The effects of changing spring temperatures on fuel use, mass loss, emergence time, and chill coma recovery in solitary mason bees (*Osmia* spp.).

By

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Abstract

Repeated cold exposures and warmer winter temperatures might negatively affect insects by depleting stored fuel reserves. I researched the effects of fluctuating thermal regimes on two species of solitary mason bees (*Osmia albiventris* and *O. lignaria*) by quantifying mass loss and reserves of lipids, free sugars, and glycogen after experimental temperature treatments during early spring. In a second season, I quantified mass loss, time to emergence, and time to recover from chill coma after bees had been exposed to one of two spring-time temperature regimes. I found that warmer temperatures in combination with greater temperature variability increases mass loss and the depletion of fuel reserves. Additionally, my results suggest that accelerated bee emergence helps to mitigate mass loss. Overall, these bees appear resilient in the face of changing spring temperatures.

Résumé

On sait peu sur la façon dont la variabilité environnementale affecte les abeilles solitaires sauvages. Ma recherche a étudié la façon dont les régimes thermiques fluctuants affectent la vigueur de deux espèces d'abeilles solitaires (*Osmia albiventris* et *O. lignaria*) en quantifiant la perte de masse ainsi que les réserves métaboliques (lipides, sucres libres, et glycogène) des abeilles suite à des manipulations expérimentales de la température printanière. Dans une deuxième saison, j'ai quantifié la perte de masse, le temps de l'émergence et le temps pour récupérer du coma froid après que les abeilles avaient été exposées à l'un de deux régimes de températures printanières. Mes résultats indiquent que les températures plus chaudes ont tendance à augmenter la perte de masse et des réserves métaboliques lorsqu'elles sont combinées avec une plus grande variabilité. De plus, mes résultats suggèrent que ces abeilles sont résilientes face aux températures printanières changeantes.

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Introduction

Warming Winter Temperatures

Owing to rising greenhouse-gas concentrations, global temperatures have been projected to increase by as much as 4.8°C in addition to becoming more variable (Hayhoe et al., 1992; IPCC, 2014) and more extreme (Fischer and Knutti, 2015; Henry, 2008; Masters, 2014). Winter temperatures have been predicted to be the most affected, especially at high latitudes (IPCC, 2014). For many ectotherms, increased variability of winter temperatures may result in increased energy expenditure as organisms will have to invest in the production of more cryoprotectants when temperatures are colder, but will also have to cope with increased energy consumption as a result of higher metabolic rates when temperatures are warmer (Williams et al., 2015). Climate change has also resulted in diminishing snow depths and durations of cold weather throughout the winter, affecting the level of protective insulation available to organisms seeking refuge under snow (Michener, 2007; Pauli et al., 2013). Snow cover, rather than macroclimatic temperature, can be the key variable determining how thermally stressful a winter will be for an organism (Simons et al., 2010; Sinclair, 2001). In particular, declining snow-pack can increase the frequency of soil frosts, which directly affects organisms overwintering near or below ground level (Pauli et al., 2013; Williams et al., 2015). These diverse aspects of changing winter climate interact with the organisms' varied ecological and physiological strategies for coping with the stress of winter conditions, which leads to disparities in each species' susceptibility to changing winter climates (Williams et al., 2015).

Paradoxically, given the generally rising temperatures, there has been an observed increase in the frequency of damaging frosts in recent decades in North America (Augspurger, 2013; Kunkel et al., 2004). This is the result of an earlier onset of the growing season (the result of warmer mean temperatures in spring), which exposes metabolically active tissues to subsequent

temperature extremes—which may still include extreme cold. Hänninen (1991) modelled bud burst in trees as a function of temperature and used the model to forecast the risk of frost damage under a future warmer climate. He calculated that bud burst could occur as early as mid-winter due to warm winter temperatures encouraging tree growth during mild spells. He concluded that below-freezing temperatures occurring in late winter would result in severe frost damage to the buds, even though late-winter temperatures were projected to increase. Indeed, spring frosts have become a common occurrence (e.g. The US 2007 'Easter Freeze' Gu et al., 2008; Kunkel et al., 2004), with the potential to affect local plant and animal species (Hänninen, 1991; Inouye, 2008). Ontario apple crops were wiped out by a sudden spring frost in 2012, and as a result, yields remained low in 2015 (CBC News, 2015).

Warmer winter temperatures can also cause a reduction in snowpack and an advance in the date of spring snowmelt (e.g. Cayan et al., 2001). There is a trend towards earlier snowmelt in the Western United States (Cayan et al., 2001; IPCC, 2014). Inouye (2008) found that earlier dates of snowmelt lead to the earlier formation of flowering buds in three montane wildflower species, resulting in greater susceptibility to damage from mid-June frosts. Similarly, increased air temperatures in winter or spring, in conjunction with reduced snow cover, could increase the frequency and the intensity of freeze-thaw cycles in the soil (Williams et al., 2015). The number of freeze-thaw cycles in a season can directly impact an insect's ability to survive subsequent low temperatures (Sinclair et al., 2003). Together, these findings suggest that increasing frequency of cold damage to overwintering organisms is a real possibility.

Diapause and Energy Reserves in Insects

Organisms in temperate locations have developed physiological mechanisms to deal with the changing seasons. One such mechanism is metabolic suppression, in which insects survive adverse conditions by maintaining a physiologically dormant state, termed diapause (Storey and

Storey, 2012). During diapause, insects have not simply lowered their metabolic rates compared to non-diapausing insects, but have actually entered an alternative developmental pathway that has its own metabolic demands, such as upregulating the production of glycerol for use as a cryoprotectant (Hahn and Denlinger, 2011). Although diapause is an adaptive method to escape physical stresses as a result of low temperatures, it is also accompanied by its own stresses that may be detrimental to the organism, such as desiccation, oxidative damage, cold injuries, or the depletion of fuel reserves (Hahn and Denlinger, 2007; Lalouette et al., 2011; Marshall and Sinclair, 2011). Because of metabolic suppression, however, these are less costly than they would be in the absence of diapause.

Even in a state of diapause, insects that have to spend the winter in cold environments must adopt a way to cope with the cold. Insects are categorized as being either freeze-avoidant or freeze-tolerant. Freeze-tolerant insects can tolerate the formation of internal ice (Storey and Storey, 1996). In contrast, freeze-avoidant insects alter their supercooling points in order to maintain body water in a liquid state at temperatures below its freezing point, thanks to the production of cryoprotectant molecules such as glycerol or sorbitol (Salt, 1969).

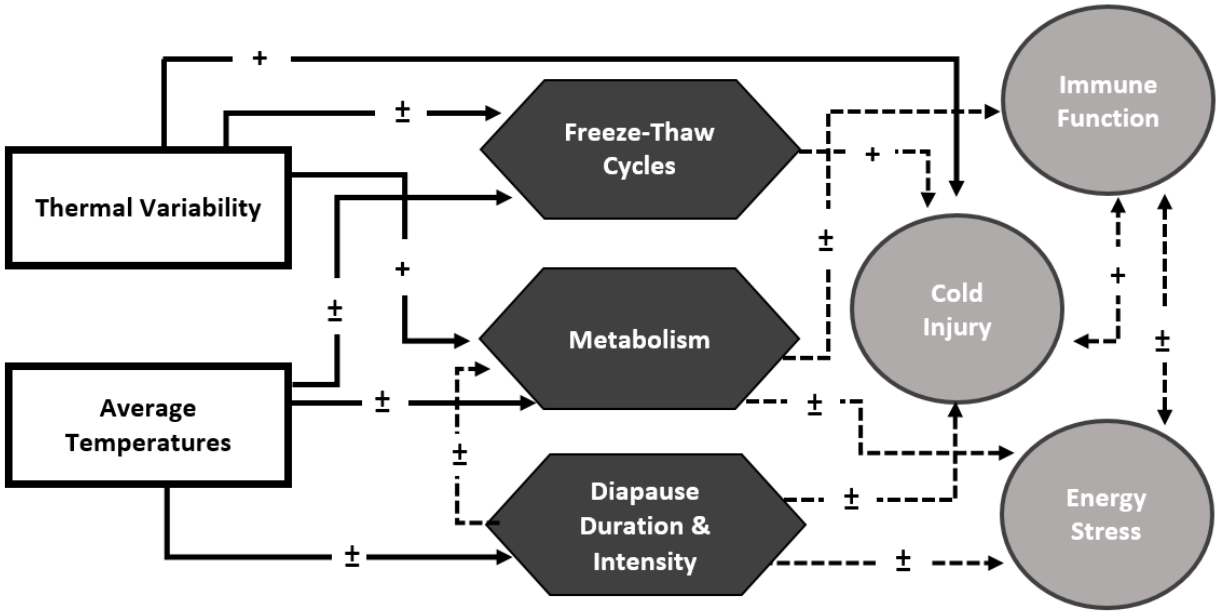

Figure 1—Conceptual flow chart of the impacts of winter climate change on insects. Climate change is manifested by abiotic drivers (white boxes) that alter environmental and biological processes (black hexagons), which in turn impact an insect's biological response (grey circles). Adapted from Figure 3 in Williams et al. (2015).

Insects convert glycerol from glycogen, their main storage reserve of carbohydrates, largely located in the fat body. Before entering diapause, many insects accumulate large reserves of glycogen (Hahn and Denlinger, 2007). The conversion of glycogen to glycerol is temperature dependent and reversible. As insects warm up in the spring, a large portion, but not all, of the glycerol is recovered and stored again as glycogen (Hahn and Denlinger, 2007). Warmer temperatures support the reconversion of glycerol to glycogen while lower temperatures drive the production of glycerol. Therefore, we might expect somewhat lower glycogen reserves after a particularly harsh winter in which an increased production of cryoprotectant molecules was needed to survive, assuming that not all of the glycerol is reconverted into glycogen in the spring. Indeed, when exposed to repeated temperature stress, spruce budworms (*Choristoneura fumiferana*) were found to increase their investment in cryoprotectants for stress resistance at the expense of their glycogen reserves during diapause (Marshall and Sinclair, 2014). The reduction in glycogen

content reduced subsequent glycerol mobilization, which resulted in higher mortality when insects were faced with subsequent cold spells (Marshall and Sinclair, 2014).

Energy utilization and storage primarily occur in the insect's fat bodies. The fat body also synthesizes most of the circulating metabolites (Vesterlund et al., 2014). Warmer temperatures increase metabolic rate and deplete energy resources quickly. The rate of depletion of a particular energy reserve varies throughout the course of diapause. Respiratory quotients measure the ratio of the volume of CO_2 to O_2, and allow researchers to determine the primary fuel being used at a specific time because different fuels produce different ratios of CO_2 to O_2 as they are metabolized. Yocum et al. (2005) measured respiratory quotients (RQ) in diapausing leaf-cutter bee larvae (*Megachile rotundata*) and found that early in diapause, the RQ was near 0.7, suggesting lipids were the primary source of energy. There was then a shift to the use of amino acids, glycogen, or a mix, later on in diapause (RQ from 0.8 to 1.0).

The main storage reserves of lipids are triacylglycerols (TAGs) located in the insect fat body. The size of the stored lipid reserves is considered vital in mitigating the metabolic demands that accompany diapause (Hahn and Denlinger, 2011). TAG fat stores are the most vital energy reserves in diapausing insects due to their high calorie content. As such, TAG stores often account for 80% to 95% of total lipids content in insects (Hahn and Denlinger, 2011). Interestingly, there is evidence from several species that even when feeding on the same diet, TAG stores of individuals undergoing diapause contain more unsaturated fatty acid reserves than non-diapausing individuals (Hahn and Denlinger, 2011). The higher level of unsaturation may be important for fat mobilization at cold temperatures since TAG stores in intracellular lipid droplets can only be mobilized when they are in a liquid form and when the droplet surface proteins are able to interact

correctly with lipolytic enzymes (Ohtsu et al., 1993). If diapause is prolonged or if metabolic demands increase as a result of repeated freeze-thaw cycles, lipid reserves are likely to be depleted.

Changing Winter Climates and Overwintering Insects

As noted above, warming winters are likely to increase the frequency of cold exposure or the frequency of freeze-thaw cycles for some overwintering organisms. The frequency of cold exposure is an important, yet frequently neglected parameter in studies of insect overwintering. Marshall and Sinclair (2014) found that the long-term survival of spruce budworm (*Choristoneura fumiferana*) was most strongly affected by the number of low-temperature stress events rather than the intensity or the duration of cold. They also noted that the timing of stressful events was significant. Larvae given a single cold exposure in March had much lower rates of survival than larvae exposed to a single cold exposure of the same duration in January. This suggests that if cold events (such as spring frosts) occur later than usual, relative to the insect's development, insects could experience reduced survival. This might be due to the decrease in the intensity of an insect's diapause as the season progresses. Budworms in March were presumably more metabolically active than budworms in January (Williams et al., 2012a).

Oxidative stress is another potential cost that may occur as a result of prolonged cold exposure. Lalouette et al. (2011) found that in darkling beetles (*Alphitobius diaperinus*), cold stress caused oxidative damage, and a warm recovery period helped them survive by activating their antioxidant system, allowing for repairs of the cold-induced damage. There may, however, be a metabolic cost to the repair phase during shifting thermal regimes (Lalouette et al., 2011; Monaghan et al., 2009). An increase in metabolism after cold exposure has been reported in a number of insects, suggesting that insects may need periods of repair in order to counteract damage due to cold temperatures (Joanisse and Storey, 1998; Lalouette et al., 2011). This period of reparation is useful, in that it may help an insect survive, though it is costly to increase metabolic

rate as well as immune function in order to repair damage as a result of cold injuries. Figure 1 provides a flow chart outlining the various drivers of winter climate change, their interactions and the possible effects on insects.

Temperature is well-known to affect metabolic rate: higher temperatures yield higher rates of energy use (Sinclair, 2014). While overwintering, metabolism decreases and yet remains responsive to temperature (Vesterlund et al., 2014). For example, bumblebee (*Bombus* spp.) queens that overwinter in warm conditions remain more active than those in colder conditions and thus utilize their energy reserves more quickly (Alford, 1978). Warm winter temperatures can thus deplete insects' fat bodies and increase weight loss, both of which compromise insects' post-diapause performance. Fründ et al. (2013) found that bees overwintered at higher temperatures (9.5°C) lost more weight than bees overwintered at lower temperatures (1.5°C). In general insects in diapause reared at lower temperatures show decreased respiration and a slower rate of reserve depletion compared to insects wintering at warmer temperatures (Williams et al., 2012a). Because body mass is positively related to fecundity in numerous insect species (Berger et al., 2008; Honěk, 1993; Knapp and Uhnava, 2014), warm winter temperatures may also reduce fecundity (Williams et al., 2014). For example, Bradshaw et al. (1998) found reduced fecundity in female pitcher-plant mosquitos (*Wyeomyia smithii*) as a result of reduced mass after stressful overwintering conditions.

Other factors that accompany diapause could also affect insect survival and subsequent reproduction. In order to reduce energy costs, unnecessary tissues such as flight or gut muscles may be allowed to atrophy (Hahn and Denlinger, 2011). If muscles were atrophied prior to diapause and then not restored due to low energy reserves, the insect's post-diapause fitness would be negatively affected. Additionally, the life stage of the insect when it enters diapause may be an important determinant of metabolic expenditures during diapause. For example, bees in the genus

Megachile overwinter as larvae whereas bees in the genus *Osmia* normally overwinter as adults. *Megachile* minimize their respiration during the winter, but *Osmia* appear to have a less intensive diapause and actually increase their respiration as the end of the winter approaches (Kemp et al., 2004). Parsivoltinism, also known as cohort-splitting (David and Geoffroy, 2011), is the coexistence of both univoltine and semivoltine individuals within a population (Sgolastra et al., 2012; Torchio and Tepedino, 1982). If *Osmia* bees display parsivoltinism, as has been found in some regions (Forrest and Thomson, 2011; Torchio and Tepedino, 1982), then the life stage (larva or adult) in which they overwinter could be a factor in determining energy expenditure during diapause.

Overwintering in Mason Bees

Bees (Hymenoptera: Apoidea) are a diverse taxon of over 20,000 species (Hedtke et al., 2013). Several solitary cavity-nesting bee species in the genus *Osmia* (Megachilidae) are colloquially called mason bees because of the mud walls they construct in their nests, which consist of a linear series of nest cells in pre-existing holes. Female *Osmia* bees provision each egg they lay with a mass of pollen and nectar that is eaten by the larva upon hatching. This will supply the young bee with enough energy to complete development and survive the winter. Pollen supplies bees with the proteins, lipids, and vitamins required for development and reproduction, while nectar is the main supply of carbohydrates (Nicholson, 2011; Roulston and Cane, 2000). Adult *Osmia* emerge from their cocoons in the spring, ready to feed and mate.

Mason bees are important pollinators in both natural and agricultural systems (Bosch and Kemp, 2001). Some large-scale almond operations, for example, have been maintaining populations of *Osmia lignaria* (blue orchard bees) as pollinators for years (e.g. Paramount Farming, California, USA). Blue orchard bees are generalist pollinators that are active in the springtime (April to June) and contribute synergistically with other bee species, including

bumble bees and honey bees, to the success of crop pollination (Brittain et al., 2013; Vicens and Bosch, 2000). These studies show that mason bees are a promising option for augmenting or replacing honey bees on important agricultural crops. Mason bees may become more important for agricultural pollination as availability of social bees, which are the current primary choice for agricultural crop pollination, becomes less reliable.

Bees adopt one of two overwintering strategies: they either stay active all winter long, albeit at a lower intensity, like honey bees (*Apis* spp.), or they enter diapause. Honey bees are active throughout the entire winter, but remain in their hives. They eat and work all winter, which requires a large cache of stored food prior to the arrival of lower winter temperatures (Burlew, 2010). In contrast, bumble bees (*Bombus* spp.) do not maintain their colonies throughout the winter. Near the end of the summer, the last brood will primarily be made up of reproductive bees (queens and males). The queens will mate and then disperse, finding a hole in the ground in which they can overwinter alone until the spring (Burlew, 2010). The strategy that is used by solitary bees of the genera *Osmia* and *Megachile* (Megachilidae) is to enter diapause.

Bees in the genus *Osmia* have an obligate diapause each winter. They typically enter diapause as adults in their cocoons, and emerge in the spring to reproduce. Some *Osmia* species (*O. cornuta* and *O. rufa*) are known to be freeze-avoidant and can survive temperatures as low as −30°C (Krunić and Stanisavljević, 2006). Like many insects (Bradshaw and Holzapfel, 2010), mason bees require a sufficient exposure to cold temperatures in order to successfully complete their diapause and survive the winter (Bosch and Kemp, 2001; Sgolastra et al., 2010). Without sufficient cold, *Osmia* spp. will extend their diapause, resulting in the possible depletion of energy reserves prior to emergence (Bosch and Kemp, 2004; Sgolastra et al., 2011). Sgolastra et al. (2010) found that *O. lignaria* bees that were not chilled, or were only chilled for a brief period in the fall,

responded to warm temperatures in the spring by depressing respiration and never reached respiration levels high enough to prompt emergence. Bees that were chilled for the longest period of time (≥196 days) over winter lost 10% of their weight, while bees that were chilled for the shortest period of time (28 days) lost 29% of their weight. In addition, bees overwintered at warmer temperatures (7°C or 22°C) lost significantly more mass than bees overwintered at colder temperatures (0°C or 4°C).

Accumulated degree days and ambient temperatures in the springtime can be used to estimate insect emergence date (Broatch et al., 2006; Forrest and Thomson, 2011) and possibly predict the termination of diapause, though the mechanisms of diapause completion remain incompletely understood. Researchers can recognize the termination of diapause in some insects (e.g. *Bombyx mori*) by measuring enzymes that are associated with the production of cryoprotectants during diapause, though the application to other insects may not be consistent (Kihara et al., 2009; Storey and Storey, 2012). Sgolastra et al. (2010) proposed two models to characterize diapause in *O. lignaria*. According to the first model, *O. lignaria* has a mid-winter diapause termination followed by a post-diapause quiescence, in which the bees remain dormant until exposed to temperatures high enough to elicit emergence. The second model proposes that *O. lignaria* has the potential to terminate diapause by mid-winter, but that further diapause is required to elicit rapid emergence from post-diapause quiescence. More detailed study will be needed to determine which model best explains the completion of diapause for mason bees. Regardless, diapause termination in all mason bees that have been studied requires a period of cold temperatures followed by a period of warmth.

Hypotheses and Predictions

I hypothesized that warmer and more variable spring temperatures would negatively affect the fitness of *Osmia* spp. bees, through the depletion of fuel reserves and the acceleration of mass loss. I conducted two sets of experiments to test these ideas.

Season 1

In the first set of experiments, my research aimed to study how fluctuating thermal regimes affected fuel reserves in solitary mason bees (*Osmia* spp.) by quantifying their lipids, free sugars, and glycogen reserves after experimental temperature treatments during early spring.

I expected that treatments with more variable, fluctuating temperatures would deplete lipid reserves and thereby increase mass loss. I also expected that treatments with a warmer average temperature would deplete lipid reserves and increase mass loss compared to treatments with a lower average temperature. I expected no change in the concentration of free sugars, since these would be replenished from stored glycogen. I expected that both warmer and more variable treatments would affect glycogen, though I was unsure of the directionality of the change. Glycogen is used to replenish free sugars, which could lower glycogen reserves in warmer and/or more variable treatments. Glycogen is also used for the production of the cryoprotectant molecule glycerol, so glycogen concentrations could be lower in colder treatments. However, observing a reduction in glycogen due to glycerol production would require measuring glycogen concentrations during cold periods since glycerol is reconverted into glycogen as temperatures increase (Marshall and Sinclair, 2014).

Season 2

Based on the first season's results, I aimed to determine how warmer spring temperatures would affect the fitness of mason bees, using mass loss and time to recover from chill coma as fitness proxies. I measured these variables, as well as emergence time, in bees that had been

exposed to one of two treatments: a warmer-than-average spring temperature treatment or an average spring temperature treatment. Historical spring temperatures are presented in Table 1. Based on the results of the first season, I expected that warmer temperatures would increase mass loss as a result of higher metabolic rates (Figure 2). I measured the chill coma recovery time upon emergence as an indication of overall bee performance (MacMillan and Sinclair, 2011). Rewarming requires the consumption of energy (MacMillan et al., 2012a; Sinclair, 2014) though it is unclear which fuel reserves are used (MacMillan et al., 2012a, 2009). Thus, I predicted that the warmer temperature treatment would result in bees having a slower recovery time from chill coma, if warmer temperatures resulted in a net increase in energy expenditure prior to emergence, and if greater energy stores allow more rapid warming. *Osmia* spp. are likely to experience cold spells after emergence in nature, since they emerge so early in the springtime; thus, ability to recover quickly from chill coma is an ecologically relevant measure of condition.

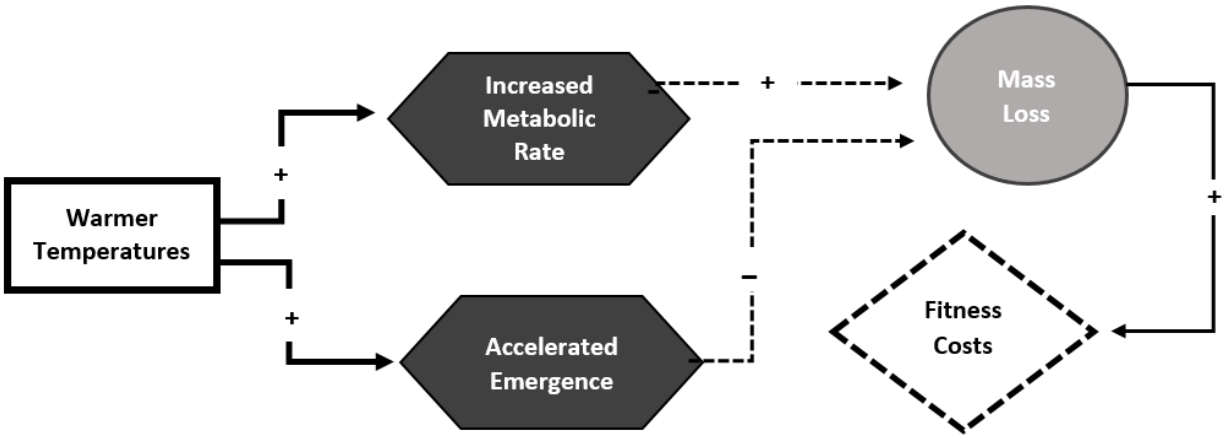

Figure 2— Proposed mechanism of the net effect of warmer temperatures on mass loss and fitness. Warmer temperatures increase metabolic rate and accelerate emergence, which in turn affect mass loss in opposite ways. Mass loss then results in increased fitness costs.

Methods

Study Organisms

The study organisms used in my experiments were solitary bees in the genus *Osmia*. Bees were obtained from artificial nesting structures established in the Ottawa–Kingston Region in 2013 and 2014. In 2013, sites were located at the National Capital Commission Mer Bleue Conservation Area, Bruce Pit, and Conroy Pit. In 2014, sites were located at the Mer Bleue Conservation Area and Queens University Biological Station. Bees were allowed to construct nests in the nesting structures during the spring. Nests were collected in the fall and brought to the laboratory. The bees were left in their cocoons and overwintered at 4°C in an incubator (Nor-lake, Model #LRI201WWW/0, Hudson, WI, USA). Early the following spring, the nests were dissected and individual cocoons were put into clear gelatin capsules for observation.

Table 1—Average and extreme daily minimum and maximum temperatures from 1981–2015 during the spring months (March–May) in Ottawa, ON. Based on data from Environment Canada (http://climate.weather.gc.ca/climate_normals and http://climate.weather.gc.ca/climateData/dailydata_e.html).

YEAR	**1981-2010**	**1981-2010**	**1981-2010**	**2012-2015**	**2012-2015**	**2012-2015**
MONTH	**March**	**April**	**May**	**March**	**April**	**May**
AVG MAX	2.5	11.6	19.0	2.3	11.5	21.9
AVG MIN	-6.9	1.0	7.5	-7.9	-0.03	8.3
EXTREME MAX (°C)	26.7	31.1	35.8	12.9	25.4	31.4
EXTREME MIN (°C)	-30.6	-16.7	-5.6	-20.5	-7.3	-0.6
DAYS WITH MAX TEMP >10°C	3.5	17	29.9	--	--	--
DAYS WITH MIN TEMPS < -10°C	9.2	0.37	0.0	--	--	--

Part 1: Energetic costs of repeated cold exposure

In early spring (March-May 2014), bees were exposed to six different fluctuating temperature regimes (Table 2). The bees were assigned alternately to treatment groups so each species and sex was represented in each treatment. The taxa included were *Osmia lignaria, O. albiventris* and *Megachile* spp., as these were the most abundant occupants of the nesting structures; only the *Osmia* spp. are considered further in this thesis. After the experimental treatments, bees were either released into a flight arena in order to quantify pollination efficiency or were used to quantify remaining energy reserves.

Experiment

I used five different incubator treatments involving repeated exposure to cold temperatures (−10°C) and interim exposure to warm temperatures (10°C). These were used to simulate fluctuating spring temperatures that are slightly more extreme than those observed locally in nature (Table 1). This was done in order to simulate what might occur as climate change progresses, potentially bringing about warmer temperatures, increased temperature variability, and increasing exposure to freeze-thaw cycles (IPCC, 2014).

I used two incubators for this experiment. The first incubator (VWR, Model #2015 2015-2, Mississauga, ON, Canada) was set at −10°C and the second (Nor-lake, Model #LRI201WWW/0, Hudson, WI, USA) was set at +10°C. Treatments were moved back and forth between the incubators to simulate fluctuating temperatures; thus, each bee experienced both incubator environments, albeit for different periods of time.

Table 2—Experimental treatments, methods, and observed temperature minima, maxima, and daily mean for Season 1. RCE=Repeated Cold Exposure. TRT=Treatment. Treatments occurred from 25 March 2014 to 23 April 2014.

TRT	TOTAL COLD DURATION	TOTAL WARM DURATION	# RCE CYCLES	METHOD	OBSERVED TEMP MAX (°C)	OBSERVED TEMP MIN (°C)	OBSERVED DAILY MEAN TEMP (°C)
C	20 days	8 days	0	20 days at -10°C, 8 days at 10°C	11.4	-9.5	-3.3
CW4	20 days	8 days	4	[5 days at -10°C, 2 days at 10°C] repeated x4	11.4	-9.5	-3.3
CW2	10 days	18 days	2	[5 days at -10°C, 2 days at 10°C] repeated x2 then 14 days at 10°C	11.4	-9.5	4.0
CW1	5 days	23 days	1	[5 days at -10°C, 2 days at 10°C] then 21 days at 10°C	11.4	-9.5	7.6
W	0 days	28 days	0	28 days at 10°C	11.4	10.6	11.2
FIELD (FLD)	28 days		NA	Uninsulated nest blocks in the field. Allowed for natural outdoor temperature variability.	27.4	-17.5	7.0

A sixth (field) treatment was used to provide a point of comparison for the laboratory treatments. Three replicate nest blocks were placed on tree trunks at the Mer Bleue site. Two HOBO data loggers (Model #UA-002-64, Onset Corp., Bourne, MA, USA), attached to different nest blocks, were used to log the temperature hourly. This treatment (FLD) served to represent bees in a natural environment experiencing natural temperature conditions.

Response Variables

Fuel Reserves

All bees experienced the full 28 days of treatment, regardless of whether they emerged or not, and then were immediately weighed and stored at −80°C until analysed. I separated the bees into mesosoma ("thorax"), including the wings and legs, and metasoma ("abdomen"), and then extracted the fuel reserves using the sulpho-phospho-vanillin method, outlined below. Optical density of the sample was measured with a "Synergy 2" spectrophotometer (Biotek, Winooski, VT, USA) in a 96-well microplate (Corning Inc., USA, 3368). I then used the Beer-Lambert law to determine the amount of each fuel reserve in both the thorax and the abdomen of each bee in reference to a standard curve. If samples fell outside of the standard curve, they were excluded from analysis as the standard curve could not accurately predict the value. For this reason, sample sizes sometimes differed between the analyzed fuels.

Following the protocol of Kaufmann (2014), adapted from Van Handel (1985a, 1985b), I extracted the lipids, glycogen, and free sugars from the bees that had undergone my experimental temperature treatments. To isolate each fuel reserve (lipids, free sugars, and glycogen), separated thoraxes and abdomens of adult bees were placed individually into a glass homogenizing mortar with 0.2 mL of sodium sulfate solution (2%, dissolved in water). Once there were no identifiable parts remaining, the extract was transferred to a centrifuge tube and the glass rod was washed into

the tube with two x 0.8 mL of chloroform/methanol solution (1:1, v/v). The extract was centrifuged for 1 minute at 3000 rpm. The supernatant, containing sugars and lipids, was transferred into a clean centrifuge tube. The pellet was retained for glycogen analysis. Then 0.6 mL DI water was added to the supernatant and mixed. The extract was centrifuged again for 1 minute at 3000 rpm. I separated the top fraction (water/methanol) for sugar analysis and the bottom portion (chloroform) was set aside for lipids analysis.

Reagents and standards: Vanillin-phosphoric acid reagent was made by dissolving 600 mg vanillin in 100 mL DI hot water and adding 400 mL 85% phosphoric acid. The mixture was stored at room temperature in the dark. Anthrone reagent was made by adding 385 mL sulfuric acid (95–98%) to 150 mL DI water. Then 750 mg anthrone was dissolved in the sulfuric acid and the mixture stored at 4°C. Lipid standards were made in triplicate using a standard solution of 100 mg per 100 mL soybean oil in chloroform. Free sugar and glycogen standards were made in triplicate using a standard solution of 100 mg per 100 mL anhydrous glucose in deionized water. All standards were made anew each day.

Lipids analysis: The portion of extract for lipid analysis was placed in a tube in a hot water bath at 90°C–110°C to evaporate the solvent. 0.2 mL sulfuric acid was added to each tube and heated for 10 minutes in the hot water bath. Vanillin reagent was added to the 5 mL level and mixed. The tubes were removed from the hot water bath to cool and allow the reddish colour to develop. Within the next 30 min, optical density was determined at 625 nm. Using the Beer-Lambert law and the standard curve, the µg of lipid per mg fresh mass was calculated.

Free sugar and glycogen analysis: The portions of extract for free sugar and glycogen analysis were placed in separate tubes in a hot water bath to evaporate the solvent. Anthrone reagent was then added to bring the solution up to 5 mL and mixed. The tubes were heated for 17

minutes at 90°C–110°C then removed from the hot water bath to cool. The optical density was determined at 625 nm. Using the Beer-Lambert law and the standard curve, the µg of sugars per mg fresh mass was calculated.

Mass Percent Change:

Mass percent change was calculated as the pre-treatment fresh bee mass minus the post-treatment fresh bee mass, divided by the pre-treatment mass and multiplied by 100%.

Pollination Effectiveness:

In March 2014, I planted and grew canola (Brassica napus) plants in the Centre for Advanced Research in Environmental Genomics (CAREG) greenhouse to assay the pollination effectiveness of experimentally treated bees. I chose these as experimental plants because they are agriculturally and economically relevant in Canada, and they are known to be pollinated by solitary bees in the family Megachilidae (Woodcock et al., 2013). Once the plants were flowering, they were moved into the Department of Psychology's bee room in the Vanier building, with the collaboration of Dr. Catherine Plowright. Bees were given a pollen source consisting of cut branches of pussy willow (Salix sp.; abundant and flowering in early spring), as well as flowering canola plants, and a honey-water mixture simulating a nectar supply (30% solution (300 g sugar, 10 g honey in distilled water to 1 L)). Upon emergence, bees (O. lignaria, female N=7; male N=8) were weighed and then released into the bee room next to vials of "nectar" in Eppendorf tubes with a surrounding yellow paper "corolla". Indoor lighting was on an 8 hour light: 16 hour dark program. Unfortunately, this project yielded little usable data since the bees were not cooperative in the bee room. Even though the windows were fully covered with black garbage bags to block out natural light, the bees flew to the windows and remained there, ignoring the food sources.

Some pollination/foraging was observed, but not on the scale needed to make any conclusions regarding pollination.

Part 2: Fitness costs of fluctuating temperatures

Experiment

Because the first season's experiments suggested exposure to warm temperatures had larger effects on bees than exposure to variable temperatures, I conducted a second laboratory experiment to simulate warmer-than-average temperatures in early spring. Unlike in the first season's experiments, in which all treatment durations were the same, treatments in the second season's experiment ended for each bee when it emerged from its cocoon. Thus, the second season's experiment simulates a situation in which different temperature regimes can impose different energetic costs, while simultaneously altering the duration of dormancy and therefore the duration of exposure to those temperature regimes. Bees (N=130) were assigned alternately to two laboratory treatment groups in order to have representatives of each species and sex in each treatment. The species included were *O. lignaria* and *O. albiventris*. Voucher specimens will be deposited at the Canadian National Collection of Insects (CNC) and in the Forrest lab bee collection.

Treatments are described in Table 3. For realism, both treatments cycled from a daytime (06:00-18:00) warm temperature (either 10°C or 20°C) to 0°C at night (18:00-06:00) to simulate daily temperature fluctuation. Each treatment was in its own incubator in the dark. Specimens were placed in Styrofoam coolers to slow the ramping times. The maximum ramping rates within the coolers for the warm spring treatment were −0.97°/min and 0.94°/min. The maximum ramping rates for the cold spring treatment were −0.32°/min and 0.63°/min. The experimental treatments continued for a maximum of 3 weeks from 15 April until 8 May 2015, during which time

specimens were checked daily for emergence. On 8 May, temperatures were increased to 20°C/10°C day/night to promote emergence, and remained at that temperature until 15 May, at which point the experiment was concluded. Emerged bees were processed, within 24 hrs of emergence, as described below. On 15 May, four weeks from the start of the experiment, bees were placed at room temperature (23°C –25°C) in the light to allow any remaining bees to emerge.

Table 3—Experimental treatments for Season 2. Mean daily temperatures were calculated from data recorded by HOBO loggers. TRT=Treatment. TEMP=Temperature.

TRT	DAILY HIGH SET TEMP	DAILY LOW SET TEMP	MEAN DAILY HIGH ACTUAL TEMP	MEAN DAILY LOW ACTUAL TEMP	INCUBATOR SPECIFICATIONS
WARM SPRING	20°C	0°C	20.5°C	0.5°C	VWR, 2015 2015-2, USA
COLD SPRING	10°C	0°C	13.4°C	0.5°C	Norlake, LRI201WWW/0, USA

Response Variables

For each bee, I recorded pre-treatment mass of the cocoon with bee inside, post-treatment masses of the cocoon and the bee separately, the date of emergence, and time to recover from chill coma (seconds). Mass percent change was calculated as described previously. Any bees that did not emerge were weighed at the end of the experiment, and included in the analysis. Emergence date was recorded as the date that the bee fully emerged from its cocoon.

Each bee's time to recover from chill coma was measured as a proxy for overall bee energy reserves and as a possible indicator of future performance. On the day that the bees emerged from their cocoons, they were weighed, and then placed in a glass jar with a HOBO temperature logger. The jar was sealed and then placed in a Styrofoam cooler full of ice water, for 3 hours, to induce chill coma. After 3 hours, the bee was removed from the jar and placed on its dorsal surface. The timer was started when the jar lid was opened. The time it took for the bee to flip onto its ventral surface was measured and recorded as the chill coma recovery time (in seconds). Any bee that did not flip over within 15 minutes has a recorded value of 900 seconds.

Statistical Methods

For the fuel reserve and mass data from Season 1, thorax and abdomen values were summed for analysis. Data were log-transformed (glycogen, lipids, and free sugar concentrations) or square-root-transformed (mass percent change) to normalize the distribution of the data. Data were subsequently analyzed using linear mixed models (LMM) in 'R' (Bates et al., 2015; Lenth and Herva, 2015; R Core Team, 2015). The random factors for each model were Site (location of bee collection) and Nest (nested within Site). The fixed factors were Treatment (6 levels, including the 5 laboratory treatments and 1 field treatment), Species, and the interaction of Treatment and Species. The model with the lowest AIC value was chosen as the best model (Table 4). If the AIC

values for two models had a difference of less than 2, the model with fewer parameters was chosen for its simplicity. Post-hoc pairwise comparisons were computed based on the best LMM in R (Lenth and Herva, 2015) and are presented in each figure. To account for the large number of separate tests (four different response variables, i.e. three different energy reserves and mass loss), I used a conservative Bonferroni adjustment to the alpha value: p-values were considered significant if they were <0.0125 (=0.05/4). A p-value between 0.0125 and 0.05 was considered marginally significant.

For Season 2, emergence dates did not require transformation. Chill coma recovery and mass percent change data were square-root-transformed to normalize their distributions. Only one bee had a chill coma recovery time of 900 seconds (the maximum recorded). Although this was an outlier, it had no significant effect on the analysis when removed, so analyses were run with the outlier included for chill coma recovery time. Data were analyzed using LMM in 'R', as above. There were no data for *O. albiventris* bees in the Cold Spring Treatment for recovery from chill coma since no bees emerged before the end of the experiment (15 May 2015). There were data on mass percent change for these bees since I weighed them after the experimental treatments despite non-emergence and death.

Table 4—Linear mixed models for response variables from Season 1. Spp = species. Trt = treatment. MPC = mass percent change. Sqrt = square root. The best model for each variable is displayed based on the lowest AIC value and fewest parameters.

VARIABLE (UNITS)	DF	FIXED FACTORS	RANDOM FACTORS
GLYCOGEN (µg/mg fresh mass)	15	Log(glycogen) ~ Trt + Trt:Spp + Spp	
LIPIDS (µg/mg fresh mass)	15	Log(lipids) ~ Trt + Trt:Spp + Spp	+ (1|Site/Nest)
FREE SUGARS (µg/mg fresh mass)	15	Log(free sugars) ~ Trt + Trt:Spp	
MASS PERCENT CHANGE (%)	14	Sqrt(MPC) ~ Trt + Spp + Trt:Spp	+ (1|Nest)

Results

Part 1: Energetic costs of repeated cold exposure

Fuel Reserves

There was a significant main effect of treatment on glycogen concentrations, with bees from the coldest treatments (C, CW4) containing more glycogen than bees from the warm, variable treatments (CW2, CW1) (Table 5, Figure 3). There was also a significant species by treatment interaction: For *O. lignaria,* the two variable, but primarily warm treatments (CW2 and CW1) had significantly lower concentrations of glycogen than the other three laboratory treatments (C, CW4, W). The warm treatment (W) had more than three times as much glycogen and the primarily cold treatments (C and CW4) had concentrations of glycogen approximately eleven times greater than those in the warm, variable treatments. For *O. albiventris*, treatment effects were less clear, although bees in treatment CW1 had 34% higher concentrations of glycogen than those in

treatment W (Figure 3). In general, *O. albiventris* had lower concentrations of glycogen than *O. lignaria*, but this difference was not significant (Table 5). Similarly, there was a significant main effect of treatment on lipids, with the more fluctuating treatments resulting in lower concentrations of lipids (Table 5, Figure 4). There was a significant species by treatment interaction: For *O. lignaria*, the colder treatments C and CW4 had more than twice the concentration of lipids than treatment CW2. Also for *O. lignaria*, the three treatments with the most time spent warm (CW2, CW1, W) had significantly lower concentrations of lipids than the natural field treatment (FLD), despite all four treatments having similar mean temperatures. For *O. albiventris*, there were no significant differences between treatments.

There was a significant main effect of treatment on the concentration of free sugars, with warmer treatments resulting in lower concentrations of free sugars (Table 5, Figure 5). The two species differed marginally in their free-sugar responses to the treatments (species x treatment interaction, $p<0.02$): For *O. lignaria*, bees in the primarily cold treatment CW4 had significantly higher concentrations of free sugars than those in treatment W. For *O. albiventris*, bees in the variable and primarily warm treatment (CW1) had significantly lower concentrations of free sugars than those in the colder treatments C and CW4 or the field treatment (FLD).

Mass Percent Change

There was a significant main effect of treatment on mass loss with warmer treatments resulting in more mass loss than colder treatments (Table 5, Figure 6). There was also a significant interaction of treatment and species (Table 5): For *O. lignaria* mass loss increased with the time spent in warm conditions (C → W). Most notably, there was substantially greater mass loss in treatment W (28 days warm, no cold treatment) than the other treatments; even 5 days of cold exposure (CW1) significantly reduced mass loss (Table 6). Bees in the most variable treatment

(CW4) lost less mass than those in treatment C, even though these two treatments had the same number of warm days and the same mean temperature. For *O. lignaria,* bees in the warm treatment (W) lost more than twice as much mass as those in the field treatment (FLD) (Table 6, Figure 6). For *O. albiventris*, all of the laboratory treatments caused significantly more mass loss than the field treatment (FLD; Table 6, Figure 6).

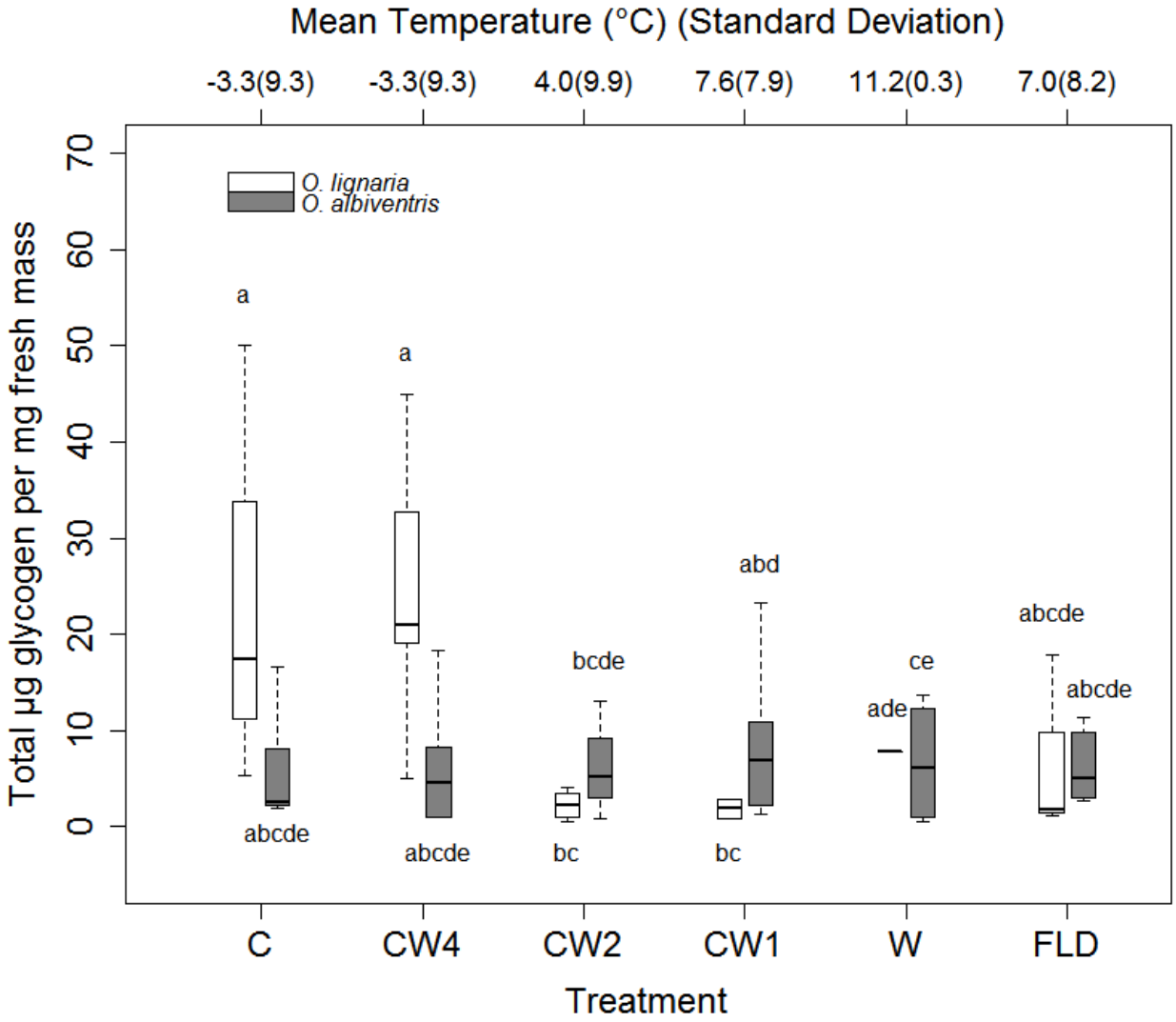

Figure 3—Total concentration of glycogen (µg/mg fresh mass) for bees in Season 1 experiment. Sample sizes for each treatment are as follows (*O. lignaria, O. albiventris).* C(4,5), CW4(5,6), CW2(4,3), CW1(2,5), W(2,5), FLD(3,6). Treatments that share letters are not significantly different from each other at $\alpha = 0.05$.

Table 5—Analysis of deviance table (Type II Wald chi-square tests) for fuels and mass loss in *Osmia* spp. bees. P-values in bold-face are considered significant at a Bonferroni-corrected α of 0.0125. χ^2 = chi-square value. DF = Degrees of Freedom.

VARIABLE	GLYCOGEN			LIPIDS			FREE SUGARS			MASS LOSS		
	χ^2	DF	p	χ^2	DF	p	χ^2	DF	p	χ^2	DF	p
TREATMENT	46.0	5	**<0.001**	30.6	5	**<0.001**	26.1	5	**<0.001**	95.4	5	**<0.001**
SPECIES	1.8	1	0.18	0.7	1	0.39	15.1	6	0.02	0.1	1	0.78
SPECIES:TREATMENT	51.8	5	**<0.001**	33.3	5	**<0.001**	NA*			44.0	5	**<0.001**

* Species: Treatment interaction was not part of the best linear mixed model for free sugars.

Table 6—Mean mass loss and standard deviation for each treatment in Season 1. Pairwise comparisons were conducted using the best linear mixed model on square-root-transformed data. *Treatments are not significantly different if they share a letter.

Species	Treatment	Mean Mass Loss (%)	Standard Deviation	Pairwise Comparisons*
O. lignaria	**C**	16.68	7.66	ab
	CW4	9.77	3.13	ad
	CW2	22.94	11.66	bc
	CW1	25.80	12.12	ab
	W	44.52	8.91	c
	FLD	19.73	12.15	abd
O. albiventris	**C**	14.68	5.57	abc
	CW4	21.08	8.00	bc
	CW2	17.96	6.22	bc
	CW1	20.11	8.11	bc
	W	17.44	13.98	bc
	FLD	8.77	5.78	d

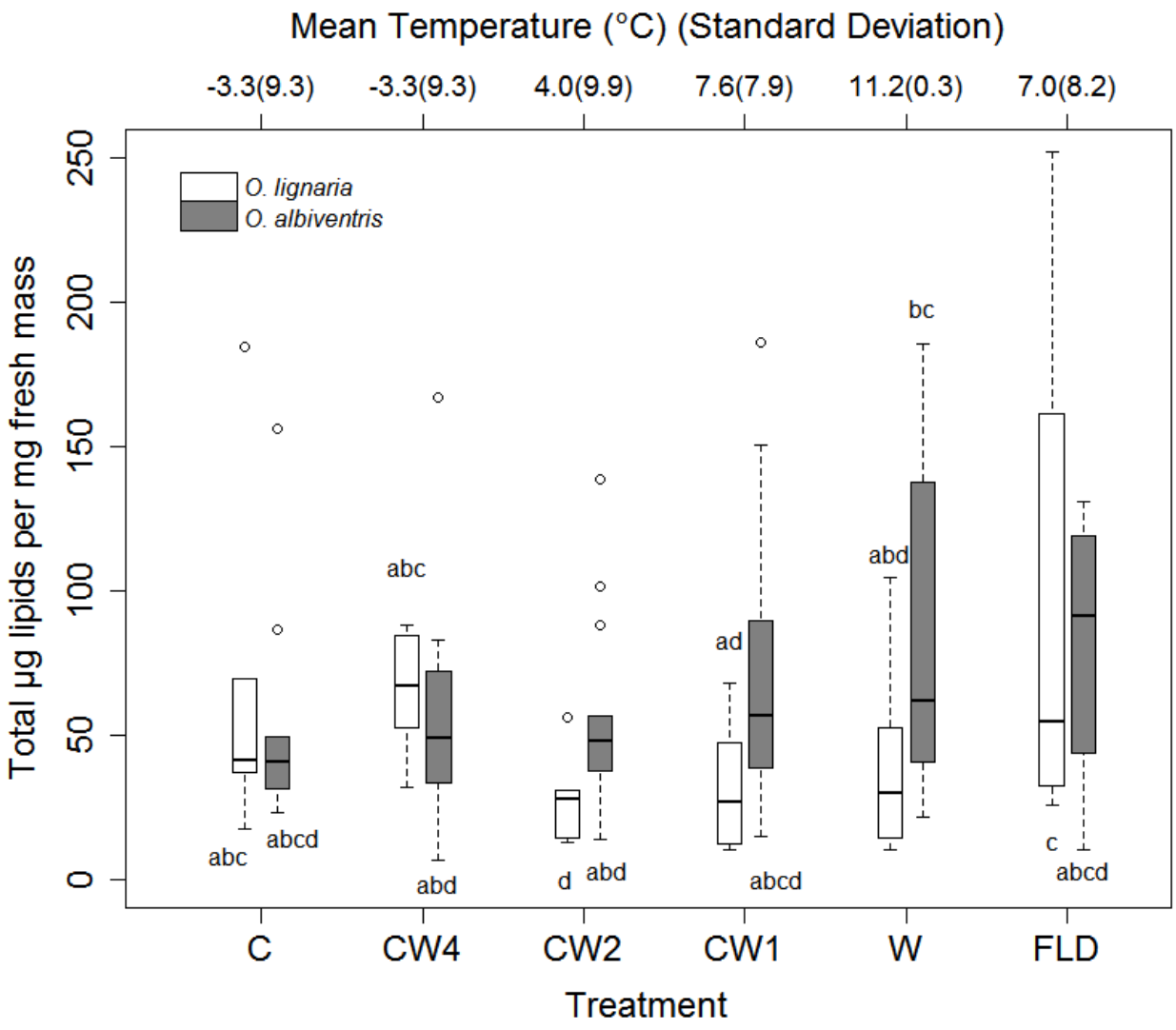

Figure 4—Total concentration of lipids (µg/mg fresh mass) for bees in Season 1 experiment. Sample sizes for each treatment are as follows (*O. lignaria, O. albiventris*): C(6,9), CW4(6,13), CW2(5,13), CW1(5,11), W(6,13), FLD(4,13). Treatments that share letters are not significantly different from each other at $\alpha = 0.05$.

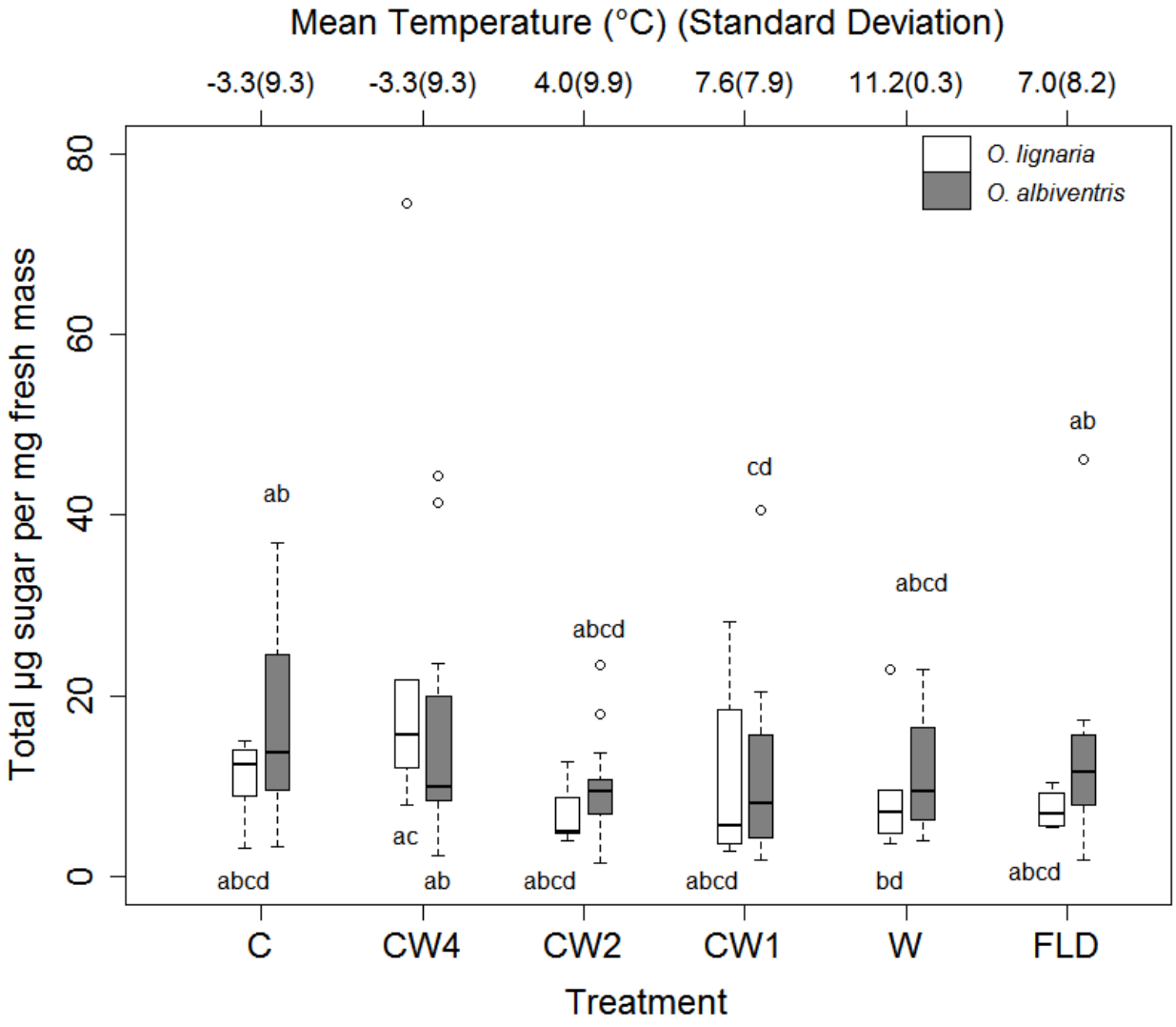

Figure 5—Total concentration of free sugars (µg/mg fresh mass) for bees in Season 1 experiment. Sample sizes for each treatment are as follows (*O. lignaria*, *O. albiventris*):C(5,8), CW4(6,12), CW2(3,12), CW1(2,8), W(3,9), FLD(4,5).

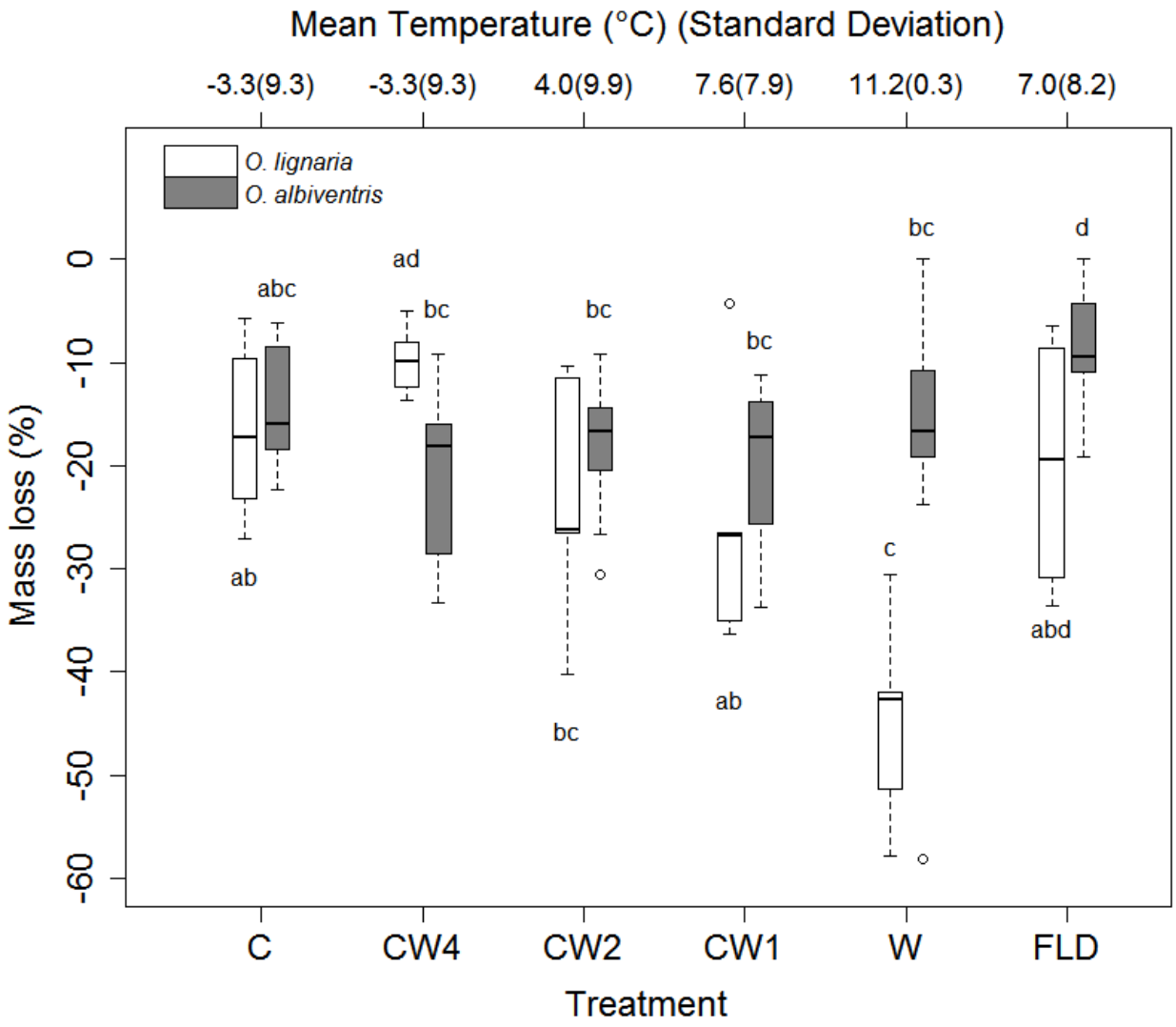

Figure 6—Mass loss (%) of bees in Season 1. Sample sizes for each treatment are as follows (*O. lignaria*, *O. albiventris*): C(6,9), CW4(6,13), CW2(5,13), CW1(5,12), W(6,13), FLD(4,13). Treatments that share letters are not significantly different from each other at $\alpha = 0.05$.

Part 2: Fitness costs of fluctuating temperatures

Emergence

Bees from the warm spring treatment emerged an average of 8.7 days earlier than bees from the cold spring treatment (Table 8, Figure 7). There was also a significant difference in emergence date between species, with *O. lignaria* emerging 6 days earlier, on average, than *O. albiventris* (Figure 7). There was also a significant interaction between treatment and sex: Males emerged an average of 6.6 days earlier than females in the cold spring treatment and an average of only 2.2 days earlier in the warm spring treatments. *O. albiventris* showed a stronger response to treatment than *O. lignaria* (significant treatment x species interaction), albeit with a small sample size. Only two *O. albiventris* males emerged from the cold spring treatment, and no female *O. albiventris* emerged from either treatment, as they died during the course of the experiment.

Mass Percent Change

There was a significant main effect of treatment on mass percent change when the non-emerged dead bees were included in analysis with the cold spring treatment losing more mass than the warm spring treatment (Table 9). Bees in the cold-spring treatment had an average mass loss of 23.6% compared to a mass loss of 15.4% in the warm spring treatment (Table 9, Figure 8). Mass loss also differed significantly between the two species, with *O. albiventris* losing 32.5% and 54.2% of their mass in the warm and cold spring treatments, respectively, compared to *O. lignaria* losing an average of 12.3% and 19.2% of their mass (Table 9, Figure 8). There was no significant difference between sexes, nor were there any significant interaction effects (Table 9). When the non-emerged bees were removed from the analysis, there was no significant effect of treatment or species (Table 9), suggesting that observed treatment effects may have been due to water loss by bees that died before the end of the experiment.

Chill Coma Recovery Time

There was a significant difference in chill coma recovery time between species, with *O. lignaria* demonstrating a faster recovery time than *O. albiventris* (an average of 193.5 seconds faster). There were no significant differences between treatments or between sexes (Table 10; Figure 9).

Table 7—Linear mixed models for response variables from Season 2. Spp = species. Trt = treatment. MPC = mass percent change. Sqrt = square root. ΔAIC calculated as difference from full model with all possible terms and interactions. DF = Degrees of Freedom. The best model for each variable is displayed. The best model for emergence had the same AIC as the full model (ΔAIC=NA), but is simpler, so the simpler model was chosen.

VARIABLE	DF	FIXED FACTORS	RANDOM FACTORS	ΔAIC
EMERGENCE (JULIAN DAYS)	8	Emergence ~ Trt + Sex + Spp + Trt:Sex + Trt:Spp	+ (1|Site)	NA
MASS PERCENT CHANGE (%)	6	Sqrt(MPC) ~ Trt + Spp +Trt:Spp	+ (1|Nest)	4.2
CHILL COMA RECOVERY TIME (SECONDS)	6	Sqrt(Chill Recovery) ~ Trt + Spp +Trt:Spp	+ (1 | Site/Nest)	2.3

Table 8—Analysis of deviance table (Type II Wald chi-square tests) for emergence date in *Osmia* spp. bees. DF=Degrees of freedom. The treatment combinations that were missing were the *O. albiventris* females in both the warm and cold spring treatments. P-values in bold-face are considered significant at α=0.05.

VARIABLE	CHI-SQUARE	DF	P-VALUE
TREATMENT	166.8	1	**<0.001**
SEX	70.0	1	**<0.001**
SPECIES	41.4	1	**<0.001**
TREATMENT:SPECIES	31.0	1	**<0.001**
TREATMENT:SEX	11.5	1	**<0.001**

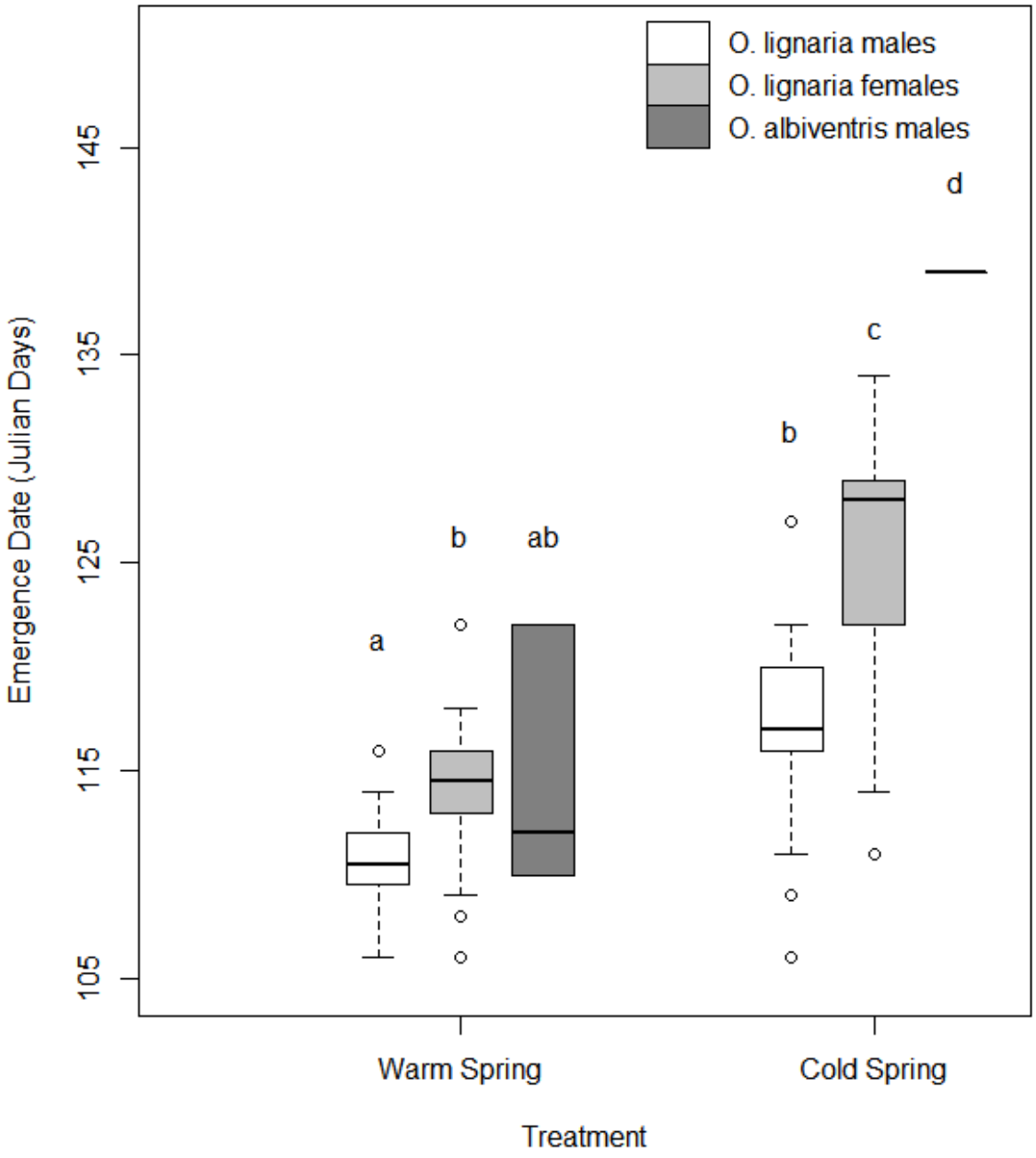

Figure 7—Emergence date (Julian days) for Season 2 experiment. Julian Dates 106 to 145 correspond to the range of 16 April 2015 to 25 May 2015. Experiment started on Julian Date 105. Warm Spring treatment was 20°C/0°C, Cold Spring treatment was 10°C/0°C daily. No *O. albiventris* females emerged. Sample sizes for each treatment are as follows (*O. lignaria* males, *O. lignaria* females, *O. albiventris* males): Warm Spring (24,18,5), Cold Spring (25,15,2). Treatments that share letters are not significantly different from each other at $\alpha = 0.05$.

Table 9—Analysis of Deviance Table (Type II Wald chi-square tests) for mass percent change in *Osmia* spp. bees in Season 2. DF=Degrees of freedom. P-values in bold-face are considered significant at α=0.05. Sample sizes including non-emerged dead bees were (*O. lignaria, O. albiventris*): Warm Spring (50,9), Cold Spring (49,7). Sample sizes excluding non-emerged bees were (*O. lignaria, O. albiventris*): Warm Spring (42,5), Cold Spring (37,0).

VARIABLE	INCLUDING NON-EMERGED BEES			EXCLUDING NON-EMERGED BEES		
	χ^2	DF	p	χ^2	DF	p
TREATMENT	5.7	1	**0.02**	0.14	1	0.71
SPECIES	20.9	1	**<0.001**	0.63	1	0.43
SPECIES:TREATMENT	1.6	1	0.21	**NA***		

*The species x treatment interaction could not be tested because there were no data for *O. albiventris* in the cold spring treatment.

Table 10—Analysis of deviance table (Type II Wald chi-square tests) for chill coma recovery time in *Osmia* spp. bees in Season 2. DF=Degrees of freedom. The treatment × species interaction could not be tested because there were zero degrees of freedom. P-values in bold-face are considered significant at α=0.05.

VARIABLE	CHI-SQUARE	DF	P-VALUE
TREATMENT	0.02	1	0.89
SPECIES	18.8	2	**<0.001**

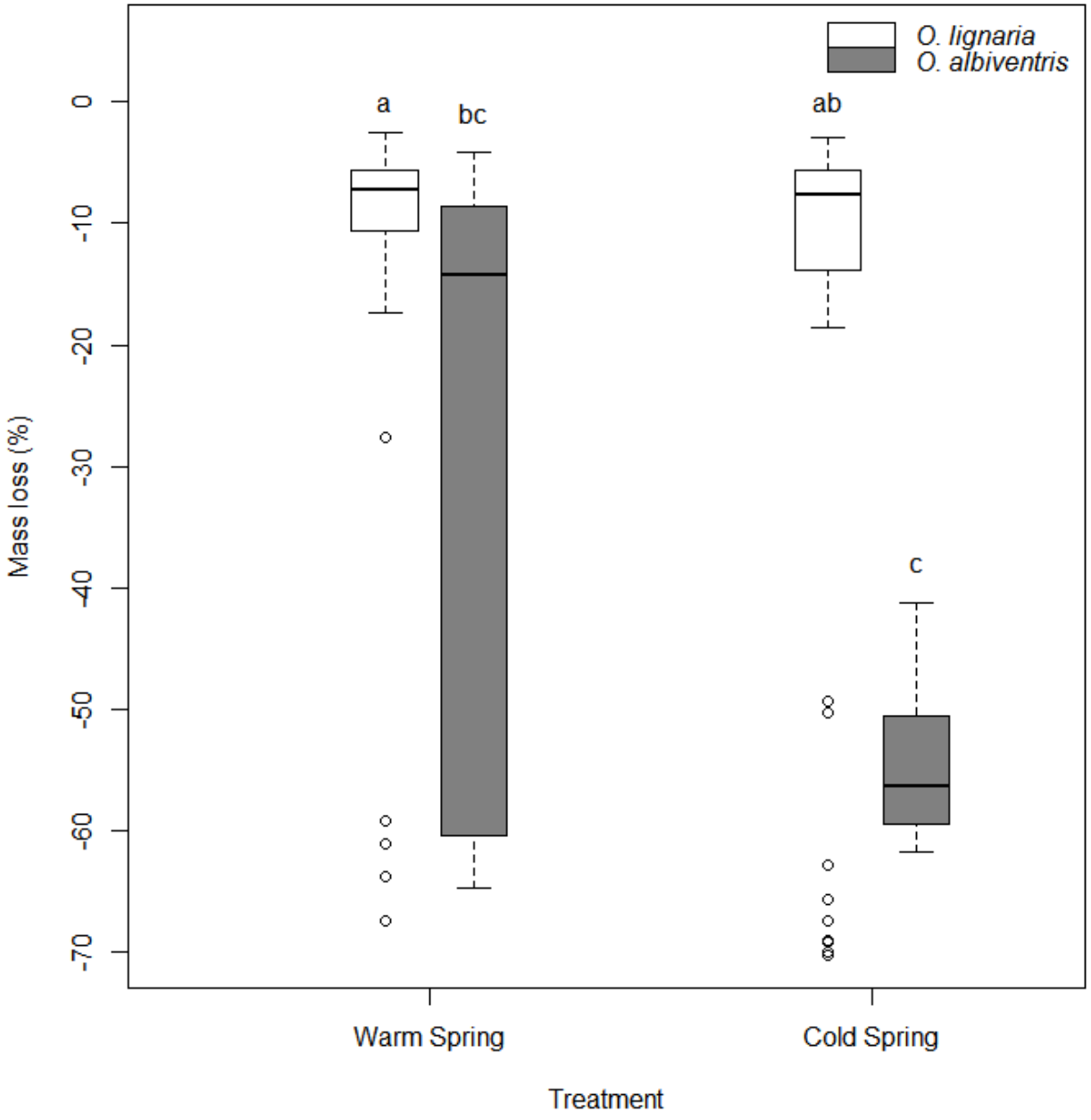

Figure 8—Mass loss (%) of bees in Season 2 experiment including non-emerged bees. Males and females are pooled together. Sample sizes for each treatment are (*O. lignaria*, *O. albiventris*): Warm Spring (50,9), Cold Spring (49,7). Treatments that share letters are not significantly different from each other at $\alpha = 0.05$.

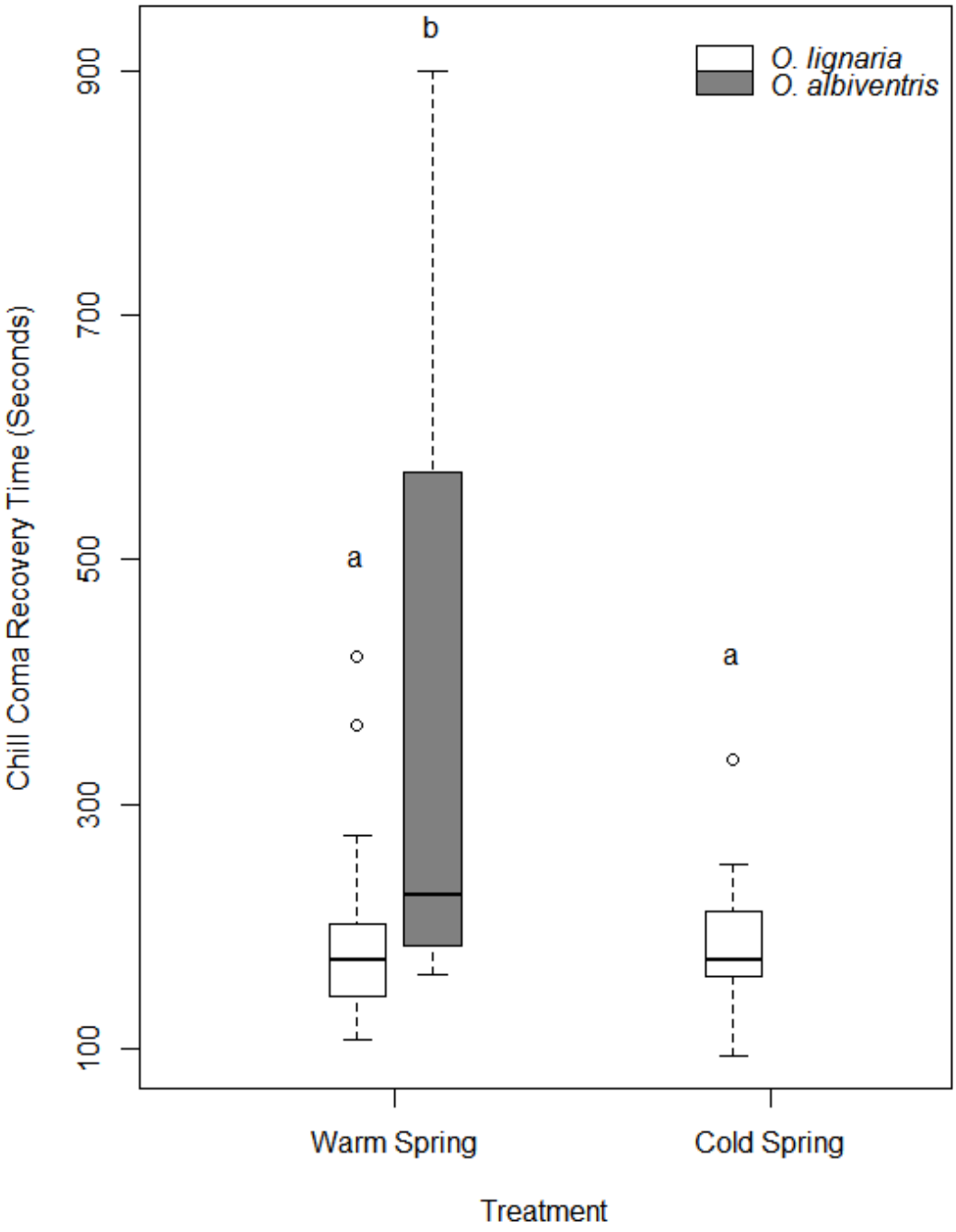

Figure 9—Chill coma recovery time (seconds) for bees after Season 2 experiment. Males and females are pooled together. Sample sizes for each treatment are as follows (*O. lignaria, O. albiventris*): Warm Spring (39,4), Cold Spring (39,0). Treatments that share letters are not significantly different from each other at $\alpha = 0.05$.

Discussion

Warmer temperatures deplete fuel reserves

In my experiment, warmer temperatures generally depleted fuel reserves more than cooler temperatures. I found that warmer temperatures depleted free sugars more than colder temperatures. For *O. lignaria,* the cold treatment CW4 had significantly higher concentrations of free sugars than treatment W (Figure 5). For *O. albiventris,* the variable and primarily warm treatment (CW1) had significantly lower concentrations of free sugars than the colder treatments C and CW4, and the field treatment (FLD) (Figure 5). It is interesting that we found any difference between treatments, since circulating sugars are replenished from stored glycogen, and the depletion of free sugars could have been masked through their replenishment from stored glycogen. In *Osmia rufa*, prolonging the bees' period of postdiapause by 3 months (from April to July) similarly resulted in significantly reduced free sugars, though the actual difference in sugar concentration may not have been biologically significant (Dmochowska et al., 2013). My results for lipid and glycogen reserves also support the notion that warmer temperatures deplete reserves. Concentrations of glycogen in *Osmia lignaria* were ten times higher in the coldest treatments (C & CW4) compared to warmer treatments (CW2 & CW1). Similarly, concentrations of lipids in this species were higher in treatment CW4 compared to CW2. For *O. albiventris,* the warm treatment (W) resulted in significantly lower concentrations of glycogen than treatment CW1, indicating that even 5 days of cold (Table 2) mitigated the loss of fuel reserves (Figure 3). Together, these results indicate that a higher mean temperature in late winter causes greater fuel depletion than a lower mean temperature.

Increased temperature variability has less of an effect than mean temperature

Fluctuating thermal regimes (i.e. more variable temperatures) tended to deplete fuel reserves more than constant temperatures under my experimental conditions, but only when

variable temperatures occurred in combination with warm temperatures. Temperature variability appears to have had a stronger effect than mean temperatures on glycogen depletion. This is supported by the lack of difference between the constant cold and constant warm treatments in Season 1 (although the sample sizes for this comparison are small). For *Osmia lignaria*, the concentration of glycogen was lowest in the warm and variable treatments (CW2 and CW1), not the coldest treatments (C and CW4), suggesting that it is warmer temperatures rather than increased temperature variability that depleted glycogen reserves. The fact that the fluctuating, but primarily cold treatment (CW4) had higher concentrations of glycogen than other treatments demonstrates that it is not necessarily the number of fluctuations or repeated cold exposures that affect the bees' glycogen reserves, but rather the overall number of warm days (Table 2), perhaps in conjunction with the variability of the temperature. In summation, the glycogen concentrations support this interpretation (however with a low sample size), whereas the results from free sugars and lipids do not—suggesting rather an effect of warm temperatures, but not of variability.

Some researchers suggest that insect development should be faster under fluctuating thermal regimes (FTR) than under constant regimes (Colinet et al., 2015). However, energetic costs incurred during the warm conditions of a FTR will be greater than the energetic savings that come as a result of the cooler conditions, so fluctuating environments can require more energy than fixed environments of the same mean temperature (Colinet et al., 2015; Williams et al., 2012b). My results for the concentration of glycogen support this idea that a warmer mean temperature in combination with a fluctuating regime (treatments CW2 & CW1) is more energetically costly than the savings that come as a result of a cooler mean temperature in conjunction with fluctuating temperatures (treatment CW4), as seen by the treatment effects in *O. lignaria* (Figure 3). Perhaps the separation of free sugars into their constituents might have

provided us with a clearer picture of responses to fluctuating thermal regimes, since it may be a particular sugar (e.g. glucose) that responds to treatments in a minute but detectable way that may be masked by measuring total free sugars.

Mass loss and fitness effects

Mass loss is exacerbated by warmer temperatures and more variable temperatures. Bees in the genus *Osmia* with a higher masses have higher winter survival rates (Bosch and Kemp, 2004; Tepedino and Torchio, 1982)**.** In the first experiment, I found that *Osmia lignaria* lost more mass as the amount of time spent in warm conditions increased (Table 2, Figure 6). The two coldest treatments (C, CW4) caused the least mass loss for *O. lignaria* and the warmest treatment (W) caused the greatest mass loss. Mass loss appears more dependent on the duration of warm temperatures than the number of repeated cold exposures, since the more variable, cold treatment (CW4) resulted in less mass loss than the variable, but warmer treatment CW2. For *O. albiventris*, all of the laboratory treatments caused significantly more mass loss than the field treatment (FLD; Table 6, Figure 6). Most of this lost mass was probably water: desiccation is a major threat for diapausing insects (Hahn and Denlinger, 2011). The greater mass loss in the laboratory treatments may indicate that there was greater humidity in the field. Additionally, in Season 1, some of the mass loss may have occurred because some bees died before the end of the experiment. In Season 2, I found no difference in mass loss between temperature treatments, likely because bees were not forced to stay in the treatments, as they were in season 1. Rather, once bees emerged, they were weighed and tested for chill coma recovery time that same day. Allowing the bees to determine when to emerge provided a more realistic treatment since in nature, they would emerge when the environmental cue was correct; but this may have reduced the magnitude of their mass loss since emerging earlier would have resulted in less water loss prior to measurement. The results from

Season 2 provide some evidence that an earlier emergence date may prevent excessive mass loss associated with remaining in their cocoons.

As an ecologist, I was interested in the effects of temperature treatments on subsequent bee performance. There is a recorded energy cost to rewarming (MacMillan et al., 2012a, 2012b; Sinclair, 2014), so the time to recover from chill coma is a valid measurement of bee energy reserves and a potential indicator of future performance. There was no significant difference in chill coma recovery time between treatments or between sexes within *O. lignaria* (Figure 10), indicating that warming spring temperatures will not significantly affect the performance of early spring emerging bees (also supported by the lack of difference in mass loss between the Season 2 treatments). However, *O. lignaria* did recover significantly faster from chill coma than *O. albiventris*, indicating that there are species-specific differences –perhaps related to *O. lignaria* being better adapted to early spring conditions (including cold spells) than *O. albiventris*. Alternatively, it is possible that recovery from chill coma may not use the same types of energy reserves as are used for enduring post-diapause conditions. It is also possible that the quantity of energy reserves does not influence recovery time, but rather the ability (or inability) to recover from chill coma at all.

Nevertheless, warm and variable spring temperatures might result in various sub-lethal effects that would affect the fitness of these bees (Marshall and Sinclair, 2012), but that were not measured in my experiments. Researchers have found negative effects of temperature variation on fitness: repeated cold exposures decreased the fecundity of *D. melanogaster* (Marshall and Sinclair, 2010). On the other hand, some researchers suggest that periods of warm temperatures during the winter may actually provide insects an opportunity to repair sub-lethal chill injuries (Colinet et al., 2015; Rinehart et al., 2011). As it stands, the exact effects of fluctuating thermal

regimes remain incompletely understood. My Season 1 results suggest that increased exposure to warm mean temperatures increases mass loss and fuel depletion and may be exacerbated by fluctuating temperatures. In contrast, in Season 2, I observed no clear effects of warmer spring temperatures.

Glycogen allocation

Glycogen is used to replenish free sugars, but also for the production of glycerol as a cryoprotectant (Marshall and Sinclair, 2014). Because we did not observe greater glycogen depletion in our colder treatments, our results could indicate that glycogen was not being converted into glycerol for cryoprotection. Since we did not directly measure glycerol, we cannot be certain of this conclusion. In the goldenrod gall moth (*Epiblema scudderiana*), the production of glycerol was largely accounted for by decreased glycogen stores (Rickards et al., 1987), which indicates that glycerol production can be inferred from measures of glycogen content, at least in some species. However, the timing of glycogen measurement is important: Rickards et al. (1987) found that glycogen content of larvae increased until mid-October, at which point it began declining; when they measured glycogen again in January, glycogen reserves were virtually exhausted. However, by March and April, some glycogen (up to 20%) had been restored. Since I measured glycogen in April, at a time when in a natural setting insects may be recovering rather than depleting glycogen, it may indicate that glycerol had already been reconverted into glycogen. Marshall and Sinclair (2014) measured glycogen in January, February, and March in *Choristonerua fumiferana* larvae and found that glycogen was highest in January and lowest when measured at the end of March. The failure to detect glycogen depletion in the cold treatments in my experiment (C & CW4) could reflect the fact that the reconversion had already taken place by the time of measurement, i.e., in the 2-8 days these bees spent at 10°C after cold exposure. An alternative interpretation would be that the production of cryoprotectants is independent of

temperature in *Osmia* spp., as it is in some other insects (Leather et al., 1993). A third possibility is that the treatments themselves may not have been extreme enough or long enough to elicit quantifiable glycerol production. In order to detect glycogen depletion, the bees may have had to have been exposed to longer and/or more extreme experimental cold treatments, rather than only 20 days of -10°C (Treatment C). Dmochowska et al. (2013) found that in *Osmia rufa*, a prolonged postdiapause at 4°C (after overwintering outside in Poznan, Poland until February, where average temperatures range from –5 to 1°C from November to February (Helicon Publishing, 2011)) resulted in significantly lower concentrations of glycogen compared to bees that experienced a shorter imposed postdiapause period. Their experiment manipulated the bees by prolonging their winter by up to 3 months, which is substantially longer than the treatments used in my experiment. They suggested that the low levels of glycogen they found in *O. rufa* may be associated with the production of glycerol before the postdiapause treatments, although they also noted that bees that were overwintered for a longer time used their glycogen reserves as an energy supply during the prolonged post-diapause from February until April or July. Thus, it remains unclear how the distribution of glycogen is attributed to the conversion to glycerol and the mobilization of free sugars, such as glucose (Dmochowska et al., 2013).

Warmer temperatures accelerate emergence

It is well known that warmer spring temperatures accelerate emergence (Bosch and Kemp, 2000; Bosch et al., 2000; Dmochowska et al., 2013; Fründ et al., 2013). In my second experiment, I expected that the warm spring treatment would result in bees emerging earlier than the cold spring treatment. However, it is possible that emergence may depend on another cue as well, such as number of days since diapause began (Sgolastra et al., 2010), in which case, bees that were kept warmer would have been using up reserves without advancing towards emergence. It is possible that emergence date is accelerated when exposed to warmer temperatures in order to mitigate fuel

depletion and water loss. I observed protandry in these bees, with males emerging significantly earlier than females, as well as differences between treatments, with the warm spring treatment accelerating the date of emergence (Figure 7). Bosch and Kemp (2001) found that females of *O. lignaria* tended not to emerge until exposed to temperatures of around 20°C. However, I observed emergence in the cold spring treatment (10°C/0°C): it simply took longer than the warm spring treatment. It is possible that *O. albiventris* females did not emerge because they never received a necessary temperature cue. Even in the warm spring treatment, bees were cycled from a daily high of 20°C to a daily low of 0°C, which may not have been a warm enough mean temperature to encourage emergence.

Species-Specific Effects/Responses

There are inherent differences between species; however, the study of only two species does not allow us to generalize to the genus as a whole (Garland and Adolph, 1994). *Osmia lignaria* tends to emerge approximately two weeks earlier in the spring than *Osmia albiventris*. In Season 2, *O. lignaria* emerged an average of 6 days earlier than *O. albiventris* (Figure 7), indicating that these species may be in different diapause intensities at the time of treatment. In their natural environment, *O. albiventris* would not be ready to emerge in April (Mitchell, 1962) whereas *O. lignaria* would be closer to their emergence date, and thus more metabolically active. In general, *O. lignaria* was more responsive to the temperature treatments, which fits with the presumed difference in diapause intensity between species: if *O. lignaria* were more metabolically active, it may simply be better prepared to emerge when faced with abnormally warm temperatures. The effect of diapause duration and intensity of various bee species necessitates further investigation.

The intensity of diapause could be an indicator of vulnerability to warming, in that we might expect that a shallow state of diapause would limit the degree of metabolic suppression

(Williams et al., 2012a) and would increase sensitivity to changing temperatures. My results lend some support to the potential for species-specific differences as a result of differences in diapause timing and intensity. Previous studies (Fründ et al., 2013; Kemp et al., 2004; Williams et al., 2012a) suggest that species-specific differences in response to temperature could be a result of differences in diapause intensity, respiration rate, and/or the life stage of the insect. For example, *Megachile* spp. bees that overwinter as larvae expend less energy in response to increasing experimental temperatures than *Osmia* spp. bees that overwinter as adults (Fründ et al., 2013). I did not measure metabolic rate in this study, so I cannot be certain whether this is true of the two species I studied, but Kemp et al. (2004) proposed that spring-emerging bees are in a less pronounced state of diapause than summer-emerging bees, based on metabolic rate. Similarly, Scriber et al. (2012) suggested that a deeper diapause and lower associated metabolic rate could explain reduced winter vulnerability in tiger swallowtails (*Papilio* spp.). Since *O. lignaria* emerged earlier in Season 1 (as little as one day after being transferred to room temperatures, vs. an average of 2 days in *O. albiventris*), we can infer that they were either out of diapause or in a less intense state of diapause at the time of the experimental treatments, whereas *O. albiventris* may have still been in diapause, providing them with a protective buffer against variable temperatures. In their natural environment, *O. albiventris* would not be ready to emerge in April (Mitchell, 1962) whereas *O. lignaria* would be closer to their emergence date, and thus more metabolically active.

There were clear differences between the two species I studied in terms of their responses to treatments, and these differences may reflect the differences in phenology (and likely diapause intensity) between these species. Of course, comparison of two species (which differ in numerous ways other than their timing of emergence) does not allow me to generalize about the effects of

diapause intensity on sensitivity to temperature treatments (Garland and Adolph, 1994). The effect of diapause duration and intensity of various bee species necessitates further investigation.

Future Studies and Implications

My research has provided valuable insights into the effects of repeated cold exposures on two species of mason bees (*Osmia lignaria* and *Osmia albiventris*), disentangling the differences in fuel reserve depletion between species and as a result of warming and fluctuating spring temperatures. Overall, my results have shown that warmer mean temperatures result in greater depletion of fuel reserves and greater mass loss, especially when variable temperatures are combined with warm temperatures. I have also explored mass loss, chill coma recovery time, and emergence time of mason bees as a result of warmer-than-average spring temperatures. Overall, the results from this study have provided an optimistic glimpse into the possible future of these species—one where solitary bees may be able to plastically adjust their phenologies in a way that mitigates the depletion of fuel reserves.

This research can add to the growing base of knowledge surrounding the management of wild bees as agricultural pollinators. My results suggest that wild solitary mason bees can adjust to warming spring climates by accelerating their emergence, thereby avoiding mass loss, and that the best course of action for these two species is to allow them to adapt naturally as temperatures change. Future studies should look at a variety of other species in the genus *Osmia* in order to determine if, in general, a deeper intensity of diapause provides protection against springtime temperature variability.

An extension of my research that I would like to delve into in the future involves constructing conceptual models of what we expect each treatment would elicit in terms of fuel depletion and then fitting the models to the data to estimate model parameters. This method has

been used by other researchers in similar fields of study (Sinclair et al., 2013; Williams et al., 2012). It would involve generating a general equation that would simulate the use of energy reserves during overwintering and then using climatological data and field observations to model our predictions. These models lend further credibility to observed results from laboratory experiments that modify the temperatures experienced by the species in question by taking climate data from multiple years and projecting the possible outcomes of various climate scenarios (Sinclair et al., 2013; Williams et al., 2012). These models would also enable forecasts of responses to future climate regimes.

Measuring the metabolic rates of bees in diapause would provide a clearer understanding regarding the depth of their diapause. Some studies (Sgolastra et al., 2011, 2010) have already characterized variations in metabolic rates of bees either pre-wintered or overwintered at varying temperatures, but have not explored the way in which metabolic rates change in response to altered post-winter temperatures, which may affect bees more acutely since diapause ends sometime within the seasonal transition of winter to spring. There is no definitive method to determine when an insect has ended its diapause. Krunić and Stanisavljević (2006) suggested that the termination of diapause is related to a rapid loss of glycerol in *Osmia* bees, but they also proposed the need for further research into the concentrations of cryoprotectant molecules in relation to diapause intensity.

Future research should investigate fitness costs associated with warming summer temperatures in order to determine if they are more metabolically costly to dormant bees than winter or springtime warming. Sgolastra et al. (2012) found that *Osmia* spp. exposed to long pre-winter periods had higher winter mortality and lost more lipids than bees exposed to shorter pre-wintering periods. This indicates that the summer diapause of *Osmia* spp. may be a factor

determining winter survival, especially in relatively warm climates (Sgolastra et al., 2012). Changing temperatures could also result in an accumulation of sub-lethal effects that may reduce capacity to reproduce later in the season. By evaluating fecundity, we could deduce how temperature affects reproductive fitness of individuals. However, studying realized reproductive output of experimentally manipulated *Osmia* bees would be challenging, since they cannot be maintained indoors long term, nor can individuals be reliably tracked in the wild. A model insect that could be reared in the lab for multiple generations would be best for analyzing the reproductive effects of changing temperatures.

Intuitively, we might predict that warmer winter temperatures would result in habitat expansion and increased survival for ectotherms inhabiting cold climates, since cold-induced stresses would be eliminated (Williams et al., 2012). However, for insects that have an obligate and lengthy winter dormancy, warming winter temperatures can be costly, as my results have supported. If global temperatures exhibit more extreme fluctuations in the future, insects that cannot adjust or adapt may not persist. By assessing the influence of changing spring temperatures on mason bees, we may be able to aid in the risk assessment of native pollinators in the context of climate change. Focusing research efforts on temperate species that are particularly vulnerable to changes in their environment may allow for the allocation of funding and research efforts to the species most at risk.

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