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Cold Tolerance, Temperature Mediated Discontinuous Gas Exchange, and Emergence of the Blue Orchard Mason Bee (Osmia Lignaria)

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COLD TOLERANCE, TEMPERATURE MEDIATED DISCONTINUOUS
GAS EXCHANGE, AND EMERGENCE OF THE BLUE ORCHARD MASON BEE (*Osmia lignaria*)

A Thesis
Presented to
The Graduate Faculty
Central Washington University

In Partial Fulfillment
of the Requirements
for the Degree
Master of Science
Biology

by
Logan Martin Kral

May 2019
CENTRAL WASHINGTON UNIVERSITY

Graduate Studies

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Dean of Graduate Studies
ABSTRACT

COLD TOLERANCE, TEMPERATURE MEDIATED DISCONTINUOUS GAS EXCHANGE,
AND EMERGENCE OF THE BLUE ORCHARD MASON BEE (*Osmia lignaria*)

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The relationship between low temperatures, emergence and supercooling point of *Osmia lignaria* were the subject of this study. One hundred sixty-eight bees were subjected to 5 pre-wintering temperature treatments (two constant temperature controls - 22°C, 14°C, one of which with and one without a 12h photoperiod, and three 12h:12h thermoperiod treatments – 14:10°C, 14:5°C, and 14:0°C) and were then evaluated in terms of emergence time and post-emergence vigor. An additional 70 bees were tested for metabolic rate and discontinuous gas exchange in response to test temperature conditions. An additional sample of 60 bees was evaluated for temperature of crystallization. Of the 168 bees which were emerged, it was found that bees held to an intermediate thermoperiod of 14°C during the “day” and 10°C at “night” emerged an average of 2 days earlier than the other treatments. During the experiment, discontinuous gas exchange was observed for the first time in this species and metabolic rates were examined in 5°C increments which ranged from 6.54 µl·g⁻¹·h⁻¹ at 0°C to 177.72 µl·g⁻¹·h⁻¹ at 20°C. Temperature of crystallization was also established as -26.4°C (±0.6°C) for Washington *O. lignaria*. 
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CHAPTER I

INTRODUCTION

Osmia lignaria or the “Blue Orchard Mason Bee” is native to the Pacific Northwestern United States, and is the focus of this thesis. In the Pacific Northwest native pollinators such as *O. lignaria* are relied on to supply not only the pollination needs of gardeners and horticulturalists, but also those of commercial orchardists and berry farmers. Nearly 100% of commercial cherry, peach, pear and roughly 70% of apple pollination is currently performed by honeybee rental in Washington state (USDA Cost of Pollination Report 2017, USDA Washington State Agriculture Overview). Unfortunately, the commercial honeybee industry is experiencing difficulty outpacing the rate of attrition in their hives due to varroa mite, fungal pathogens and Colony Collapse Disorder. It is speculated that native bees would be ideally suited to fill the coming shortfall in pollination productivity (Dushoff 2011). Recent studies however, have shown that native pollinator numbers are suffering effects of a primarily anthropogenic origin (Kluser 2007, Bosch & Kemp 2000, Bosch et al. 2000, Bosch & Kemp 2003).

*Osmia lignaria*

*Osmia* are a cosmopolitan genus of hole-nesting solitary hymenopterans within the Megachilidae commonly referred to as “mason bees”. *Osmia* spp. provide a component of native pollination services and can be found on nearly every continent.
ranging from the Palearctic to the Nearctic. *Osmia lignaria* as a species are distributed across most of the western United States and southwestern Canada. Across its native range there are two phenotypes which have been described, an “early-flying” California phenotype, and a “late-flying” Utah phenotype, of which the northwestern *O. lignaria* are considered to be a geographic extension of (Pitts-Singer, 2014). During their most active period in the spring, *O. lignaria* subsist on a diet which often consists of tree pollen and nectar from the *Prunus, Malus, and Pyrus* genera of the Rosaceae family (Kemp, 1997), although *Ribes* and *Taraxacum* pollen has also been observed in the nesting cavities in prior studies (Torchio, 1982).

Much of what is known regarding the lifecycle of *Osmia lignaria* was described by P. Rau in 1937. The species is univoltine (undergoing one reproductive cycle per year) developing through the summer into an adult before an obligate winter diapause. Considered a “spring” bee (Bosch & Kemp, 2004), it emerges as a fully formed adult from its cocoon when many native flowering plants begin blooming in the early to mid spring, once average daily temperatures reach 14°C. Males emerge first followed by the females shortly thereafter. Upon emergence, the males and females mate soon after the female completes a short pre-nesting period, possibly to allow for complete sexual maturation (Bosch & Kemp 2002).

Following emergence and mating, the females immediately begin provisioning a nesting site. Suitable nesting sites have been extensively studied and although many varieties exist, the fundamental quality required is a small hole roughly 8mm in diameter, such as an old beetle bore or the abandoned nests of wasps or *Xylocopa* bees
(Cane 2007). Once found and cleaned, the female will begin storing a pollen and nectar mass into the rear-most space of the hole (Rau 1926, Rau 1937). Once adequately filled with provisions, she fertilizes her first egg (making it female) and lays it atop the pollen mass. She then retrieves some soft clay from a nearby clay source, caps the chamber with the egg inside and then repeats the process for a second chamber and egg (Torchio 1989). The female fertilizes the first few eggs she lays in the nest to produce females, before finishing laying with unfertilized eggs which result in haploid males. She will continue depositing eggs in this manner until she runs out of space to create new egg chambers. Once the tube is filled, the female will form a thick plug of clay in the end of the tube and leave to find a new nesting site where she can repeat the process (Rust, 1974).

After one to two weeks, a larva hatches from the egg and begins eating the pollen and nectar package left by its mother. Over the ensuing month the larva eats and grows and molts into progressively larger instars, culminating as a 5th instar which sets about spinning a cocoon shortly before onset of the heat of summer. Roughly one to two months after finishing the cocoon, the prepupa develops into a pupa and enters a short summer diapause lasting around one month (Bosch & Kemp 2000, Kemp et al. 2004, Bosch & Kemp 2005). Once diapause is complete, the pupated adult bee emerges from its old pupal case, but remains within the confines of its cocoon as it begins to enter winter diapause (Bosch et al. 2010). The bee will spend nearly 200 days in its cocoon, through fall and into winter before finally emerging in the spring, just as its parents did the prior year, to repeat the mating and nesting process for itself.
Diapause

The process of diapause is defined as the decrease in metabolic activity at the cellular level, suspending physiological growth and development (Andrewatha 1952, Tauber 1986, Chapman 1998) the purpose of which is to conserve energy and synchronize development. Another key element of diapause is that it can occur at any time of the year. The process of diapause occurs over a several distinct phases beginning with an induction phase, followed by an initiation phase, a maintenance phase, and finally a termination phase. During induction, the insect readies itself for diapause following exposure to a token stimulus (Tauber 1986). These stimuli can be a change in photoperiod or thermoperiod, or simply a predetermined temporal period, depending on the organism in question. During the initiation phase, lipids and carbohydrates accumulate in the tissues of the insect to improve cold-hardiness and to provide fuel for development after termination of diapause (Kostal 2004). Following this phase, the insect undergoes a diapause maintenance phase during which oxygen uptake and nervous system activity slows and it becomes less responsive to token stimuli (Kostal 2006). The diapause process finally reaches a termination phase where the insect will once again respond to a stimulus and then enters a post-diapause quiescent period (Kostal 2006). Quiescence is similar to diapause in that metabolism is slowed, although it lacks the physiological suspension of development. Because this termination may occur while unfavorable conditions exist, the quiescent period helps to mitigate resumption of normal development until such time as conditions become more favorable.
*Osmia lignaria* must undergo two separate diapause events to complete their life cycle (Sgolastra 2009). They undergo a short summer diapause following their final larval molt into a pupa, after which the now fully-formed adult bee chews itself out of its pupal carapace and rapidly begins dropping its metabolic activity in preparation of for a second winter diapause (Sgolastra 2011). *Osmia lignaria* require a cold period of at least 185 days to completely overwinter and emerge successfully (Bosch & Kemp 2000, Bosch et al. 2000, Bosch & Kemp 2003), and exhibit a characteristic rapid lowering of their metabolic activity post-eclosion. This decrease in metabolic activity indicates the initiation of winter diapause in the species (Sgolastra 2011). As the onset of winter temperatures occur, metabolic activity rises sharply at first but then plateaus as the insect completes winter diapause and begins a long and slow temperature-mediated quiescence (Kemp 2004). During this quiescence, metabolic rates remain low, but only until temperatures rise. In the case of *O. lignaria*, this comes after exposure to 22°C for a few hours (Bosch & Kemp 2000). Following this, the bee begins chewing its way out of its cocoon and then rapidly increases its respiration rate in anticipation of its first flight. Previous studies have examined the relationship between high temperatures and development of *O. lignaria* during the summer, winter and spring (Bosch & Kemp 2000, Bosch 2003, Sgolastra 2011), but so far the potential for a possible low temperature stimulus to initiate diapause has been as yet unexplored.
Climate Change

As the effects of anthropogenic global warming have taken hold, the inland Pacific Northwest climate has changed from historical patterns. Summers have become longer with more pronounced droughts and higher daily maximum temperatures, while winters have become shorter and milder, often breaking above freezing temperatures for weeks at a time before returning to winter-like conditions (Littell et al., 2009, IPCC 2014). What effect these changes will have on the native flora and fauna is only just beginning to be understood. Prior research has shown that laboratory-managed populations of mason bees can be over-wintered at 3°C for 120 days, resulting in more efficient and lower mortality when compared to naturally over-wintered bees (Bosch 1994). This could be explored as a potential avenue for mitigating any potential asynchronicity (Forrest 2011) between flowering of the host plants which are primarily controlled by photoperiod, and the insects which depend on them which are primarily controlled by thermoperiod. Related species of *Osmia cornuta and rufa* have demonstrated pronounced cold temperature characteristics (-30°C and -31°C respectively, Krunic & Stanisavljevic, 2006), while Utah populations of *O. lignaria* have also shown strongly depressed freezing points (Kemp, unpublished data). As of yet however, no research on the freezing properties of Washington-based *O. lignaria* has been explored.

Anthropogenic effects are also altering the arthropod landscape in new and frightening ways. Recently, several studies have been released which more thoroughly explore the complex relationship between insect and environment (Kremen 2007,
Broussard 2011, Cane 2001, Dushoff 2011). Many of these studies are reaching similar conclusions: that the outlook for insects in a post-climate change world are dire. Threats to *Osmia* include invasive species outcompeting native bees for forage and nesting sites, increasing rates of parasitism, competition with non-native species, disease, as well as a host of anthropogenic threats to bee survival like monocrop-style agriculture, habitat fragmentation and loss, increasing rates of pesticide use and overharvesting of natural resources (Kluser 2007). As demand for specific crops continues to grow, additional native habitat will be converted into plantation, which aggravates and may amplify the preceding threats mentioned. With less available wildlands to forage from, native bees are forced to source pollen and nectar from a much less diverse set of plants, some of which can be found on the edges of crops and on the sides of roads (Hopwood 2008). Unfortunately, roadways have also been shown to be major barriers to insect diversity and movement, not only due to the typically lethal contact with the vehicles themselves, but also as a result of behavioral preferences to avoid roads entirely (Torres 2015).

Currently there are many organized efforts underway to protect both commercial and native bees. Conservation has been a rapidly burgeoning topic recently, with honeybees taking the spotlight, spurring the creation of film and political campaigns alike. Recent reports have linked neonicotinoid pesticides to various adverse effects in honeybees (Troxler 2017, Chensheng 2014). Neonicotinoids like Clothianidin and Imidacloprid are not only highly toxic to *O. lignaria*, but have also been shown to inhibit the development of larvae after ingesting sublethal amounts of the toxin from
their pollen provisions (Hopwood 2016), while fungicides like Rovral 4F, Pristine, and N-90 have been linked to decreased nest recognition in female *O. lignaria* (Artz 2015).

As a result of the multitude of issues facing native pollinators, a systematized approach for brood management must be developed to enhance the continued success of the species. Currently, commercial suppliers, orchardists and hobbyists control the largest populations of artificially managed *O. lignaria* in the Pacific Northwest, with very little in common in terms of brood raising strategies. Much of the data concerning optimal wintering and prewintering conditions was explained in Bosch & Kemp 2000 and Bosch 2000, such as the need for sufficient degree-day accumulation as well as the effect of temperature on development. For instance it is known that higher temperatures can shorten development time during the summer (albeit at the expense of springtime longevity) and that fluctuating temperatures can also accelerate development with minimal increases in mortality (Bosch & Kemp 2000, Bosch 2000). It is also known that with an extended duration of high late-summer temperatures (above 20°C) during the pre-wintering development window results in accelerated fat body depletion, which in turn leads to higher mortality (Sgolastra 2011).
CHAPTER II

STUDY DESIGN

Although the data regarding the development and respiration rate of *O. lignaria* during key developmental stages are relatively comprehensive, some areas remain to be explored including the effects of low temperature cues during winter and prewinter. Bosch (2000) noted that bees exposed to fluctuating temperatures developed faster than control groups (Bosch 2000). Thus, the question was raised whether a low temperature could potentially influence the rate of metabolism, and the onset of diapause, either positively or negatively. In addition to this, it is currently assumed that Washington *O. lignaria* differ phenologically from Utah populations. To explore potential differences here, and to better understand how prepared Washington bees are for potentially severe winter extremes in the future, a series of supercooling runs were completed on a sample of individuals from the reserve population of bees which had been collected. Once it became apparent that discontinuous gas exchange was taking place, respirometry was conducted on random bees selected from the first experiment. The results of these experiments will assist conservationists and growers alike in better managing their own populations of *O. lignaria* to ensure lower winter mortality and higher rates of successful springtime emergence.
Metabolic rates

Using what is already known about diapause and post-diapause quiescence, I originally sought to better clarify the exact cue by which these bees initiate diapause following eclosion using methods previously established in earlier studies (Kemp 2004, Sgolastra 2011). In effect, this information could then be used to better inform methods for advancing emergence in the early-bloom scenarios often sought after by commercial orchardists. *Osmia lignaria* not exposed to wintering temperatures do not enter diapause at all, which renders them unable to survive winter to emergence in the spring. Therefore, it was hypothesized that if a warmer average daily temperature delayed or inhibited induction of diapause, a cooler thermal regime might have the opposite effect of accelerating diapause initiation. A system of pre-wintering thermoperiod treatments were modeled on prior respirometry studies (Kemp 2004, Sgolastra 2011) which would simulate increasingly variable day/night temperature cycles that one may see under a future climate scenario. Following this, a deeper exploration of *O. lignaria* respiration rates at differing test temperatures using flow-through respirometry was also explored. I hypothesized that metabolic rate and the rate of discontinuous gas exchange cycling (hereafter referred to as DGE) would rise with exposure to higher temperatures.
Emergence of Pre-Wintered Groups

Differences between the pre-wintering thermal regimes of the prior fall were examined by emerging the test subjects and analyzing for differences between treatment and control groups. It was hypothesized that bees which had experienced lower “nighttime” temperatures (and thus lower average daily temperatures) would enter diapause slightly earlier than bees held at warmer temperatures, and would thus emerge earlier as well.

Temperature of crystallization of Washington O. lignaria

*Osmia lignaria* from Utah have shown a capability to depress their freezing point to -18.4°C (±2.5°C) (Sheffield 2008), and it was hypothesized that Washington *O. lignaria* could have a freezing point lower than that seen in the phenology from other areas (Pitts-Singer 2014), potentially as a result of genetic connectivity to populations in colder environments or from a shared evolutionary history.
CHAPTER III

METHODS & ANALYSIS

*Osmia lignaria* used in this study came from Crown Bees of Woodinville, Washington. Only nests from the Puget Sound area were used for the purposes of this study to avoid any potential subtle geographic differences which may exist. The bees used for all experiments in this study were sourced from nests in and around the Puget Sound region of Washington State from a mixture of previously released as well as wild naturally-occurring parentage. The nests used in the Metabolic Rate experiments were A02, N46, M09, A03, W34, B16, and V118 and found on the map below (Fig 1). The Emergence and Temperature of Crystallization experiments used bees from all of the above nests as well as nest L09, which had not been included in the Metabolic Rate experiments.

All bees which were used in these experiments were first prewintereed for 34 days before wintering for 143 days, and originated as eggs deposited in April to May of 2017 in mixed tree fruit orchards. Drilled wooden blocks with paper straw inserts were provided as nesting materials for the bees in several small shelters at each orchard. Following the conclusion of nesting in mid-June, the blocks were left undisturbed until late July to allow the larvae to reach 5th instar, at which point they were brought in from the field to an unheated warehouse.
On August 16, a random sample of wooden nest blocks was split apart and the straw nest inserts were dissected. Individual cocoons were sexed, weighed, and serialized. Cocoons were then brought into a laboratory held at 23°C and ~75% RH for approximately two weeks, during which time they were monitored for eclosure. Monitoring was accomplished by dissecting 10 random cocoons every 5 days until at least 9 of the 10 cocoons sampled contained eclosed adults. Eclosure is characterized by
the tongue retracting and the adult bee fully emerging from the pupal carapace with fully formed wings. Eighty-four bees in total of both sexes (42 male, 42 female) were set aside for use during the first metabolic rate experiment which began on September 5 (Fig. 2). On September 22, another 84 bees (42 male and 42 female) were similarly treated. This second group of bees were used in the emergence experiment to better gauge what effect, if any, a longer “summer” would have on the development of diapause after having been exposed to same test conditions as the first 84. All remaining bees from the reserve population which had not been assigned to the treatment groups of the early winter metabolic rate experiment were prewintered at 23°C and ~75% RH for approximately 5 weeks from August 16 until September 22 at which point they were transferred into a constant 14°C temperature-controlled incubator and then held for an additional week and before being finally transferred to a constant 4°C cold room with ~70% RH on September 29. These bees were kept in complete darkness at all times and were used for the temperature of crystallization and effect of testing temperature on metabolic rate experiments.
Figure 2. Distribution of bees used in experiments of Washington *O. lignaria*

Respirometry methods

All respirometry was conducted using a positive pressure flow-through system made of plastic air lines and Sable Systems RC glass respirometry chambers (30mL) to measure carbon dioxide using a Li-Cor LI-6251 CO₂ analyzer. The device was set to operate in differential mode with a flow-rate of 100mL/min. Incoming air was scrubbed of CO₂ and water vapor through a Drierite/Ascarite column. All respirometry tests were conducted following a 60-minute acclimation period during which time all respirometry chambers were flushed with air scrubbed of CO₂. Each chamber was closed and respiration was allowed and recorded sequentially, followed by 5 minutes of flushing the following chamber in sequence before recording began. Respiration rate was expressed as CO₂ produced per ml·g⁻¹·h⁻¹ of fresh weight.
Early Winter Metabolic Rates

Earlier studies have shown that males and females do not significantly differ in respect to respiration pattern and weight loss (Kemp et al., 2004), so only females were used for respirometry and analysis in this study as had been done in prior studies (Bosch et. al. 2010, Kemp et. al. 2004, Sgolastra 2010, Sgolastra 2011).

Following along the methods used previously (Bosch et al., 2010) to select and distribute bees amongst treatment groups, 42 females were randomly selected from the assortment of nests so that no thermoperiod treatment received more than one individual from the same nest. The six thermoperiod treatments were structured in a way so that each treatment had seven females in each of 3 post-eclosion thermoperiod treatments (14:10, 14:5, and 14:0°C, on a 12h:12h regimen to simulate day:night cycles, and all in complete darkness), as well as three controls: two held at a constant 14°C, with one having fluorescent cabinet light exposure for 12 hours a day, and one without, as well as a third control held at 22°C, also in complete darkness, which had been shown previously (Bosch et al., 2010) to inhibit diapause development (Fig. 3). Two weeks later on September 22, 2017 42 additional females and 42 males were also started into the same thermoperiod treatments as before but were only retained as backups for the first group and were not analyzed for respirometry, but were used for emergence.
The females used for respirometry were measured for CO₂ production every 3 days from adult eclosion until their metabolic activity demonstrated the characteristic leveling-off (Bosch et al., 2010; Sgolastra et al., 2011) which indicates the initiation of winter diapause. All respiration measurements were conducted at 14°C in complete darkness for all groups except for the photoexposed 14°C control which was tested at 14°C in full light, and the 22°C control group which was tested at 22°C in the dark. Chambers were flushed for five minutes prior to recording metabolic data for five minutes per chamber. All individuals were weighed at regular intervals throughout the wintering process, including before every respirometry measurement and at the beginning of the
emergence experiment. These were then compared among the different treatment and control groups.

Effect of testing temperature on metabolic rate

Seventy individuals were randomly selected and tested from November 14 to November 29 of 2017 to measure their metabolic response to temperatures from 0°C to 20°C in 5°C increments (n=14 per treatment). A one-hour acclimation period was allowed for the testing chambers to reach the necessary temperature before recording metabolic rates, and rates were measured for one hour per chamber. The data was statistically analyzed for significance in R 3.5.1 using the “lattice”, and “nortest” packages. An Anderson-Darling test was used to confirm normality and a stepwise regression was carried out to produce a one-way ANOVA.

Effect of temperature treatments on emergence

Following the methods used by Sgolastra (2011) to emerge *Osmia*, all 84 females and 84 males from each of the 6 treatment groups used in the Metabolic Rate experiments were placed individually into ventilated glass tubes and incubated in a chamber set at a constant 20°C to induce emergence. The tubes were checked daily and the date of emergence and days survived was recorded, which has been shown previously to be an accurate measure of vigor (Bosch 2003). Bees which failed to
emerge were excluded from the analyses. The effect of photoperiod on emergence time and vigor was analyzed for significant differences using a Welch’s two sample t-test. Pre-wintering duration also was analyzed using a Wilcoxon Rank-sum test to determine whether it was appropriate to combine all datasets into a single larger emergence dataset. Differences in the time to emergence and post-emergence vigor between treatment groups was analyzed using a Kruskal-Wallis test. A Wilcoxon rank-sum pairwise post-hoc test was used to identify differences among groups. All analysis was conducted in R 3.5.1.

Temperature of crystallization of Washington O. lignaria

Sixty random individuals in total were selected and tested on October 24 & 28, November 27 & 29, January 2 and April 15 after having been exposed to their 4°C “wintering” treatment for approximately 4, 9, 14, and 28 weeks respectively. These individuals were dissected from their cocoons in a 4°C cold room, taking care to remove any remnants of larval carapace attached to their setae, and then inserted into a 2mL centrifuge tube. A type-T thermocouple was inserted into each centrifuge tube and placed in contact with the sternum of the insect and then packed with a stiff open-cell foam to inhibit movement. These tubes were then placed into 15mL test tubes and lowered into a Neslab RTE 740 ethanol cold bath (4°C). Temperature was then allowed to equilibrate with the cold bath, at which point recording began as the bath was cooled at ~1°C/minute to -40°C. Temperature of crystallization ($T_c$) of the bee was recorded at
the first exotherm measured for each bee. Afterwards, individuals were removed from their tubes and dried to constant mass for 72 hours (40°C) and then weighed a second time to calculate water content. These data were then analyzed for potentially significant predictor variables (sex, month sampled, nest location, dry body mass, and water content) using stepwise AIC as part of a one-way ANOVA, and modeled with a linear model using R 3.5.1. All results reported are given as means plus or minus their standard error.
CHAPTER IV

RESULTS

Early Winter Metabolic Rates

Five minutes of data sampling using flow-through respirometry was inadequate for an accurate estimation of metabolic rate in *O. lignaria*. Metabolic rate data obtained from the 5 treatment groups tested at 14°C was more than 2 orders of magnitude below that seen in prior studies, and ultimately deemed inconclusive for the purposes of this experiment. Data collected for the 22°C control group appeared outwardly similar to prior studies, but couldn’t be compared with the other treatments of the experiment due to the discovery of discontinuous gas exchange.

Effect of testing temperature on metabolic rate

When tested for at least one hour, metabolic rates of *O. lignaria* were shown to be highly influenced by testing temperature, demonstrating a positive linear relationship between the log of weight-normalized metabolic rate and test temperature conditions, ranging from a minimum of 6.54 μl·g⁻¹·h⁻¹ at 0°C to 177.72 μl·g⁻¹·h⁻¹ at 20°C. As the temperature at which the test was conducted increased, metabolic rate increased (Fig. 4). Stepwise regression revealed that the respiration rate was a function of body mass, test temperature, and the frequency of the periodic cycling “bursts” of CO₂ activity (AIC= -86.65). Temperature was the most significant predictor variable (F = 38.9, p<0.001, r² = 0.86). Although mass and breathing cycles per hour were included in
the final respiration rate model, their effects were not statistically significant by themselves.

The number of discontinuous gas exchange cycles per hour was recorded and analyzed using a Kruskal-Wallis nonparametric test, which showed that breathing cycles per hour was significantly different ($F = 18.9, p < 0.001, r^2 = 0.75$) across the tested temperatures. A Wilcoxon Rank-sum test confirmed this significance in both cases (Fig. 5&6).
Figure 4. Log of metabolic response to testing temperature in adult wintering Washington Osmia lignaria

\(F = 38.9, \ p < 0.001, \ r^2 = 0.86\)
Figure 5. Respiration rate in relation to test temperature in Washington *Osmia lignaria*.
Figure 6. Discontinuous gas exchange (DGE) cycling rate in relation to test temperature in Washington *Osmia lignaria.*
Emergence of Pre-Wintered Groups

Photoperiod had no significant effect on time to emerge ($t=0.797, p=0.431$) nor post-emergence vigor ($t=-0.692, p=0.4944$). Emergence time ($t=-1.203, p=0.232$), post-emergence vigor ($t=-1.914, p=0.057$) and mass ($t=-0.935, p=0.351$) was not significantly different between pre-wintering experimental conditions, be it 17 or 35 days in total, so the datasets were combined for the purposes of further analysis. *Osmia lignaria* in the 10°C prewintering treatment group emerged the earliest of all tested groups, significantly earlier than the 22°C control, and 5°C treatment groups with an average emergence time of 5.8 days ($\chi^2=17.4, p=0.001$). The time to emergence increased as prewintering thermal conditions deviated from 10°C (Fig. 7). Post-emergence vigor on the other hand, showed no significant differences ($\chi^2=3.9, p=0.415$) across the treatment groups (Fig. 8).
Figure 7. Average number of days *Osmia lignaria* took to emerge after exposure to an intermediate (14:10°C) prewintering thermal treatment were significantly different from other treatment and control groups.
Figure 8. Post-emergence vigor (as measured by number of days survived after emergence) was unaffected by prewintering treatment in Washington *Osmia lignaria*.

**Temperature of crystallization**

Washington *O. lignaria* had an average temperature of crystallization ($T_c$) of -26.4°C ($\pm0.6°C$). Over the course of winter and into spring, supercooling capability
weakened only slightly (Fig. 9) although not significantly. Stepwise regression (AIC=56.2) showed that body mass alone had the greatest effect on freezing point depression of all tested variables. This was then developed into a linear model which showed strong statistical significance ($F = 7.795$, $p=0.007$), on an adjusted $r^2$ of 0.1033. No other interactions tested were statistically significant. Temperature of crystallization was lower in larger bees, which can be seen below (Fig. 10).

![Figure 9. Temperature of crystallization by month in Washington O. lignaria averaged -26.4°C (±0.6°C SEM) throughout winter and into the following spring](image-url)
Figure 10. Relation of body mass to temperature of crystallization exhibits an inverse relationship in Washington *O. lignaria* ($F=7.8$, $p<0.001$, $r^2=0.1033$)
CHAPTER V

DISCUSSION

Effect of Prewintering Temperatures on Emergence

Although it is understood that warmer temperatures during winter and spring can advance emergence (Bosch & Kemp 2000, Bosch 2000), and that individuals must have completed a requisite number of degree-days to successfully emerge (Sgolastra 2010), the possible effects of a low thermal cue during the short prewintering period after adult eclosion on emergence was until now unexplored. Only the intermediate thermoperiod of 10°C had a statistically significant effect on reducing time to emergence ($\mu=5.8$ days). The 22°C control group on the other hand, experienced what is effectively a longer “summer” which in turn lengthened the time to emergence ($\mu=12.9$ days). This partially disagrees with the findings of prior studies which saw that bees held at summer temperatures for a longer period of time emerged sooner than bees wintered earlier by more than two days (Sgolastra 2011). None of the groups tested in this study had any effect on post-emergence vigor.

In prior studies, higher summer temperatures resulted in decreased prepupal development and earlier emergence, with emergence time decreasing until reaching an average summer temperature of 32°C, at which point it began increasing again (Bosch & Kemp 2000, Kemp 2005). However, longer durations of summer temperature post-eclosion resulted in lower survival rates (77.6% for long summer vs 98.2% and 96.4% for short and middle-length summers respectively) in other studies (Sgolastra 2011). This
can lead one to conclude that there exists a need of an intermediate temperature which is required to most effectively initiate winter diapause and progression through wintering development. This also lends further credence to the findings in Bosch 2004 that winter diapause in *Osmia* is similar to the dynamic model proposed by Sawyer (1993). The dynamic model posits that in earlier stages of development, response to temperatures is low, and that as development continues this responsiveness to thermal conditions increases. This information could be extremely useful to growers and agriculturalists who wish to better synchronize bee emergence and post-emergence vigor in anticipation of earlier blooming seasons.

**Evidence for Discontinuous Gas Exchange**

The form and physiology of respiratory systems found in insects consist of trachea extending from valve-like spiracles located along the external surface of the abdomen, branching repeatedly as it reaches towards the interior of the insect’s body until it interfaces directly with intracellular tissues (Chapman 1998). In this morphology, a respiratory pattern has been observed across the taxonomic orders such as honeybees, ants, flies, beetles and moths, called discontinuous gas exchange (DGE). DGE is defined as a characteristic sequence of three respiration events induced by spiracle activity and CO$_2$ concentration (Quinlan 2006). These three phases are commonly referred to as “open”, “closed” and “fluttering” gas exchanges. The periodic bursts of CO$_2$ activity seen while performing respirometry testing in this study were described by
Lighton (1996) as classical DGE. Over the years, multiple potential hypotheses have been put forward to explain the phenomena ranging from a method to conserve water loss (Kestler, 1985) or reducing oxidative stress (Hetz 2005), or even as a means to facilitate breathing in low oxygen environments such as those found underground (Lighton, 1998). However more recent studies have shown that water loss during open respiration cycles is negligible, and that many species of insects which experience extreme desiccation do not exhibit DGE at all (Lighton 2007).

In the experiments on metabolic rates in response to prewintering temperature treatments it was observed that groups tested below 22°C were more than 2 orders of magnitude below the rates reported in prior studies (Sgolastra 2011). The only conclusion one can draw from this is that the data collected were more than likely a measure of simple gas diffusion through the body of the insect. Despite increasingly lower prewinter treatment conditions, the differences in metabolic activity seen across those treatments was not statistically significant. The only exception was the 22°C constant temperature control group, which was also the only group tested at higher temperature. To explore this connection between testing temperature and observed metabolic rates, a series of tests was performed in a subsequent experiment which showed that observed metabolic rates can be drastically altered by the temperature at which the test is conducted. Because of this, when performing metabolic experiments at temperatures below 22°C, flow-through respirometry will likely be insufficient to accurately capture the true metabolic data unless performed over the course of many hours. In preliminary tests I performed, it was seen that at lower temperatures
(between 0°C and 14°C), breathing events might only occur once every 8 hours. Because of this very long delay between breathing cycles, the potential to sample data from the time in between DGE cycles is high.

Furthermore, this also implies that care must be taken when conducting flow-through respirometry experiments on *O. lignaria* at temperatures below 22°C. When respirometry data were collected for a longer duration as part of the “effect of testing temperature on metabolic rate” experiments shortly after the beginning of winter temperatures, it was observed that metabolic rate is tightly correlated with the number of open breathing events per hour. There appears to be an exponential relationship between the metabolic rate and the temperature at which the data itself were collected. With the discovery of temperature-mediated DGE in *O. lignaria*, the number of open breathing cycles at a given temperature must be considered before accepting that metabolic data collected is in fact representative of the true metabolic rate.

**Washington *Osmia lignaria* supercooling capabilities**

As winters reach their coldest points in the Pacific Northwest, ectotherms like *O. lignaria* are subjected to temperature extremes well below the point at which water freezes. To survive, insects use a combination of survival methods, both behavioral as well as physiological. Washington *O. lignaria* had an average temperature of crystallization (T_c) of -26.4°C (±0.6), which is below the values reported for Utah *O. lignaria* (-18.4°C ±2.0, Sheffield 2008). Although this difference could be simply
explained by genetic drift or gene flow with populations from nearby colder climates, the fact that progeny from California were found to differ developmentally from progeny from Utah in a common-garden experiment (Pitts-Singer, 2013), necessitates further exploration of the various freezing points of these insects.

Temperature of crystallization was seen to be lower in larger bees (Fig. 10), although this runs counter to findings in other Hymenoptera which showed that supercooling capability decreased with larger body sizes (Hahn, 2008). The best model given the tested parameters resulted in an $r^2$ of 0.1033, and it is possible that this slight negative trend could change with additional factors that were not previously considered like geographic origin, thermal hysteresis, or the simple osmotic concentration of fluids within the body of the insect.

Implications for growers and need for additional research

By taking the information already available on *O. lignaria* into account, such as the required number of degree days to complete diapause, and the optimal temperature conditions during summer and winter development, this study can help better explain the effect of low temperature cues on this beneficial native pollinator. The fact that bees which experienced a prewintering thermoperiod of 14:10°C emerged 2 days sooner on average than both the other thermoperiods as well as controls shows that a period of intermediate temperature may help facilitate early emergence. Climate change has presented a very real danger of disrupting the mechanisms that insects rely
on, resulting in reduced fecundity (Irwin 2000), and decreased ability to establish adequate energy reserves to survive warmer winters (Musolin 2003). With the onset of cold temperatures serving as a kind of check to ensure all members of the species can more successfully emerge in time to pollinate and reproduce the following year, *Osmia lignaria* appears to have some resistance to the effects of longer summers (Sgolastra 2011). Although warmer winter temperatures could potentially accelerate the time to emergence, a cold temperature is required at some point after adult eclosion to successfully terminate the maintenance phase of diapause (Bosch 2010). We can therefore conclude that an intermediary temperature condition between warm summer and cold winter temperatures will yield the best results in terms of emergence for growers and agriculturalists alike, at no expense of longevity.

Additionally, due to the very low freezing point (-26.4°C) seen in Washington *O. lignaria*, the potential for subfreezing cold storage may be possible as a means for long term storage with minimal mortality. The findings of Bosch 2003 showed that bees which had been overwintered at 0°C could withstand wintering durations of at least 210 days, which was longer than any above freezing condition tested in the same study. Considering that these tests were performed on Utah bees with higher freezing points than Washington bees, I feel it is not unreasonable to suppose that the lethal temperature for Washington *O. lignaria* may also be similarly depressed. Future experiments could investigate this potential avenue for extreme cold-holding.

Furthermore, there is scant research into what exactly drives the low freezing point of these bees. The strong significance, but relatively low $r^2$ due to body size alone suggests
that there is another, stronger contributing factor to the low freezing point seen in Washington *O. lignaria*. Whether this is simply a matter of sugar concentration or thermal hysteresis remains to be seen and is an excellent opportunity for further research.
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