple sugars (Figure 2; $F_{2,53} = 7.22$, $p < 0.011$) and trehalose (Figure 2; $F_{2,53} = 11.04$, $p < 0.001$). No effect of treatment was observed in glycogen (Figure 2; $F_{2,35} = 2.10$, $p > 0.130$) or lipids (Figure 2; $F_{2,35} = 0.01$, $p > 0.99$).

2.4.2. Length of Interrupted Development

2.4.2.1. Macromolecule Analysis

The result showed significant differences for time point (Pillai's Trace = .07, $F_{12,153} = 3.99$; $p < 0.0001$) and time point x treatment (Pillai's Trace = .07, $F_{12,153} = 3.68$; $p < 0.0001$). The

discriminant analysis showed a large driving factor for group differences being driven by the lipid content of the insect, followed by trehalose (Table 1). Most notably, the FTR control was significantly different from the FTR 1 day treated bees ($p < 0.05$), but this trend was not observed within STR-interrupted bees. The FTR control was also significantly different from the 1 week treated bees ($p < 0.05$), but the control was not different from the 2 weeks treated bees. The reverse was observed within STR treated bees, with the control being significantly different from 2 weeks in treatment but not 1 week. For all treatment lengths except for the control, FTR and STR-interrupted bees were significantly different. In FTR treated bees, 1 day, 1 week, and 2 weeks in treatment, were not significantly different from one another, but in STR treated bees the opposite was true.

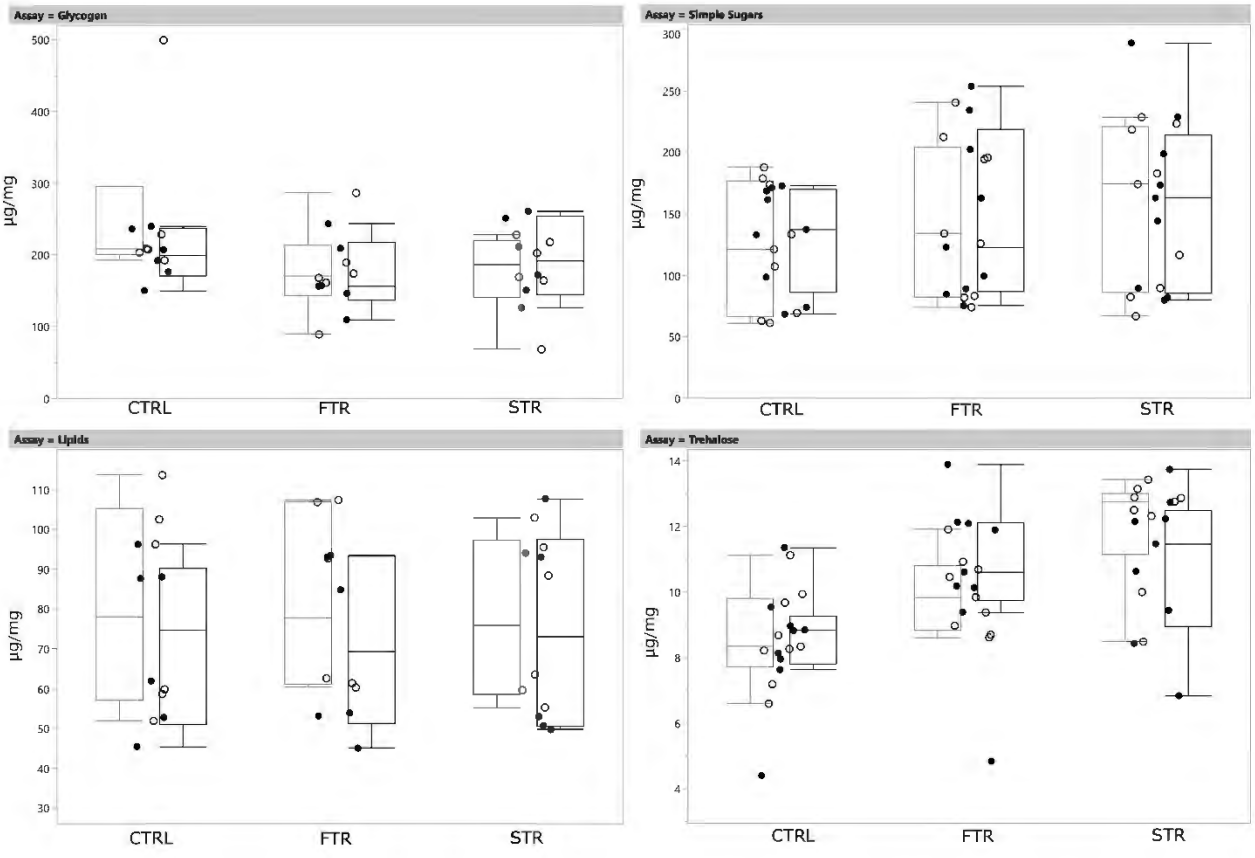

Figure 2. Bees with interrupted development treated in September (filled circles and black lines) and November (open circles and gray lines).

Table 1. Relative importance of each macronutrient towards group separation. Standardized scoring coefficients from the discriminant analysis. Absolute value of the value denotes its relative significance to the separation of treatments and time points. Sign indicates positive or negative correlation.

	Trehalose	Lipids	Glycogen	Simple Sugars
Canon1	0.541903869	0.7802163	-0.44003	0.3581448
Canon2	-0.357996601	0.6213215	0.432682	-0.512225
Canon3	0.854095092	-0.202539	0.396218	-0.676299
Canon4	-0.358589595	-0.094172	0.815574	0.5945714

Table 2. Macronutrient values. Mean value of each macronutrient for the combined treatments and time points.

Count	Treatment*Time Pt	Trehalose	Lipids	Glycogen	Simple Sugars
5	FTR Control	17.99±2.44	5.96±0.89	199.03±33.83	83.93±7.74
9	FTR 1 Day	22.42±1.74	12.51±1.01	208.19±26.56	107.05±3.45
9	FTR 1 Week	24.86±1.40	12.24±0.78	192.13±18.01	141.40±19.74
9	FTR 2 Weeks	25.19±2.28	8.08±0.97	197.45±22.98	132.16±7.79
4	STR Control	20.02±3.62	8.64±0.61	221.80±37.50	88.67±9.63
9	STR 1 Day	15.07±1.83	7.15±0.84	164.26±26.44	97.62±9.21
7	STR 1 Week	26.25±2.72	8.97±0.43	151.00±22.02	133.98±7.67
8	STR 2 Weeks	31.14±1.82	14.35±1.15	177.68±8.43	151.16±8.57

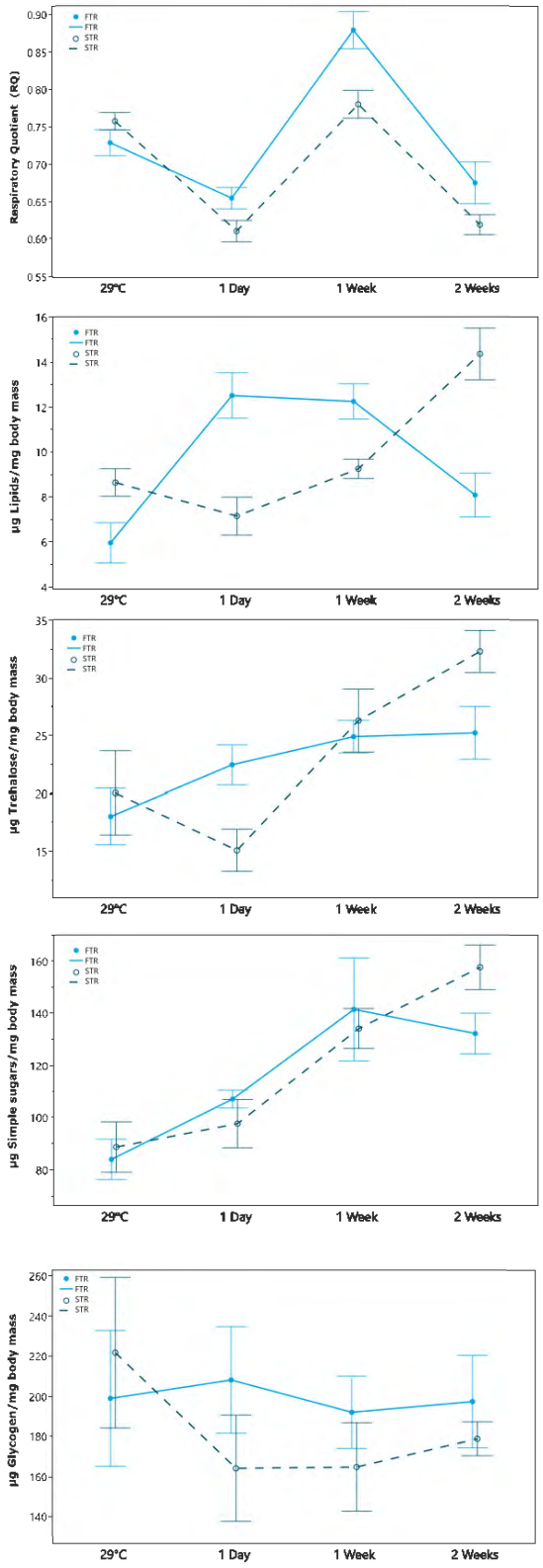

Figure 3. Respiratory quotient and macromolecule concentrations over time. FTR shown with a solid line and filled circles and STR shown with a dashed line and open circles.

2.4.2.2. Respirometry

Absolute CO_2 emission (Figure 4A; $F_{3,27} = 281.131$; $p < 0.0001$), absolute O_2 consumption (Figure 4C; $F_{3,27} = 205.185$; $p < 0.0001$), and respiratory quotients (Figure 3A; $F_{3,28.7} = 31.5590$; $p < 0.0001$) all significantly changed as time passed. Mass-specific CO_2 (Figure 4B; $F_{3,27} = 350.9438$; $p < 0.0001$) and O_2 (Figure 4D; $F_{3,27} = 246.6486$; $p < 0.0001$) also varied with time. Both absolute and mass-specific CO_2 emissions and O_2 consumption, as well as RQ all were significantly lower than the 29˚C control ($p < 0.05$). When accounting for mass, O_2 consumption varied by treatment with FTR treated bees consuming more O_2 ($F_{1,29} = 5.1586$; $p = 0.0307$). The RQ was affected by an interaction between the time and treatment ($F_{3,27} = 4.3029$; $p = 0.0132$). Observed interactions of note were the significant differences in 1 week of FTR being higher than the control, 2 weeks, and 1 day in FTR ($p < 0.05$). In STR, 2 weeks in treatment was significantly lower than the control and 1 week in treatment, and 1 day in STR was significantly lower than the control and 1 week in treatment ($p < 0.05$).

When removing the 29˚C control from the analysis, O_2 varied by time point (Figure 4G; $F_{2,28} = 12.8390$; $p = 0.0001$), with 1 day being significantly higher than 1 week ($p < 0.0001$), as well as when accounting for mass (Figure 4H; $F_{2,28} = 13.1510$; $p < 0.0001$), with 1 day being significantly lower than 1 week ($p < 0.0001$) and 1 week being significantly higher than 2 weeks in treatment ($p = 0.0270$). Respiratory quotient also varied by time point when the 29˚C control was removed from the analysis ($F_{2,28} = 47.3153$; $p < 0.0001$), with 1 day being significantly lower than 1 week ($p < 0.0001$) and 1 week being significantly higher than 2 weeks in treatment ($p < 0.0001$). When removing the 29˚C control, both absolute and mass-specific CO_2 and O_2 and RQ all varied with treatment ($p < 0.05$). The respiratory quotient varied between bees in 1 week of FTR and both 1 day and 2 weeks in FTR ($p < 0.05$). In STR treated bees, the respiratory

quotient varied between 1 week of STR and both 2 weeks, and CO_2 emission ($F_{3,27} = 281.131$; $p < 0.0001$), O_2 consumption ($F_{3,27} = 205.185$; $p < 0.0001$), and respiratory quotients ($F_{3, 28.7} = 31.5590$; $p < 0.0001$) all significantly changed as time passed, as well as when accounting for mass in both CO_2 ($F_{3,27} = 350.9438$; $p < 0.0001$) and O_2 ($F_{3,27} = 246.6486$; $p < 0.0001$). Bees treated with STR had CO_2 emission, O_2 consumption, and respiratory quotients all were significantly lower than the 29˚C control, as well as when accounting for mass in CO_2 and O_2 ($p < 0.05$). The RQ varied differently depending on the time point and treatment (time x treatment interaction, $F_{3,27} = 4.3029$; $p = 0.0132$). Observed interactions of note were the significant differences in bees after 1 week of FTR being higher than the control, 2 weeks, and 1 day in FTR ($p < 0.05$). In STR treated bees, 2 weeks in treatment was significantly lower than the control and 1 week in treatment, and 1 day in STR was significantly lower than the control and 1 week in treatment ($p < 0.05$). When accounting for mass, O_2 consumption varied by treatment ($F_{1,29} = 5.1586$; $p = 0.0307$).

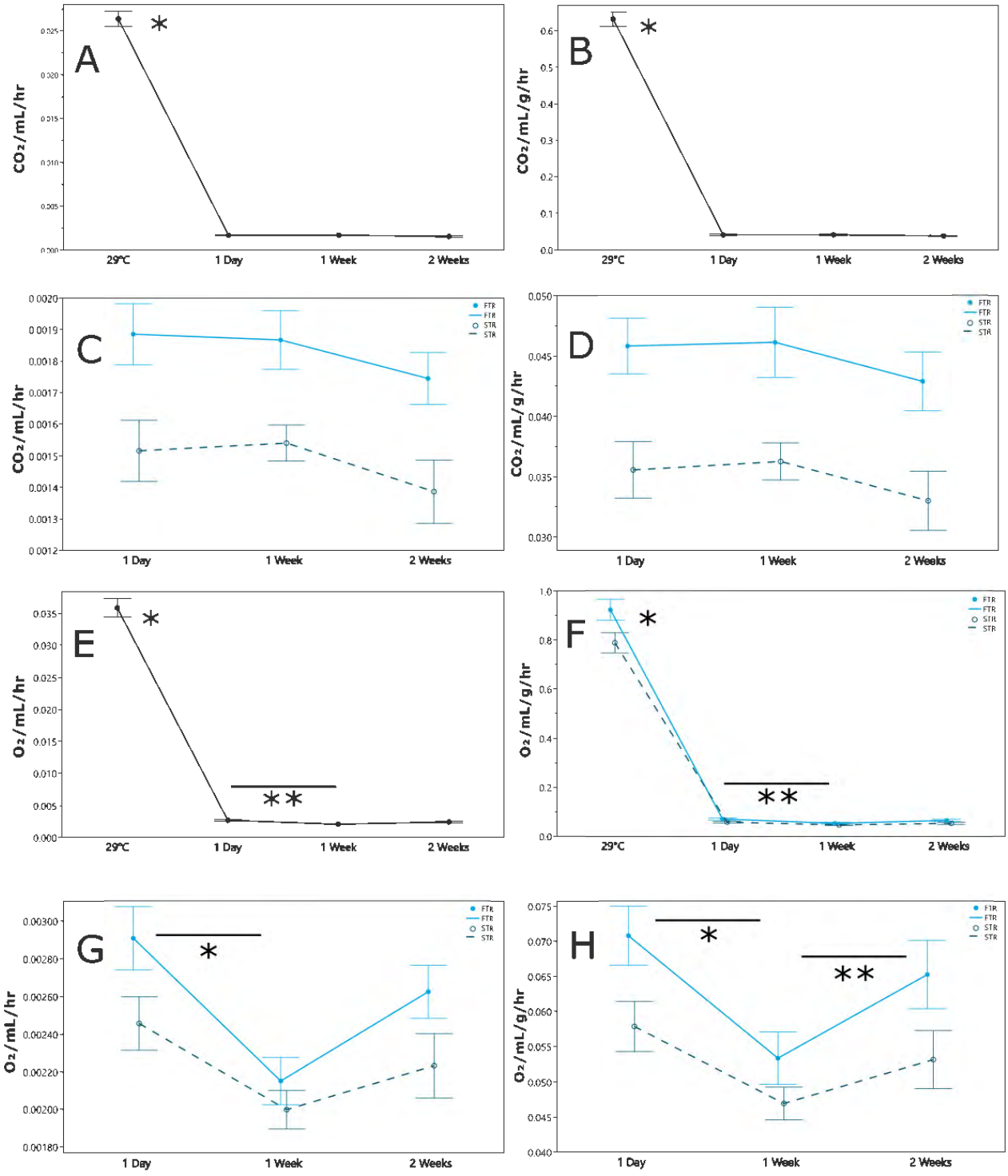

Figure 4. Absolute and mass-specific CO_2 emissions and O_2 consumption. Graphs shown with and without the 29˚C control included. FTR shown with a solid line and filled circles and STR shown with a dashed line and open circles. FTR and STR are shown separately when significantly different ($p < 0.05$).

2.5. Discussion

This study aimed to use the respiratory quotient (RQ) and macromolecular composition of bees whose development was interrupted by different thermal regimes to gain insight into the reason why insects exposed to fluctuating temperatures during interrupted development have better survival. My hypothesis that there would be differences between type and duration of low temperature stress during development was supported, however the nuances regarding the macronutrient composition and RQ is more complicated.

In insects, the respiratory quotient usually ranges from 0.7-1.0 (Nunes et al., 1997). Respiratory quotients around 0.7 indicate the oxidation of lipids, 1.0 indicates the oxidation of carbohydrates, and 0.8 indicates the oxidation of proteins or amino acids (Hall & Hall, 2020). While the RQ is not a definitive measurement of macronutrients being oxidized in metabolism, it is a helpful tool to gain insight on the processes occurring. The trend observed in the data show that bees developing at 29˚C had RQs between 0.7 and 0.75 (Figure 3), which indicates the relative usage of lipids for energy during pupal development, which has been observed previously (Damos et al., 2011). Lipids provide almost twice the metabolic water upon oxidation as carbohydrates, therefore producing more energy which is useful for long term non-feeding periods like overwintering in *Megachile rotundata*, but also providing water during a time when insects could become dehydrated due to lack of water intake (Downer, 1981). Use of lipids is expected as the overwintering process, diapause, characteristically uses lipids for energy to sustain long term developmental arrest (Adedokun & Denlinger, 1985; Batz & Armbruster, 2018). Relative to other time points, lipids are also low at the initial measurement which supports the assumption that low levels indicate the usage of a macromolecule.

At both 1 day and 2 weeks in treatment, FTR and STR-treated bees had RQs below 0.7. Respiratory quotients below 0.7 can indicate the occurrence of ketogenesis or gluconeogenesis (Kerner & Hoppel, 2004). Ketogenesis is the synthesis of ketone bodies which can operate as fatty acids for energy where there are low levels of sugars (Kerner & Hoppel, 2004). Gluconeogenesis is the formation of glucose from sources other than other carbohydrates, typically proteins or fats, but gluconeogenesis can also occur from the products of ketogenesis (Engelking, 2015). The products of ketogenesis can lead directly into gluconeogenesis, showing a direct transition from lipids to sugars (Kerner & Hoppel, 2004). Gene expression of proteins related to gluconeogenesis are upregulated after 48 hours of low temperature stress in fish, which supports this idea during low temperature stress (Qian & Xue, 2016). The increase in simple sugars in both STR and FTR treated bees was observed after 1 week of low temperature stress. This indicates ketogenesis initially at 1 day followed by gluconeogenesis. This trend is not reflected in the RQ, which indicates protein metabolism after 1 week of low temperature stress, but gluconeogenesis or ketogenesis again after 2 weeks. This variation would not be expected if ketogenesis and gluconeogenesis would be occurring. The lower amounts of trehalose in STR-interrupted bees when compared to FTR-interrupted bees is not reflected in the simple sugars analysis. This has been found previously in insects treated with fluctuating temperatures that showed increased levels of trehalose when compared to static temperatures, though this trend is not sustained over time (Overgaard et al., 2007). This could indicate that ketogenesis to gluconeogenesis is the primary source of energy for STR treated bees, and the decrease in RQ at 2 weeks occurs as bees are creating cytoprotective sugars through gluconeogenesis because of the long-term stress rather than utilizing it for energy.

FTR and STR-interrupted bees show contrasting levels of lipid content during all periods of low temperature stress. Although they start in a similar range, lipids in FTR-interrupted bees increase at 1 day and 1 week in treatment and lipids in STR-interrupted bees decrease. After that, lipids in FTR decrease again at 2 weeks, and lipids in STR-interrupted bees increase. This trend has also been observed previously with insects treated with FTR having higher a higher lipid content than insects treated with STR (Overgaard et al., 2007). A possible explanation for this is that after 1 day in low temperature stress, FTR treated bees switch from using lipids for energy during development to using ketones and then sugars because of the initial stress, which would explain why lipid content increases so drastically after 1 day in low temperature stress. Lipogenesis in FTR-interrupted bees would also be expected based on previous studies showing gene expression changes indicating lipid creation which would explain the increased lipid content but not usage (Melicher et al., 2019). Energy expenditure likely would also not be as high as during active development, resulting in any created lipids potentially remaining in excess when compared to active development. This is also somewhat reflected in the RQ, which decreases to levels that would be expected during ketogenesis, but not the eventual usage of sugars for energy after gluconeogenesis (Kerner & Hoppel, 2004). Protein metabolism is indicated after 1 week in treatment which could additionally contribute to lipogenesis and eventual ketogenesis. After extended low temperature stress to maintain membrane potential, FTR treated bees may then resort to using additional lipids for creating sugars to power ion pumping, since genes encoding V-type ATPase pumps are upregulated in other species during low temperature stress (Koštál et al., 2007; Qian & Xue, 2016). This could be why FTR-interrupted bees show a steady amount of simple sugars from 1 to 2 weeks in treatment and a decrease in lipid, because prolonged repair takes more energy. However, some previous data has

suggested that metabolic water and lipid contents are unchanged during fluctuating temperatures (Boardman et al., 2013). This disparity may be due to life history differences between species because *M. rotundata* are overwintered prior to low temperature stress and *Thaumatotibia leucotreta* as used in that study are not an overwintering species.

Respiratory quotients over 1.0 can indicate anaerobic processes or lipogenesis (Guenst & Nelson, 1994; Kovacs & Westerterp-Plantenga, 2006). As previously stated, RQs of 0.7 indicate primarily metabolism of lipids (Nunes et al., 1997). Previous research has indicated that lipid metabolism, specifically beta-oxidation of long chain fatty acids, can be important for low temperature stress resistance in vertebrates, and gene expression for such increases (Melicher et al., 2019; Sun et al., 2019). Combining the RQ and lipid composition results from our experiments, neither lipogenesis or significant usage of metabolized lipids is indicated which would be visible by a RQ of 0.7 in Figure 4A and a decrease in lipids in 4B. This could potentially indicate that lipogenesis is not a large factor contributing to low temperature stress tolerance in insects, or increased survival in FTRs. More likely, however, is that the RQ is the sum of all metabolic reactions taking place, and we may not be able to resolve the creation of lipids when there may be many metabolic processes occurring simultaneously.

2.6. Conclusions

While fluctuating temperatures and static temperatures showed significant differences in both metabolic rates and macronutrient compositions, our initial predictions were not supported. Glycogen levels did not contribute significantly to the differences between bees interrupted with FTR and STR, but lipid levels did. It appears likely that a more complex combination of macronutrients and processes are being used for energy and cryoprotection at different time points. The most likely explanation is that bees interrupted with FTRs are using the warming

periods for lipogenesis which for energy, likely ion balance which is largely fueled by amino acid metabolism. This could be further evidence towards the hypothesis that fluctuating temperatures allow for repair mechanisms to occur rather that cryoprotection usage which increases survival. The observed results also provide evidence for more in-depth non-destructive exploration of macronutrient usage during interrupted development because RQ is potentially not providing the nuance that is required to understand the ongoing biochemical processes. A largely unexplored area of this study is the potential metabolic water created and how that affects bees that have been non-feeding for an extended period of time such as overwintering. Incorporating a protein assay and water content analysis could be a potential way to expand upon this research to help better understand the benefit of FTRs.

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