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Maternal Effects and Management of Alfalfa Leafcutting Bees

Makenna May Johnson
Utah State University

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MATERNAL EFFECTS AND MANAGEMENT OF

ALFALFA LEAFCUTTING

BEES

by

Makenna May Johnson

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biology

Approved:

Karen Kapheim, Ph.D.
Major Professor

Theresa Pitts-Singer, Ph.D.
Committee Member

Kezia Manlove, Ph.D.
Committee Member

D. Richard Cutler, Ph.D.
Vice Provost for Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2022
ABSTRACT

Maternal Effects and Management of Alfalfa Leafcutting Bees

by

Makenna Johnson, Master of Science

Utah State University, 2022

Major Professor: Dr. Karen M. Kapheim
Department: Biology

Solitary bees are recognized for their ability to pollinate native plants and crops efficiently, but wide-ranging variations in life history between species make them difficult to manage, especially at commercial scales. Pollination occurs as females provision pollen and nectar for offspring in mass provisions. The outcome of the offspring then affects pollination the following year. Most bees, and other insects, halt their development during periods of unfavorable climate or lack of resources through a process called diapause. Timing of when offspring complete development, and emerge as adults, is variable within and between species. Variations occur via external factors like photoperiod, temperature, and weather cues, as well as internal factors from genetic cues. Management techniques can also have an impact on reproduction and offspring outcome.
though. This issue is particularly relevant in the management of the alfalfa leafcutting bee (ALCB), *Megachile rotundata*, the most highly managed solitary pollinators. In some populations of ALCBs, not all offspring enter diapause, but emerge as adults in late summer, the same year they were laid as eggs. It has been posited that this loss of pollinators is due to maternal effects, though the effects and mechanisms behind it is unknown. We investigated this loss using a common management practice of cool stalling to align ALCB emergence and crop bloom. We hypothesized this practice of cool stalling bee emergence to align with crop bloom, along with external factors like photoperiod and time of season, has led to the loss of offspring through a maternal effect.

Using a field experiment, we found strong effects of photoperiod and time of season on the number of offspring produced and proportion of offspring that entered diapause. We found that cool stalling affected maternal nutritional condition by significantly reducing abdominal lipid stores. Females that experienced longer cool stalling produced fewer offspring, constructed smaller mass provisions, and produced fewer diapausing offspring. Females that experienced longer periods of cool stalling produced fewer female diapausing offspring. We found one mechanism of maternal effects to be mass provisions. Maternal nutritional condition was evident in offspring via size differences of mass provisions. Our results provide insight into how management techniques affect these solitary bees and the next generation of pollinators.

(79 pages)
PUBLIC ABSTRACT

Maternal Effects and Management of

Alfalfa Leafcutting Bees

Makenna May Johnson

Most bees are solitary and important contributors to pollination of a variety of crops. Solitary bees do not live in colonies. Instead, females work individually to construct nests and provision nutrients for each offspring. However, solitary bees can be difficult to manage, especially on a large scale like honey bees, because of differences in how species provision for their offspring and construct nests. Variations in the timing of offspring development to adulthood even within species makes commercial management difficult. Variations in reproductive strategy, like how many offspring to make, how much provision to give each, and when to make them can be influenced by external factors like changes in day length or temperature. Variations in reproductive strategy can also occur based on the maternal environment, or condition of the mother and what she experiences. Some bees halt their development when resources are scarce or weather is not favorable entering a state called diapause. In one species of solitary bees, the alfalfa leafcutting bee (ALCB), *Megachile rotundata*, some offspring forgo pausing their development and instead emerge as adults the same generation they were born. This results in a loss of pollinators for the following growing season.

ALCB are the most managed solitary species and one common management practice for ALCBs is to slow their development in the spring to better align crop bloom
and bee emergence, though the effects of this practice are not well known. This is done by placing bees, after they complete diapause, into cool temperatures to slow their development. From preliminary data we found that cool stalling decreases maternal nutritional condition by reducing the amount of lipids. We hypothesized that the loss of bees in the same season they were laid is due to a maternal effect as a result from the practice of cool stalling, but what this maternal effect is and how it passes to offspring is unknown.

We set up an experiment to investigate the effects of maternal nutritional condition on offspring outcome and diapause. We found that external factors like photoperiod and time of season were always significant predictors of offspring development and outcome. We found that females that experienced longer times in cool stalling had less lipids, produced fewer offspring and made smaller provisions for their offspring. These females that experienced longer cool stalling also made fewer diapausing offspring, specifically fewer female diapausing offspring. We also found maternal nutritional condition reflected in offspring development and outcome through differences in mass provision sizes. Cool stalling decreases maternal nutritional condition and the poor condition leads to less offspring overall and specifically fewer female bees, pollinators, for the next season.
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I want to thank my advisor and mentor Dr. Karen Kapheim for giving me the resources, encouragement and space to grow into the scientist I am today. Thank you for being my biggest advocate and dear friend. Dr. Kezia Manlove taught me how to approach statistics for biological research and was an invaluable intellectual resource as a member of my committee. I would also like to thank Dr. Theresa Pitts-Singer for her unwavering support both mentally and often times physically, by providing the boots on the ground and equipment for this research to transpire.

I would like to thank every undergraduate at USU who helped in the field and in the laboratory with this project. I would like to thank the USDA Bee Lab for their hard work and support, in the field, laboratory, and cheering me on. I would like to recognize USDA NIFA for funding my project and NSF GRFP for funding me personally.

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CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>PUBLIC ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>I. Background: Alfalfa Leafcutting Bees, the Most Managed Solitary Bee</td>
<td>1</td>
</tr>
<tr>
<td>Thesis Background: Experimental Design</td>
<td>5</td>
</tr>
<tr>
<td>II. Maternal Effects and Management of Alfalfa Leafcutting bees</td>
<td>8</td>
</tr>
<tr>
<td>Introduction</td>
<td>9</td>
</tr>
<tr>
<td>Methods</td>
<td>15</td>
</tr>
<tr>
<td>Analysis</td>
<td>23</td>
</tr>
<tr>
<td>Results</td>
<td>23</td>
</tr>
<tr>
<td>Discussion</td>
<td>47</td>
</tr>
<tr>
<td>Conclusion</td>
<td>52</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>54</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>55</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Experimental design</td>
<td>7</td>
</tr>
<tr>
<td>1.2. Nest cells assigned to reproductive females</td>
<td>7</td>
</tr>
<tr>
<td>2.1. Pollen balls</td>
<td>46</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Timing of field projects</td>
<td>6</td>
</tr>
<tr>
<td>2.1. Life cycle of the Alfalfa Leafcutting Bee</td>
<td>14</td>
</tr>
<tr>
<td>2.2. Floral resources</td>
<td>22</td>
</tr>
<tr>
<td>2.3. Abdominal lipids of newly emerged female bees</td>
<td>31</td>
</tr>
<tr>
<td>2.4. Abdominal lipids of reproductive female bees</td>
<td>33</td>
</tr>
<tr>
<td>2.5. Total nest cells per female</td>
<td>34</td>
</tr>
<tr>
<td>2.6. Mass provision size</td>
<td>36</td>
</tr>
<tr>
<td>2.7. Relationship between offspring body size and mass provision size</td>
<td>38</td>
</tr>
<tr>
<td>2.8. Frequency of diapausing offspring</td>
<td>40</td>
</tr>
<tr>
<td>2.9. Relationship between mass provision size and offspring sex</td>
<td>41</td>
</tr>
<tr>
<td>2.10. Offspring sex ratio</td>
<td>42</td>
</tr>
<tr>
<td>2.11. Offspring abdominal lipids</td>
<td>44</td>
</tr>
</tbody>
</table>
CHAPTER ONE

Background: Alfalfa Leafcutting Bees,

The Most Managed Solitary Bee

Humans have been utilizing bees, particularly honey bees (*Apis mellifera*), since early Neolithic times (Roffet-Salque et al., 2015). Most of this has involved honey bees, but solitary bees compose the majority of bee species and are better pollinators of native plants and some crops (Calderone, 2012; Cane, 2002; Cane & Schiffhauer, 2003; Garibaldi et al., 2014; Javorek et al., 2002; Richards, 1995; Soroka et al., 2001). Managing solitary bees can be difficult due to the many differences in life history between species. One solitary bee however, the alfalfa leaf cutting bee (ALCB), *Megachile rotundata*, is used around the United States as a pollinator of alfalfa crops. ALCB are the most intensely managed solitary bee and are second only to honey bees in agricultural importance (Calderone, 2012; Pitts-Singer & Cane, 2011). Native to southeastern Europe and southwestern Asia, ALCB are thought to have been introduced into North American sometime in the 1930s, possibly multiple times (G. E. Bohart, 1970a; Kemp & Bosch, 2000). Around this same time, the use of alfalfa as feed for cattle grew along with the need for an efficient pollinator of the crop for seed production, and management of the solitary ALCB began.

ALCB are efficient pollinators of alfalfa. Pollination of the alfalfa flower occurs when a pollinator triggers the staminal column, causing the stamen to slam into the pollinator, ‘tripping’ the flower and covering the pollinator in pollen. The pollinator can then access the nectar and continue on to other flowers (Cane, 2002; Tysdal, 1940; Vansell & Todd, 1946). Honey bees are not efficient pollinators of alfalfa, being large
enough to avoid triggering the staminal column of the alfalfa flower and steal nectar without receiving the pollen load (Cane, 2002). In a previous study, female ALCB tripped 78% of alfalfa flowers they visited while honey bee foragers tripped only 22% of flowers visited (Cane, 2002a).

In addition to their pollination efficacy of alfalfa, the solitary ALCBs nest in aggregations, above the ground in small hallowed out cavities (Kemp & Bosch, 2000). This grouped nesting can be replicated in artificial nesting areas, such as holes drilled into wooden boards (G. E. Bohart, 1970a) and can be scaled up to a commercial scale for production of ALCBs to be sold as pollinators.

Female ALCB pollinate alfalfa to provision for their offspring. As their name implies, females use their specialized mandibles to cut semicircular pieces from alfalfa leaves. They use these leaf pieces to line a nest cavity, creating a small capsule of leaves referred to as a nest cell (Wedmann et al., 2009). The leaf lined capsule is then filled with a mixture of pollen and nectar, known as a mass provision. The mass provision is made of a mix of nectar and pollen. At the beginning of making the mass provision, it is composed mostly of protein rich pollen (80% pollen to 20% nectar). By the time of completion however it is composed mostly of nectar (64% nectar and 36% pollen). This is done by the mother to account for the changing protein needs of developing offspring as it goes from egg, needing mostly sugars, to larvae, needing more protein (Cane et al., 2011; Klostermeyer, E.C., Mech, Stephen J., Rasmussen, 1973). An egg is then laid directly on the mass provision and a circular leaf piece used to seal the leaf capsule, completing one nest cell. The process is repeated until the nest cavity is filled and is plugged with many circular leaf pieces (Michener, 2007; Pitts-Singer & Cane, 2011).
Based on timed floral trips from previous studies (Gerber & Klostermeyer, 1972; Klostermeyer & Gerber, 1969) in order for females to make the average of 1-2 nest cells each day (Klostermeyer & Gerber, 1969), they need to visit, and end up pollinating, thousands of flowers. It takes an average of 7 hours 37 minutes to make one complete nest cell, 2 hours 27 minutes of nest construction and 5 hours and 3 minutes of provisioning (Klostermeyer & Gerber, 1969). Estimations by Cane et al. 2011 found a minimum of around 400 flowers is required to provision just one nest cell, however they posit that this number might be an underestimation with as many as 2000 flowers required for construction of a single nest cell (Cane et al., 2011).

In addition to large amounts of floral resources necessary for nest cell construction, external factors such as photoperiod, temperature, and weather play a crucial role in variations in reproductive strategy and thus offspring development and outcome (Beck, 1968; Jain & Brockmann, 2018; Pitts-Singer, 2020; Pitts-Singer & James, 2008; Sgolastra et al., 2010; Tasei & Aupinel, 2015; Villagomez et al., 2021; Walton et al., 2011). Previous studies investigating photoperiod, temperature, resource availability, and diapause conditions show that these are crucial in offspring development and outcome across insect species (Yocum et al., 2018; Hobbs & Richards, 1976; Kemp & Bosch, 2001; Klostermeyer, E.C., Mech, Stephen J., Rasmussen, 1973; Peterson & Roitberg, 2006; Pitts-Singer, 2020; Pitts-Singer & Bosch, 2010; Pitts-Singer & Cane, 2011; Tepedino & Parker, 1986; Undurraga & Stephen, 1980; Wilson et al., 2021; Yocum et al., 2021). Other factors such as maternal condition play a major role in offspring outcome and development (Fox & Savalli, 2000; Kim & Thorp, 2000; Leftwich et al., 2019; Moore et al., 2019; Mousseau & Dingle, 1991a, 1991b; Mousseau & Fox,
Mothers influence their offspring in a number of ways, for example, in some cases, determining the number of offspring, resources an offspring receives, time of season offspring are produced and the environment that they are produced. These maternal decisions that affect multiple generations are known as maternal effects (Mousseau & Fox, 1998a, 1998b). In our study we investigated the influence of external factors and maternal effects on offspring development and outcome possibly induced by commercial management practices of ALCB.

One issue with managing solitary species like ALCBs is the variability of if they enter diapause or not. Diapause is a cessation in development to account for poor environmental conditions like unfavorable weather or lack of resources. (Denlinger, 2003, 2008; Santos et al., 2019). Populations of ALCB in the Western United States, some offspring do not enter the period of diapause and instead complete development to adulthood and emerge in late August instead of entering diapause to emerge the following spring (Tepedino & Parker, 1986). This summer generation could be useful in extending the current season, but does not appear to add overall to the population as it is a loss of potential pollinators for the following year (Bohart, 1963).

One management practice in ALCB is to stall development by placing developing adults into cool temperatures (18 °C) for a period of up to 3 weeks when there is going to be a miss match in bee emergence and crop bloom (Tepedino & Parker, 1986). From a pilot study, we found that nutritional condition of the mother was affected by this management practice to align bee emergence with crop bloom (Pitts-Singer & Boyle, unpublished data). We hypothesized that loss of generation in the summer is due to
maternal effects of poor maternal nutritional condition induced by the management practice of cool stalling.

**Thesis Background: Experimental design**

We spent three field seasons investigating the possible maternal effects of nutritional condition induced by the management practice of cool stalling during adult development for crop alignment and bee emergence. After the first year, we conducted the experiments in two parts using a 2x2 design to better capture changes in photoperiod (see table 1.1). The first two years of the study photoperiod did not align, so no direct comparisons could be made between the two. The final year encompassed a larger photoperiod range (see figure 1.1).

A critical step in determining the maternal effects on offspring development and outcome were the behavioral observations of reproductive females in our cage study. After the first year, 2018, we were unable to assign a majority of offspring to mothers and so increased the frequency of our observations the following years (see table 1.2).

Using the information from 2018 and 2019, we were able to conduct a more comprehensive experiment in 2020. The results discussed in chapter 2 are from 2020 only.
Figure 1.1

Timing of Field Projects

Figure 1.1. The photoperiods from 2018 and 2019 did not overlap and we cannot compare them to each other. The final year 2020 aligned to encompass photoperiods near the summer solstice (represented by the sun) and then decreasing photoperiods. Across the experiment in 2018 photoperiod increased by 95 minutes, in 2019 photoperiod decreased by 88 minutes and in 2020 photoperiod decreased by 104 minutes.
Table 1.1

Experimental Design

<table>
<thead>
<tr>
<th>EARLY SEASON</th>
<th>LATE SEASON</th>
</tr>
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<tbody>
<tr>
<td>Control (1 day)</td>
<td>Control (1 day)</td>
</tr>
<tr>
<td>Cool-stalled (14 days)</td>
<td>Cool-stalled (14 days)</td>
</tr>
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A 2x2 design of the experiments in the 2019 and 2020 field seasons to encompass changes in photoperiod.

Table 1.2

Nest Cells Assigned to Reproductive Females

<table>
<thead>
<tr>
<th>YEAR</th>
<th>TOTAL NEST</th>
<th>NO. NEST CELLS</th>
<th>PERCENT ASSIGNED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CELLS</td>
<td>ASSIGNED</td>
<td></td>
</tr>
<tr>
<td>2018</td>
<td>2,790</td>
<td>1,521</td>
<td>54.5%</td>
</tr>
<tr>
<td>2019</td>
<td>1,194</td>
<td>1,113</td>
<td>93.2%</td>
</tr>
<tr>
<td>2020</td>
<td>1,563</td>
<td>1,277</td>
<td>81.7%</td>
</tr>
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</table>

Total number of nest cells assigned to reproductive females. 2018 had poor nest cell ownership assignment. Frequency of behavioral observations were increased in 2019 and 2020 and more nest cell ownership assignments were made.
CHAPTER TWO

Maternal Effects and Management of

Alfalfa Leaf Cutting Bees

Abstract

Solitary bees are important pollinators of not only native plants, but also crops. However, variations in life history across species make it difficult to manage solitary bees commercially. For example, species differences in reproductive strategy affects pollination performance and many environmental factors can affect pollination efficacy. In solitary bees, females pollinate crops when feeding themselves, but mainly provisioning for their offspring. In turn, offspring development and outcome have impacts on the next generation of pollinators. One development outcome that varies between species is whether or not offspring diapause and the timing relative to crop production. Offspring sex ratio can also impact pollination positively or negatively, depending on which way the sex ratio is skewed. Both diapause timing and sex ratio are influence by environmental factors, such as photoperiod and resource availability, but are also likely influenced by maternal effects. The issues of pollination efficacy are particularly relevant to the management of the solitary alfalfa leafcutting bee (ALCB), *Megachile rotundata*. ALCBs are the most economically valuable and highly managed solitary bees. In some populations of ALCBs, some offspring skip diapause and complete their development to adulthood the same year that they were laid as eggs. It has been hypothesized that this is due to a maternal effect, though the mechanisms are unknown. We investigated the effects of a management practice called cool-stalling,
which is used to align bee emergence and crop bloom in ALCBs that we found to reduce maternal lipids. We hypothesized that poor nutritional condition, induced by managed cool-stalling, caused the loss of progeny. We found overall that photoperiod was always a significant predictor of offspring outcome. We also found females that experienced prolonged cool stalling had lower fecundity by producing significantly fewer nest cells overall. Females that experienced prolonged cool stalling made significantly smaller mass provisions for their offspring. Mass provisions appear to be one mechanism by which maternal nutritional condition is passed to offspring. Females that experienced the prolonged cool stalling made significantly fewer diapaus ing offspring, affecting the number of pollinators available the next year. Of those offspring that did enter diapause from females that experienced prolonged cool, there were significantly fewer females. Managing solitary bees as pollinators is difficult and management techniques can have negative consequences on generations of pollinators.

**Introduction**

Solitary bees are critical pollinators, but management of them for wide-spread pollination is difficult. There has been a recognition that we need other pollinators, in addition to honey bees, particularly solitary bees that are better suited to pollination of some crops and native plants (Bänsch et al., 2021; Batra, 1997; Bohart, 1970a, 1970b; Garibaldi et al., 2014; Kline & Joshi, 2020; Wood et al., 2017). Managing solitary pollinators however has come with a host of obstacles due to variations in life history across species and plasticity within species of reproductive strategy. Environmental cues
cause plasticity within individual species in reproductive strategy, which can affect pollination.

One of the most common cues from the environment that affect insect reproductive patterns is photoperiod. Increasing or decreasing daylengths can lead to shifts in behavior (feeding, mating, and oviposition), development (emergence, egg eclosion, pupation, and ecdysis), physiological rhythms (central nervous system, sensory system, neurosecretory, and endocrine systems functions), overall growth (growth rates, pigmentation and body form) to diapause (Beck, 1968; Bradshaw & Holzapfel, 2007; Jain & Brockmann, 2018; Pitts-Singer, 2020; Villagomez et al., 2021; Walton et al., 2011). In *Bombus terrestris* queens, photoperiod caused delayed oviposition. This resulted in a lower proportion of queens that produced offspring that then became queens (Tasei & Aupinel, 2015). Longer photoperiods, adequate weather, and resources, means more time to forage and provision resources to offspring in social bees like *Apis mellifera*, bumble bee species and solitary bees like *Megachile rotundata* and *Osmia cornuta* (Nielsen et al., 2017; Peterson & Roitberg, 2006; Pitts-Singer & Bosch, 2010; Vicens & Bosch, 2000). Most bees go through a halt in development during inclement weather or lack of resources, referred to as diapause, that is particularly variable between species (Denlinger, 2008; Santos et al., 2019; Tepedino et al., 2022). For example, bees can diapause at any life stage from egg, larvae, pupa or adult. Diapause is highly variable and plastic not only between species, but within species in response to cues like photoperiod (Denlinger, 2003).
In addition to photoperiod, resource availability affects offspring outcome in many bee species. The size of the provision, mass provisions, is linked to offspring sex ratio in *Osmia lignaria*, solitary bees utilized for orchard pollination, smaller mass provisions were correlated with males (Torchio & Tepedino, 1980). Mass provision size is also correlated with diapause outcome in alfalfa leaf cutting bees (ALCB), *Megachile rotundata*, with offspring that diapause being found on larger mass provisions (Fischman et al., 2017). Maternally gathered mass provisions have lasting effects on offspring.

Other factors in addition to photoperiod, temperature and resource availability influence variation and plasticity of offspring development and outcome within species. In the red mason bee, *Osmia bicornis*, used for pollination of orchards, photoperiod has no effect on diapause outcome (Wasielewski et al., 2013). While in alfalfa leaf cutting bees (ALCB), *Megachile rotundata*, used for alfalfa pollination for seed production, temperature within nest cells has no effect on diapause outcome (Wilson et al., 2021). Other factors are maternal environment. In some species, the mother influences offspring safety, nutrition and provisioning, and when and where they will develop (Mousseau & Fox, 1998b). This influence from mother to offspring happens physiologically, behaviorally, morphologically, or anatomically (Russell & Lummaa, 2009). It is not only the “decisions” made by the mother during offspring care and development, but also, the condition of the mother, such as body size and nutritional condition. For solitary bees that mass provision for their offspring, the condition of the mother has lasting effects on the outcome of the offspring because the environment the offspring experiences is the mass provisions left by their mother (Räsänen & Kruuk, 2007). In ALCB particularly, the offspring outcome to diapause has been found to be influenced by maternal effects.
(Parker & Tepedino, 1982; Parker, 1988; Pitts-Singer & Cane, 2011). However, it is unknown what the maternal effects are and the mechanisms by which maternal environment and condition are passed to the offspring.

The ALCB is the most intensively managed solitary bee species (Calderone, 2012; Pitts-Singer & Cane, 2011) and is an effective pollinator of a wide variety of crops, but are particularly utilized as pollinators of alfalfa crops for seed production (Cane & Schiffhauer, 2003; Javorek et al., 2002; Pitts-Singer & Cane, 2011; Richards, 1995; Soroka et al., 2001). One common management practice to maximize their pollination efficiency is to align crop bloom with bee emergence. This is done by placing post-diapausing offspring into a cool temperature (18°C), thus stalling their development, but not causing them to enter a state of diapause again (Johansen & Eves, 1973) (see figure 2.1A) to better align with alfalfa bloom. ALCBs are a univoltine species, meaning having one generation per season, but in the Western United States, some offspring do not halt their development and instead complete development to adulthood the same season they were laid as eggs (Tepedino & Parker, 1986) (see figure 2.1B). Previous studies of ALCB diapause have found photoperiod and temperature to influence offspring outcome and development, but conclude that the second generation of offspring in the same season is due to a maternal effect (Earls et al., 2021; Parker & Tepedino, 1982; Pitts-Singer & Cane, 2011; Wilson et al., 2021). We hypothesized the management practice of cool-stalling influences offspring outcome and development via maternal nutritional condition (see figure 2.1B).

We used the well-established system of ALCB to investigate how maternal effects interact with environmental conditions to influence reproductive strategy and offspring
developmental outcomes. Specifically, we investigated how nutritional condition is altered by cool-stalling practices, and what outcome this has on reproductive strategy and offspring development. We investigated the maternal effects of cool stalling up to 14 d, as the extreme amount of time required to account for asynchrony of crop bloom and bee emergence, to fill the knowledge gap about the mechanisms of maternal effects in solitary pollinators. We predicted that 1) photoperiod, time of season and nutritional condition of the mother will have an effect on reproductive strategy, offspring development and diapause outcome. 2) a mechanism for maternal effects would be able to be detected through effects of photoperiod and time of season.
Figure 2.1. Alfalfa leafcutting bees overwinter as prepupae. Once warmer temperatures are experienced, development continues to pupa and then adult. Adult bees are released into fields to pollinate crops and construct nests cells and lay eggs. A) the management technique of cool-stalling happens during this period of post-diapause development in the life cycle. B) Some offspring do not remain a prepupae and enter diapause, but instead continue development to an adult, emerging in late summer or early fall of the same year they were laid as eggs.
Methods

Pre-field study preparation

Prepupae diapausing in cocoons were received in October 2019 from farms near Logan, Utah in Cache and Box Elder counties. The cocooned bees were examined using x-radiography and sorted to remove parasites, prior to cold storage for diapause through the winter season. Bees were placed into cold storage, a walk-in refrigerator at 4°C for 8 months. Diapausing bees were then removed from cold storage and placed into incubators at 29°C for 17 days. After 17 days of development at 29°C, bees were moved to an incubator for cool stalling treatment at 18°C for either 1 or 14 d, with the control being 1 d cool interruption. After the allotted time of cool stalling, bees were moved back into 29°C incubation until emergence. Removal from winter temperatures was staggered for all bees. This was done so that bees that experienced 14 d in cool stalling emerged around the same time as the 1 d control. Then all bees could be released into field cages simultaneously. Bee emergence was staggered so that the field experiment took place in two parts, early season and late season, to encompass changes in photoperiod.

Upon emergence, females were collected from both the 14 d cool stalling and the 1 d control. They were all photographed, then frozen at -80°C for analysis of lipid content. A total of 232 newly emerged females were collected for lipid analysis from the same group of emerging bees that were released into field cages. In early season, 66 females were collected from the control and 14 d treatment groups each. In late season, 45 females were collected from the control and 55 females from 14 d.
Field study

In our study, photoperiod encompassed the changing dark and light regimes that existed at the study site, while “time of season” encompassed other external factors like temperature, floral resources, and pest presence that change with the progress of time through the season (Allen et al., 2018; Pellissier et al., 2017; Strickler & Freitas, 1999). We used 16 mesh field cages (6.1 X 6.1 X 1.8 m) wet up in a alfalfa field located in Logan, Utah (41.7652291, -111.8145105). Cages were arranged in two rows of 8, on the east and west of the field. We used half of these cages for experiments in the early season (22 June-8 July 2020) and half in the late season (8 July-18 July 2020). Active cages were staggered between the east and west rows to account for possible differences in placement, though nest blocks all faced east. The field was treated with generic chlorpyrifos pesticide and Lambdastar pesticide 17 days prior to bee release. Newly emerged females were randomly assigned to field cages (1-16) and colored marked using enamel paint (Testors Enamel Paint) with 2 horizontally aligned colored dots on the thorax for a unique identification. This color coding allowed the treatment groups to be kept unknown to observers (with the exception of a few observations done by myself). In each field cage (16 total cages), a total of 16 females were released, half from 1 d and half from 14 d cool stalling treatment. Bees were released for the first two days of early and late season. Males and females were released simultaneously at a rate of 2:1 male to female. This ratio was used to allow for adequate chance of fertilization, but without increased risk of male harassment (Rossi et al., 2010).

Every cage had a navy-colored box (23 cm x 25 cm) open on the east facing side, secured in the center of the cage, for protection from sun and rain for the nest block. Nest
blocks (8 cm x 8 cm) consisted of 7 Styrofoam wafers with 7 holes that fit paper straws (9.5 cm long, 2 cm diameter). Each of the 7 wafers were stacked on one another and secured while in the field to form a 7x7 hole grid, with each hole filled with a paper straw to allow for removal of nests for data collection. These nest blocks could be collected in the evenings, the wafers disassembled, and imaged using x-radiography. These images provided an image of developing offspring with minimal disturbance. X-rays of nest blocks were done three times a week.

Behavioral observations were conducted 3 times per day at 10 am, 1 pm, and 4pm every day for the duration of the field study. We recorded all occurrences of the following behaviors within a 20 min period: leaf-a female carrying a cut leaf piece into the nest hole, pollen- female entering nest hole with pollen visible in the scopa hairs, located on the ventral abdomen, capping- female seen plugging the end of the paper straw in the nest hole with a cut leaf circle, investigating- female sitting on the nest block, in the nest hole or entering into the nest hole with no pollen or leaf pieces. We observed females marking their nests in the days immediately following release. Bees were seen rubbing their abdomen around the opening and inside of the paper straw.

To determine which female constructed each nest cell, nest progression was documented each day after bee activity ceased between 1930-2130. Otoscopes were used to shine through the paper straws and a sharpie used to mark the progression of nest construction for that date. These dates were used in conjunction with the behavioral observations to identify which reproductive female constructed each cell.

We monitored floral resource availability throughout the study using the same method as Pitts-Singer and Bosch 2010 (Pitts-Singer & Bosch, 2010). We avoided treating
alfalfa with additional pesticides once bees were in the cages which resulted in a gradual decrease of available floral resources due to an increase in pests over time (see figure 2.2). As a result, thrips and aphids became so pervasive that alfalfa was watered nightly in every field cage for the duration of the field experiment. The plants were watered with a backpack sprayer, after sunset, to physically remove the insects from the plants, without disturbing the bees.

Temperature and humidity were recorded hourly for the duration of the field experiment using Onset HOBO Data Loggers. During early season, several rainy days disrupted nesting behavior. This is reflected in the temperature and humidity data, as well as nest ownership and a delay in nesting behavior.

*Post-field offspring rearing*

Upon completion of the field study, nest blocks were removed from field cages when activity ceased around sunset, and placed in a protected outdoor area to continue development. After removal of adult bees, nest blocks were placed into a large plastic storage tub and placed outdoors in an open-air structure so the offspring were never exposed to direct sunlight or precipitation, but experienced the outdoor temperatures. The plastic tub was altered to have 2 sides removed and fitted with multiple layers of fine mesh fabric to exclude predators or parasites while allowing air flow. Offspring remained in this outdoor shelter until October when offspring were prepared for overwintering.

In August 2020 there was an influx of parasitic wasps that subsequently parasitized the nest blocks. There is some emerging evidence of plant powders, such as turmeric, as a viable deterrent against *Pteromalus* (Ong et al., 2020). To avoid a complete loss of progeny to the parasitic wasps, turmeric was lightly sprinkled on the bottom of the
storage tub and remained until offspring were placed into cold storage for overwintering. Offspring cells were never in direct contact with turmeric. We did not have many occurrences of parasitism after the turmeric was applied.

At the end of the field experiment, nest blocks were removed from field cages. Each nest block was placed in an individual plastic lidded container. The following day, nest blocks were examined in the laboratory and all adults were removed. Females were placed into labeled 1.5 ml centrifuge tubes and stored at -80 °C until further analysis. After adults were collected from nest blocks, additional females were collected from field cages using nets. These females were placed in individually labeled 1.5 ml centrifuge tubes and placed in a cooler, nestled between layers of cardboard to avoid direct contact with ice packs. Coolers were then transported back to the lab where females were placed into -80 °C until further analysis. All males collected from nest blocks and field cages were released outdoors.

We used x-ray to check the development and life stage of each offspring until October. From the outdoor storage tub, nest blocks were examined every other day by x-ray, until all summer emergences ceased, so that we could remove them before they chewed through other developing cells. Frequency of x-ray examinations of nest blocks decreased to twice a week and then once a week until October to remove any parasites. Offspring that developed into adults and emerged in the summer were identified using x-ray once they pupated. These were then excised from the nest block by carefully slicing open the paper straw directly over the nest cell, extracting the cell and placing it in a gel capsule. The gel capsule was then fixed to a labeled board and placed in incubation at 29 °C. These gel capsules containing developing adult offspring were checked each day for
emergence. Emerging bees were photographed and put into uniquely labeled 1.5 ml centrifuge tubes. Tubes were moved to -80 °C until further analysis.

In October, all remaining offspring in the outdoor storage tub were excised from nest blocks and placed into gel capsules, labeled and attached to boards. These boards were then put into cold storage at 4°C for overwintering. Offspring remained in this cold storage in diapause until June the following year. Once removed from cold storage the following year, bees were placed into incubation at 29 °C until emergence. Developing offspring were checked daily and processed in the same way as described above for summer emergence offspring.

*Laboratory data collection*

**Intertegular width**

Newly emerged females for lipid assays and females to be released into field cages were photographed upon emergence and intertegular width was measured and used as a size metric (McCabe et al., 2021). Images were measured in fiji imageJ (Schindelin et al., 2012) calibrated to 1 mm using a ruler present in each image. Intertegular widths were also obtained for all surviving offspring that emerged in the summer or diapausing offspring the following year, using the same methods.

**Mass provision size**

Images from x-rays were used and each provision was measured from x-rays taken on the date immediately following when it was complete to ensure measurement of the provision at its largest size before beginning to be consumed by the developing larva. Mass provision size was measured using the same methods described in Fischman et al., 2017 using fiji imageJ (Schindelin et al., 2012). Measurements were done using the
“Freehand selections” tool and measured in square pixels. Each mass provision was traced 3 times and an average taken to produce an average square pixel size (Fischman et al., 2017).

Lipid analysis

Newly emerged, reproductive females, and offspring were removed from the freezer and abdomens cut on dry ice. Due to bees being paint marked, only abdomens were used for the lipid extraction and assay. Lipid extractions were performed using a 2-day chloroform suspension protocol (modified Folch extraction method) (Perkins, 1975; Toth & Robinson, 2005) and then assayed using a spectrophotometer to determine abdominal lipids in grams. Newly emerged bees and reproductive females that were collected from the field were pooled by treatment group, with 2 bees per sample. All offspring were single samples due to the fact that by pooling we would lose many samples from needing to remain within cage, mother and sex of the offspring.
Figure 2.2

Floral Resources

Figure 2.2. Floral resources in early and late season 2020. a) early season floral resources were counted every five days for the duration of the early field experiment. Bloom reached the peak around 27 June 2020. b) late season floral resources measured on the day of bee release. When a sharp decline in resources was recorded at the five-day count, a final count was conducted two days later. In most cages, floral resources were well below the threshold for sufficient resources and the project was ended.
Analysis

All statistics were run using R (R-4.1.1) (R Core Team, 2021). Mixed effects models that included the random effects variables of cage and mother were tested first for all analyses. When the variance of random effects variables was near zero, they were removed and the model was rerun with only fixed effects. Akaike information criterion scores were used to determine the best model fit. When scores for two models were close ($\Delta_{\text{AIC}} < 2$), the simplest models were selected. Linear models were used for all lipid analyses. Negative binomial generalized linear models were used for analyzing the time, in days, it took for reproductive females to begin construction of their first nest cell, as well as analyzing the total number of nest cells constructed. Mass provision areas in square pixels were analyzed using linear mixed effects models. Proportion of offspring diapausing or non-diapausing were analyzed using binomial generalized linear models, while the sex ratios of offspring were analyzed using binomial generalized linear mixed effects models. All graphs were generated using ggplot2 in R (Wickham, 2016).

Results

Cool stalling treatment

To test if the prolonged cool stalling treatment was effective in changing female condition, we collected females at emergence for lipid analysis. We found cool-stalling treatment and time of season affected lipid levels at emergence (Figure 2.3; $F_{2,112}=13.02$; multiple R-squared = 0.19; $p = 8.26e-06$; early season $N_{1}=33$, $N_{14}=33$; late season $N_{1}=22$, $N_{14}=27$). Females that experienced prolonged (14 d) cool stalling had an average of 24 % lower total lipids in the abdomen at emergence than females that experienced the 1 d control (Figure 2.3, $t = -4.32$, $p = 3.46e-05$). Females emerging in the late season had 19.6
% more lipids than those emerging in the early season (Figure 2.3, \( t = -2.94, p = 4.02 \times 10^{-3} \)). In early season, 14 d cool stalling led to a 25.9\% decrease in average amount of abdominal lipids, and in late season, a 23.4\% decrease in the average amount of abdominal lipids (Figure 2.3).

The lipid depletion caused by 14 d cool stalling that was evident at emergence did not persist throughout the nesting season. We modeled abdominal lipids in females collected from field cages at the end of the experiment as a function of cool-stalling treatment and time of season (Figure 2.4; \( F_{2,147} = 15.4; \) multiple R-squared = 0.17; \( p = 8.50 \times 10^{-7}; \) early season \( N_{1d} = 46, N_{14d} = 30; \) late season \( N_{1d} = 40, N_{14d} = 34 \)). Females from each treatment collected from field cages at the end of the nesting period did not have significantly different total abdominal lipids (\( t = -1.634, p = 0.10 \)). However, nesting females at the end of the late season cage experiment had 24.4\% lower abdominal lipids than those from the early season (\( t = -5.18, p = 7.11 \times 10^{-7} \)). Likewise, including photoperiod on the day of release in the model instead of time of season yielded similar results. Photoperiod was a significant predictor of total abdominal lipids at the end of the study (\( F_{2,147} = 14.78; \) multiple R-squared = 0.17; \( p = 1.42 \times 10^{-6}; \) early season \( N_{1d} = 46, N_{14d} = 30; \) late season \( N_{1d} = 40, N_{14d} = 34 \)).

**Reproductive Females**

Reproductive females that experienced a long period of cool stalling adjusted their reproductive strategy in several ways. First, females that experienced 14 d of cool stalling had decreased fecundity. In a model that included time of season, cool stalling treatment, and thorax width as a metric for body size, time of season and cool stalling treatment were significant predictors of total number of nest cells (Figure 2.5; \( X^2 (3, \))
N=256) = 42.38; p = 3.34e-09; early season N₁₄d= 52, N₁₄d= 48 reproductive females; late season N₁₄d= 47, N₁₄d= 42 reproductive females). Females that experienced 14 d of cool stalling during development constructed 18% fewer nest cells than females that experienced the 1 d control (z = -2.37, p = 0.02). Within early season there was a 20.3% decrease, and in late season, a 13.7% decrease in the number of nest cells made by females that experienced 14 d of cool stalling compared with those females exposed to only 1 d of cool stalling (z = -6.49, p = 8.73e-11). When using this same model with photoperiod on day of female release in place of time of season, we find that photoperiod on day of female release and cool stalling treatment are both significant factors in determining the number of nest cells constructed (X² (3, N=256) = 41.30; p = 5.64e-09; early season N₁₄d= 52, N₁₄d= 48; late season N₁₄d= 47, N₁₄d= 42). Number of pollen balls, nest cells that had an offspring that died in the early egg and larval stages, were low in both early and late season and similar between cool stalling treatments (see table 2.1).

This difference in fecundity was not driven by differences in the time it took to initiate nesting when comparing females from 14 d cool stalling to females from the control. In a model that included time of season, cool stalling treatment, and thorax width, time of season and thorax width, but not cool stalling treatment, were significant predictors of the time it took females to lay their first egg (X² (3, N= 187) = 37.61; p = 3.42e-08; early season N₁₄d= 52, N₁₄d= 48; late season N₁₄d= 46, N₁₄d= 41). Females in late season laid their first egg an average of 2 days sooner than those in the early season (p = 8.39e-08; early season average 5.61 days to first cell ± 0.34 standard error; late season average 3.52 days to first cell ± 0.12 standard error). Time to first egg decreased with increasing body size, as measured by thorax width (z = -1.98, p = 0.048). Results were
similar when photoperiod on day of release was included in the model instead of time of season, with photoperiod on date of release being a significant predictor of nest initiation, while treatment was not a significant factor, but body size was nearly significant ($X^2 (3, N= 187) = 38.38; p = 2.34e-08$; early season $N_{1d}= 52$, $N_{14d}= 48$; late season $N_{1d}= 46$, $N_{14d}= 41$).

Second, females who experienced 14 d cool stalling produced fewer diapausing offspring than females that experienced the 1 d control. In a model that included time of season and cool stalling treatment, both were significant predictors of the proportion of diapausing offspring ($X^2 (2, N = 124) = 86.082; p < 2.24e-16$; early season $N_{1d}= 36$, $N_{14d}= 33$ reproductive females; late season $N_{1d}= 28$, $N_{14d}= 27$ reproductive females).

Across the entire experimental period, females who experienced 14 d cool stalling had an average of 37.6% ($\pm 0.06$ standard error) diapausing offspring, while control females had 52% ($\pm 0.06$ standard error) diapausing offspring ($z = -2.26; p = 0.024$). Late-season females had a higher proportion of diapausing offspring than early season females ($z = 6.52; p = 7.15e-11$; average proportion diapausing = 0.84 $\pm 0.05$ standard error), but the effect of the cool stalling treatment was significant in both early and late season. In early season there was an 8.2% decrease in the average proportion of diapausing offspring compared to proportion of diapausing offspring from females that experienced 1 d of cool stalling ($z = -2.26; p = 0.024$; average proportion diapausing 1 d = 0.23 $\pm 0.06$ standard error; average proportion diapausing 14 d = 0.84 $\pm 0.05$ standard error). While in late season there was an 8.6% decrease in the average proportion of diapausing offspring from females that experienced 14 d of cool stalling during development compared with those that did not ($z = -2.26; p = 0.024$; average proportion diapausing 1 d = 0.89 $\pm 0.06$ standard error).
standard error; average proportion diapausing 14 d = 0.79 ± 0.08 standard error). In a model with photoperiod on day of female release and cool stalling treatment, both photoperiod and cool stalling treatment were again significant predictors of proportion of offspring that entered diapause \((X^2(2, N = 124) = 86.21, p < 2.2e-16; \text{cool stalling treatment } z = -2.31, p = 0.021; \text{photoperiod } z = -6.51, p = 7.61e-11)\).

Third, females that experienced prolonged cool stalling did not provision their offspring as well as control females. In a model that included time of season, cool stalling treatment and thorax width, the time of season and cool stalling treatment were significant predictors of offspring mass provision size \((X^2(3, N = 1053) = 32.00; p = 5.24e-07)\). Overall, there was a 5% decrease in the average mass provision size made by females that experienced 14 d cool stalling compared to those made by females that experienced 1 d cool stalling \((t = -3.87; p = 1.6e-04; 1 \text{ d average provision mass} = 2747.70 \text{ sq. pixels ± 17.08 standard error, } N_{1d} = 663; 14 \text{ d average provision mass} = 2611.01 \text{ sq. pixels, } N_{14d} = 483)\). Females in the late season made 8.8% larger mass provisions on average than those in the early season \((t = 5.25; p = 4.41e-07; \text{early season average provision mass} = 2629.70 \text{ sq. pixels ± 14.76, } N_{\text{early}} = 847; \text{late season average provision mass} = 2861.16 \text{ sq. pixels ± 24.18, } N_{\text{late}} = 299)\). However, the effects of cool-stalling were consistent across seasons, with mass provisions being 5.12% and 5.14% smaller from experimental females, as compared to control females in the early and late season, respectively. Offspring size reflected mass provision sizes with larger mass provisions leading to larger offspring (see figure 2.7, \(F_{3,300} = 26.82; p = 2.142e-15\); for mass provision \(t = 8.89, p < 2e-16\); for time of season \(t = -3.57, p = 4.19e-04\)). In a model with photoperiod on day of cell construction instead of time of season, photoperiod and
cool stalling treatment were significant predictors of provision size ($\chi^2(4, N = 1053) = 29.42; p = 6.41e-06$).

**Offspring**

Diapause outcome for individual offspring was predicted by mass provision size, time of season and photoperiod, but not maternal cool stalling directly. Among individual offspring, late season and larger mass provisions significantly increased the probability of diapause ($\chi^2(3, N = 526) = 76.30; p < 2.2e-16$). In early season, 8.2% of offspring entered diapause (62 diapausing, 692 not diapausing) while in late season, 58.1% of offspring entered diapause (158 diapausing, 114 not diapausing). This was a significant seven-fold difference in percent of offspring that entered diapause (Figure 2.8, $z= 5.717$, $p = 1.08e-08$). When the same model was analyzed with photoperiod on day of cell construction, photoperiod and mass provision size were the only significant factors in determining offspring diapause outcome ($\chi^2(3, N = 526) = 68.32; p = 9.76e-15$).

We did not find any sex differences in factors influencing diapause outcome. When looking at female offspring only, we found that time of season, and mass provision size were the significant predictors for diapause ($\chi^2(3, N = 194) = 52.40; p < 2.2e-16$). When the same model was run with photoperiod on day of cell construction instead of time of season, photoperiod and mass provision size were significant factors for female diapasing offspring ($\chi^2(3, N = 194) = 43.76; p < 1.70e-09$). When looking at male offspring only, we found that time of season and mass provision size were significant factors in determining diapause outcome ($\chi^2(3, N = 332) = 76.71; p < 2.2e-16$). Similar results were seen when the same model was run with photoperiod on date of cell construction in place of time of season ($\chi^2(3, N = 332) = 72.75; p = 1.10e-15$).
Offspring sex was influenced by mass provision size, along with time of season and photoperiod, but not maternal cool stalling treatment directly. From the perspective of individual offspring, time of season and mass provision size were both significant predictors for offspring sex (male or female) \( (X^2(3, N = 526) = 123.39; p < 2.2e-16) \). In late season 70.3% of offspring were males, while in early season 59.6% of offspring were males (Figure 2.9; early season: 143 females, 211 males; late season: 51 females, 121 males). Similarly, when analyzing photoperiod on day of cell construction in place of time of season, photoperiod and mass provision were significant predictors of offspring sex determination \( (X^2(3, N = 526) = 125.94; p < 2.2e-16) \). Overall, the sex ratio was male skewed with 63.2% of offspring that could be sex identified were male (194 female: 333 male). Probability of offspring sex correlated to provision size with larger mass provisions more likely to have female offspring (figure 2.9). Males comprised 67.9% of all diapausing offspring and 60.5% of all non-diapausing offspring (Figure 2.8; diapausing N = 190 (61 females, 129 males); non-diapausing N = 337 (133 females, 204 male)). When only looking at the subset of diapausing offspring in a model with cool stalling treatment, time of season, and mass provision size, mass provision size was the only significant predictor of sex among diapausing offspring (male or female) \( (X^2(3, N = 190) = 39.37; p = 1.45e-08) \). Similarly, when the same model was run with photoperiod on day of cell construction in place of time of season, mass provision was the only significant predictor of offspring sex outcome in diapausing offspring \( (X^2(3, N = 190) = 42.57; p = 3.04e-09) \).

Mass provisions were a significant predictor of abdominal lipids in grams for both diapausing and non-diapausing offspring at the time of emergence. For diapausing
offspring, both time of season and mass provision size were significant predictors of total lipid content at emergence (Figure 2.11b, $F_{4,192} = 13.56$, multiple $R$-squared = 0.22, $p = 9.34 \times 10^{-10}$). Diapausing offspring from late season had 24% higher average lipids in grams than diapausing offspring from early season (Figure 2.11; $t = 4.58$, $p = 8.43 \times 10^{-6}$; early season $N = 55$, average grams lipids early = $0.03 \pm 0.001$ standard error; late season $N = 176$, average grams lipids late = $0.04 \pm 0.001$ standard error). Results were similar when photoperiod on day of cell construction was included instead of time of season ($F_{4,192} = 13.83$, multiple $R$-squared = 0.22, $p = 6.27 \times 10^{-10}$).
Figure 2.3

Abdominal Lipids of Newly Emerged Female Bees

Figure 2.3. In early (a) and late (b) season each biological replicate is a pool of two bee abdomens. Samples were only combined within the same cool stalling regime. Letters represent statistically significant differences within each season (p < 0.05). (a) early season control N = 33, 14 d cool stalling N = 33. Mean = 0.024, min = 0.008, max =
0.056 grams of lipids. (b) late season control N = 22, 14 d N = 27. Mean = 0.029, min = 0.009, max = 0.053 grams of lipids.
Figure 2.4

Abdominal Lipids of Reproductive Female Bees

Figure 2.4. Grams of abdominal lipid stores in reproductive females collected at the end of the field experiment in early (a) and late (b) season. Letters represent statistically significant differences within each season (p < 0.05). (a) early season control N = 46, 14 d cool stalling N = 30. Mean = 0.0084, min = 0.0026, max = 0.013 g lipids. (b) late season control N =40, 14 d N = 34. Mean = 0.006, min = 0.002, max = 0.01 g lipids.
**Figure 2.5**

*Total Nest Cells Per Female*

*Figure 2.5.* Total nest cells made per female according to cool stalling treatment in (a) early and (b) late season. Total nest cells included all cells that produced living offspring, parasites, dead eggs or larvae, and cells that were unfinished. Only cells that could be assigned to a reproductive female were used. Letters indicate a significant difference (p < 0.05). (a) early season control N = 52, 14 d cool stalling N = 48 females. Mean = 6.41,
min = 0, max = 18 nest cells. (b) late season control N = 47, 14 d N = 42 females. Mean = 2.78, min = 0, max = 10 nest cells.
Figure 2.6

Mass Provision Size

Figure 2.6. Mass provision size allocated to offspring by reproductive females that experienced either 1 d or 14 d of cool stalling treatment in (a) early and (b) late season. Letters indicate significance within each season (p < 0.05). (a) early season control N = 495, 14 d cool stalling N = 352, mean = 2625.2, min = 829.3, max = 4365.3 average sq.
pixels. (b) late season control $N = 168$, $14 \ d \ N = 131$, mean $= 2849$, min $= 973.3$, max $= 4141$ average sq. pixels.
Figure 2.7

*Relationship Between Offspring Body Size and Mass Provision Size*

*Figure 2.7.* Relationship between offspring body size (thorax width) and mass provision size (average sq. pixels). a) Larger mass provisions produced larger bodied offspring. b) How the relationship between body size and mass provision relate to male versus female
offspring c) How the relationship between offspring body size and mass provision relates to diapause outcome.
Figure 2.8

*Frequency of Diapausing Offspring*

(Figure 2.8. Females that experienced 14 d cool stalling produced fewer diapausing offspring. Offspring from females that experienced the control produced 41.1\% diapausing offspring, while offspring from females that experienced 14 d cool stalling produced 29.7\% diapausing offspring.)
Figure 2.9

*Relationship Between Mass Provision Size and Offspring Sex*

*Figure 2.9.* Mass provision size as it correlates to probability of sex of offspring. Larger mass provisions produced female offspring; smaller mass provisions produced male offspring. This is for all offspring from both early and late season that could be identified to sex.
Figure 2.10

Offspring Sex Ratio

Figure 2.10. Sex ratio of (a) all offspring (b) diapausing offspring (c) non-diapausing offspring. (a) Overall offspring of females that experienced the control produced 38.7% female and 61.3% male, while those that experienced 14 d cool stalling produced 34.5% female and 65.5% male. (b) Diapausing offspring from females that experienced the control were 38.5% female and 61.5% male, while those diapausing offspring from
females that experienced 14 d cool were 20.6% female and 79.4% male. (c) non-diapausing offspring from females that experienced the control produced offspring that were 38.6% female and 61.4% male, while those non-diapausing offspring from females that experienced 14 d cool were 40.4% female and 59.6% male.
Figure 2.11

Offspring Abdominal Lipids

Figure 2.11. Abdominal lipids of all offspring, collected immediately following emergence, of (a) non-diapausing and (b) diapausing offspring. Letters indicate significant differences (p < 0.05). (a) early season N = 133, late season N = 8. Mean = 2.48e-02, min = 2.90e-05, max = 6.65e-02 lipids in grams. (b) diapausing offspring early
season $N = 55$, late season $N = 176$. Mean = 0.04, min = 0.012, max = 0.065 average sq. pixels.
Table 2.1

*Pollen balls*

<table>
<thead>
<tr>
<th>Cool stalling treatment</th>
<th>Season</th>
<th>No. pollen balls</th>
<th>Total cells</th>
<th>Percent pollen ball</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>Early</td>
<td>40</td>
<td>529</td>
<td>7.56%</td>
</tr>
<tr>
<td>1 day</td>
<td>Late</td>
<td>22</td>
<td>211</td>
<td>10.43%</td>
</tr>
<tr>
<td>14 days</td>
<td>Early</td>
<td>38</td>
<td>376</td>
<td>10.11%</td>
</tr>
<tr>
<td>14 days</td>
<td>Late</td>
<td>15</td>
<td>161</td>
<td>9.32%</td>
</tr>
</tbody>
</table>

Number of pollen balls, nest cells that contain dead eggs or early instar larva, in both early and late season according to cool stalling treatment.
**Discussion**

Variations in reproductive strategy among female bees directly affects pollination efficacy and are major challenges when developing management strategies for alternative pollinators. In our study, we replicated the management practice of cool stalling post-diapause ALCBs in order to align their emergence with alfalfa bloom. We hypothesized that poor nutritional condition induced by this cool stalling practice, in conjunction with environmental factors like photoperiod and time of season, would influence reproductive strategy in ways that influence pollination performance. We found multiple ways by which variation in reproductive strategy influenced offspring development and outcome through photoperiod and time of season. We also saw an effect of maternal nutritional condition induced by cool stalling. Females that experienced prolonged cool stalling had significantly lower abdominal lipid stores than control females. This poor body condition led to fewer nest cells and smaller provisions, translating to fewer offspring (especially females) that entered diapause that would become the pollinators of the following season. Reproductive strategy was also influenced by environmental cues, such as time of season and photoperiod, but the effects of female body condition were consistent throughout the season. These results suggest common management practices may interact with environmental variation in ways that have substantial impacts on pollinator performance. They also provide important insight into how external and internal factors influence reproductive strategies and offspring outcomes in a solitary bee.

Photoperiod and time of season (temperature, weather, floral resources, and pests) were the most consistent significant predictors of the reproductive strategies of females and their offspring outcome in our study. Over the whole experiment, a total of 104-
minute reduction in daylength, and a one-month difference in time of season, led to significantly reduced fecundity (nest cells produced), larger allocations to offspring (mass provision sizes), a significant increase in the proportion of male offspring, a reduction in offspring abdominal lipid stores, and a significant increase in the probability of diapause among offspring. Our results support other findings that photoperiod strongly influences behavior, function, reproduction, and pollination in all insects, including ALCBs (Beck, 1968; Denlinger, 2003; Pitts-Singer, 2020; Strickler & Freitas, 1999). The mechanisms underlying these differences due to photoperiod and time of season could be foraging time (day length), floral resource availability, nutritional condition of mothers, and weather.

Due to factors like pests, weather, and floral resources (see figure 2.2), early season lasted 14 d while late season lasted 8-11 days. This limited the amount of time, and quantity and quality of forage in late season. However, since there were no significant differences in abdominal lipid levels between treatment groups in late season, and similarly in early season, the nutritional deficit caused by cool stalling was regained relatively quickly, at least in the first 8-11 days of foraging. Our finding that reproductive females having lower lipids overall at the end of the season, whether early or late, compared to abdominal lipids of newly emerged females supports that lipid stores of female ALCBs decrease as the nesting season progresses (O’Neill et al., 2015).

Female bees in early season experienced longer photoperiods at day of release and on day of nest construction. Early season day of release was 15 hours 53 minutes of daylight while late season day of release was 15 hours 3 minutes of daylight. Females released in early season had 50 more minutes of foraging time to replenish lipid stores.
This could explain why females collected at the end of late season had lower lipid stores on average than those collected at the end of early season due to differences in foraging time because of changes in photoperiod. Photoperiod can also explain why females in late season made mass provisions that were 8.8% larger than those made by females in early season, despite the fact that there were fewer resources available. Diapause is the best option for offspring survival late in the season. Females ALCBs produce more diapausing offspring in response to shortening photoperiod and offspring that enter diapause are found on larger mass provisions (Denlinger, 2003; Fischman et al., 2017; Klostermeyer, E.C., Mech, Stephen J., Rasmussen, 1973; Pitts-Singer, 2020). We found that females reproducing during shorter photoperiods produced larger provision and more diapausing offspring, but at the cost of fewer nest cells.

Differences in floral resources between the early and late season played a large role in these results. It is known for ALCBs that fewer cells are made when resources are low (Peterson & Roitberg, 2006; Pitts-Singer & Bosch, 2010) which was reflected in our study in the total number of nest cells made by females in late season compared to early season when floral resources were more than sufficient (see Figure 2.2). The nutrients available in floral resources change as the season progresses and are essential for ovary development in honeybees and oocyte maturation in Osmia (Cane, 2016; Cane et al., 2011; Lan et al., 2021; Strickler & Freitas, 1999; Vaudo et al., 2020). Pests were more pervasive in late season than in early season and destroyed much of the floral resources contributing to a decline in forage in late season compared to early season (see Figure 2.2). In our study we found that females in late season had significantly higher lipid content overall at the start of the experiment than those in early season. However, after
the shortened duration of the late season, reproductive females had lower lipids overall than those reproductive females from early season. This shows that floral resources have a major impact on the nutritional condition of reproductive females. In addition, diapausing offspring in late season had significantly more lipid stores than diapausing offspring from early season. This indicates it is a maternal strategy rather than a passive effect of resource availability.

Weather events strongly influence the ability to forage and build nests (Nielsen et al., 2017; Vicens & Bosch, 2000). In early season, there was a period of rainy weather following female release that caused an interruption of nesting activities. This is likely why the time to nest construction in early season is significantly longer than the time it took to begin nest construction in late season. We found that female body size was a significant factor in addition to photoperiod and time of season in the time it took to begin nest initiation.

We found that poor condition of the mother could limit their ability to pollinate and allocate resources for offspring, in that female that experienced prolonged cool stalling made significantly smaller mass provisions for their offspring compared with those made by females that experienced the control, despite external factors of photoperiod and time of season. The main environment an offspring of a mass provisioning bee larvae like ALCB experiences is the mass provision the mother has given it. Until it emerges as an adult, in either the summer generation or overwinters to the following spring, the developing ALCB is completely encapsulated in the leaf cocoon inside of a cavity. The offspring experience temperature fluctuations, but little light penetrates. A recent study found that photoperiod was a cue for diapause but that the
temperature inside the nest cell did not affect diapause outcome in ALCBs and it was concluded that there was a maternal effect for diapause, independent of temperature the offspring experienced, but tied to photoperiod (Wilson et al., 2021). We found that offspring outcome is determined by not only external factors, but also by internal factors determined by the provision size of a mother. A mother that provisions to the best of their ability and body condition, in response to external cues.

We found evidence that the mass provision is a mechanism for maternal effects. It has been shown in a previous study that mass provision size is linked to offspring sex, with males being found on smaller provisions in *Osmia lignaria* (Torchio & Tepedino, 1980) (see figure 2.9). Mass provision size has also been found to be indicative of offspring diapause outcome, with those offspring that diapause found on larger mass provisions (Fischman et al., 2017) (see figure 2.7). Cool stalling treatment of the mother was not directly significant however, mass provision size was a significant predictor of sex. Our results reveal that mass provision is one mechanism through which maternal condition is passed to offspring and how poor nutritional condition effects pollination efficacy by affecting the sex ratio of the next generation of pollinators. We found that females that experienced prolonged cool stalling produced fewer diapausing offspring and specifically fewer female diapausing offspring via differences in mass provision size.

We found that the common management practice of cool stalling results in poor body condition of reproductive females, which are the pollinators. This poor body condition could lead to less pollination, through smaller mass provisions made for offspring and fewer offspring, though further study is needed to directly measure this. Poor body condition also potentially leads to less pollination the following year because
less offspring diapause and specifically there are fewer female diapausiing offspring.

From previous studies it is known that the second generation of offspring in the summer, that do not diapause to become pollinators the next year, proves as a loss of up to 50% of progeny, since some findings suggest that this summer generation is female skewed (Hobbs & Richards, 1976; Krunic, 1972; Pitts-Singer & Cane, 2011).

Poor body condition of pollinators can happen through a variety of management strategies. In honey bees, overwintering storage conditions affect lipid stores and affect head proteins in workers, that are indicative of brood food producing glands (Hopkins et al., 2021; Kucharski et al., 1998). For wild Bombus species, mimicking natural overwintering conditions for queens is difficult and artificial overwintering has a high mortality rate (Lindsay, 2020). In ALCBs, due to their solitary nature, emergence as adults after diapause is the critical life stage to manage. While fluctuating thermal regimes have proven able to prolong adult emergence up to 2 years in ALCBs, the limitations of commercial refrigerators make the cool stalling at constant temperature still the best option for alignment of bee emergence and crop bloom (Yocum et al., 2021). The effects of this cool stalling on pollination and on the generations of pollinators to come are critical in understanding how management techniques influence pollinators and can lead to novel management approaches moving forward.

**Conclusion**

Management of solitary bees for pollination is difficult due to variations and plasticity in life history and reproductive strategy between and within species. Reproductive strategy can affect pollination because the female bees collect pollen for their offspring. Using the most managed solitary bee species, the alfalfa leafcutting bee,
we tested a common management practice to investigate the effects on aspects of reproductive strategy and offspring development that are most likely to impact pollination performance. We found that photoperiod and time of season, encompassing pest presence, weather and resource availability, are significant predictors of offspring diapause outcome. We also found that the practice of cool-stalling post-diapausing females negatively impacted their abdominal lipid stores and that this poor body condition was translated to the next generation through a maternal effect. One mechanism we identified as a means of translating poor body condition to offspring was via the pollen mass provision. Females that experienced a longer period in cool stalling made fewer nest cells and smaller pollen mass provisions. Females that experienced a longer period of cool stalling also had less offspring enter diapause, and specifically, fewer female diapausing offspring. Further studies are needed to determine what the impacts of cool stalling are on pollination efficacy.
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