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RESEARCH ARTICLE

Thermal history of alfalfa leafcutting bees affects nesting and diapause incidence

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ABSTRACT

Variable spring temperatures may expose developing insects to sublethal conditions, resulting in long-term consequences. The alfalfa leafcutting bee, *Megachile rotundata*, overwinters as a prepupa inside a brood cell, resuming development in spring. During these immobile stages of development, bees must tolerate unfavorable temperatures. In this study, we tested how exposure to low temperature stress during development affects subsequent reproduction and characteristics of the F1 generation. Developing male and female *M. rotundata* were exposed to either constant (6°C) or fluctuating (1 h day^{-1} at 20°C) low temperature stress for 1 week, during the pupal stage, to mimic a spring cold snap. Treated adults were marked and released into field cages, and reproductive output was compared with that of untreated control bees. Exposure to low temperatures during the pupal stage had mixed effects on reproduction and offspring characteristics. Females treated with fluctuating low temperatures were more likely to nest compared with control bees or those exposed to constant low temperature stress. Sublethal effects may have contributed to low nesting rates of bees exposed to constant low temperatures. Females from that group that were able to nest had fewer, larger offspring with high viability, suggesting a trade-off. Interestingly, offspring of bees exposed to fluctuating low temperatures were more likely to enter diapause, indicating that thermal history of parents, even during development, is an important factor in diapause determination.

KEY WORDS: Trade-offs, Solitary bees, Sublethal effects, *Megachile rotundata*, Low temperature stress, Insect development

INTRODUCTION

In temperate regions, insects are exposed to fluctuating temperatures throughout the seasons. At some latitudes in winter, ambient temperature fluctuates around temperatures that may be lethal to some species. Suboptimal microclimates during the winter can be metabolically costly and have long-term negative effects on reproduction (Irwin and Lee, 2003; Turnock and Fields, 2005). During winter, insects survive extreme low temperatures in a state called diapause through various physiological mechanisms, including metabolic depression and production of cryoprotectants (Bale and Hayward, 2010; Hahn and Denlinger, 2007; Hahn and Denlinger, 2011). However, such physiological protections may be absent during the spring, when insects are actively developing (Chown and Sinclair, 2010; Huey, 2010).

Unfavorable spring weather events can include sudden bouts of low temperatures in the form of cold snaps or freeze–thaw events (Kellomäki et al., 1995; Roitberg and Mangel, 2016; Sambaraju et al., 2012). Abrupt temperature changes may be too severe to induce a hormetic response (Mattson, 2008) or may expose insects to suboptimal conditions that could lead to detrimental effects, because they no longer have the physiological protections afforded during diapause (Lee et al., 1987). In general, insects may seek to limit thermal stress by physiological and/or behavioral methods, such as escaping to a more favorable microclimate and activating flight muscles (Kingsolver et al., 2011; Heinrich, 1975; Heinrich and Esch, 1994; Heinrich and Vogt, 1993). However, options for behavioral thermoregulation are limited for insects during immobile life stages, such as those undergoing metamorphosis within brood cells or cocoons that must survive the temperatures to which they are exposed (Kingsolver et al., 2011). Experiencing sublethal thermal stress during metamorphosis could have negative, long-term consequences that persist into the next generation.

Many parameters of a thermal stress event are important to consider, such as the duration of exposure, frequency and magnitude (Marshall and Sinclair, 2015). Insects that survive a stressor may have changes in offspring quantity and/or quality (Stearns, 1989). Downstream effects of stress are not ubiquitous and may differ across taxa. Furthermore, responses of some offspring characteristics, such as mortality, sex ratios and diapause incidence, may be more affected than others depending on species (Mousseau and Fox, 1998). For instance, aphids (*Rhopalosiphum padi*) had reduced fecundity and increased offspring mortality after they were exposed to low temperatures as either first instar larvae or newly molted adults (Hutchinson and Bale, 1994). In contrast, adult fruit flies (*Drosophila melanogaster*) that experienced repeated low temperature stress versus one long continuous stress had increased survival, decreased fecundity and altered offspring sex ratios, suggesting a trade-off and reallocation of diminished energetic stores (Marshall and Sinclair, 2009). After experiencing a low temperature stress as adults, pea leafminers (*Liriomyza huidobrensis*) produced fewer eggs, although the viability and sex ratios of the offspring were not affected (Huang et al., 2007). The wide range of responses suggests there may not be a universal pattern. Therefore, future studies need to incorporate more non-model species to understand and develop more robust patterns.

Whether an insect enters diapause or continues direct development has been found to be maternally regulated in a variety of species (Coleman et al., 2014; Denlinger, 2002; Mousseau and Dingle, 1991; Simmonds, 1948). For many of these species, offspring are more likely to enter diapause when their parents are reared under low temperatures and short day lengths (Saunders, 1966; Tougeron et al., 2020; Vaghina et al., 2014). Also, the sensitive stage of mothers for thermoperiod effects can differ among species, which makes testing the effects of stress on multiple life stages important (Mousseau and

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Dingle, 1991). Long-term effects on offspring diapause from temperature stress of the parental generation has not been studied.

Changes in offspring diapause incidence could indicate that a female may be interpreting environmental cues differently than predicted (Mousseau and Dingle, 1991). Entering diapause at the correct time is important for offspring to avoid exposure to an array of detrimental stressors that could negatively affect their survival and the overall population composition (Tougeron et al., 2020; Tuljapurkar and Istock, 1993). For instance, summer weather can expose immobile insects to lethal heat stress (Pitts-Singer and James, 2008). The effects of timing on diapause termination has been well studied in terms of physiological mechanisms and ecological and applied agricultural importance (Danforth, 1999; Denlinger, 2008; Forrest and Miller-Rushing, 2010; Menu et al., 2000; Rajon et al., 2014). However, the consequences of poorly timed diapause induction have been vastly understudied.

Megachile rotundata, commonly known as the alfalfa leafcutting bee, is a solitary bee that has limited mobility during overwintering and subsequent development to adult. The female parent determines offspring sex, body size, and potentially whether the offspring enters diapause (Krunic, 1972; Hobbs and Richards, 1976). Alterations in offspring quality and quantity occur through parental care provided to offspring. Female parents construct brood cells made of leaf pieces glued together with nectar and saliva and provisioned with pollen and nectar inside (Cane et al., 2011; Trostle and Torchio, 1994). At lower latitudes *M. rotundata* is bivoltine with non-diapausing bees that are predominantly female, compared with the overwintering cohort, emerging in the summer (Tepedino and Parker, 1988). The sex ratio of overwintering bees is skewed toward males, which require less of an energetic investment, receiving 17% less provisions compared with female siblings (Klostermeyer et al., 1973). Offspring investment by female *M. rotundata* can be altered by environmental stressors, including increased flight distance to forage and reduced resource levels (Peterson and Roitberg, 2006a,b; O'Neill et al., 2010). However, it is unclear whether temperature stress can also cause changes in female offspring investment.

Owing to extensive research on the effects of low temperatures for its long-term and short-term storage, *M. rotundata* is an excellent model for understanding how insects respond to thermal stress during development (Rinehart et al., 2011, 2013, 2016; Torson et al., 2019; Yocum et al., 2010). For instance, *M. rotundata* exposed to low temperatures for durations as short as 1 week (both constant and fluctuating) during development do not differ in survival from controls (Bennett et al., 2015; Rinehart et al., 2011; Yocum et al., 2010). However, those exposed to either constant or fluctuating low temperature stress had delayed adult emergence, and males had reduced longevity (Bennett et al., 2015). Adults that survived had reduced flight ability and wing deformities (Bennett et al., 2015). Thermal stress may also negatively affect reproduction or reduce the quality and/or quantity of offspring. The effects of low temperature stress during development on future offspring production in *M. rotundata* is currently unknown.

In this study, we tested the hypothesis that exposure to low temperature stress during development alters offspring characteristics. To test this hypothesis, pupal *M. rotundata* undergoing metamorphosis were exposed to two different types of low temperature stress (fluctuating or constant) for 1 week. A constant temperature was chosen as a baseline stressor that may be relevant for individuals buffered in their microhabitat (Irwin and Lee, 2003), while fluctuating low temperature stress would be representative of potential spring temperature changes. All bees were allowed to resume development, and emerging adult bees were transported to tents in an alfalfa field to track offspring production. Nests containing offspring were brought back to the laboratory to determine the number of offspring produced and their characteristics, including sex, dry mass and diapause state. Changes in the number of offspring and their characteristics would show a shift in parental investment owing to a long-term response to low temperature stress.

MATERIALS AND METHODS

Megachile rotundata rearing

Diapausing prepupae, of Canadian origin, were purchased from JWM Leafcutters (Nampa, ID, USA) as single, loose brood cells. *Megachile rotundata* (Fabricius 1787) prepupae were kept in a 6°C incubator (Percival, Perry, IA, USA) in constant darkness. To initiate development, brood cells were moved to a 29°C incubator in 16 oz plastic containers covered with a fine mesh lid allowing air flow throughout the container.

Low temperature treatments

After 14 days at 29°C, when the majority of developing *M. rotundata* are at the eye-pigmented stage (Bennett et al., 2015; Yocum et al., 2010), brood cells were randomly placed into one of three treatment groups: (1) constant low temperature stress, (2) fluctuating low temperature stress and (3) control. The constant low temperature stress was a constant 6°C, while the fluctuating low temperature stress had a baseline of 6°C with a daily pulse at 20°C for 1 h with 1 h ramps up and down, as previously described (Rinehart et al., 2011). We chose these temperature treatments based on previous work showing that exposure to fluctuating low temperature stress reduces mortality (Rinehart et al., 2011) and some sublethal effects (Bennett et al., 2015) compared with constant low temperature stress. Constant and fluctuating bees remained in their respective treatments for 7 days. At day 21, both low temperature treatment groups were returned to the 29°C incubator until adults emerged. Bees designated for the control group were kept in the 29°C incubator to allow for continuous development with adult emergence timed to coincide with the other treatments. All bees finished development and emerged as adults in the 29°C incubator before being marked and released.

Field design

Each treatment was assigned three replicate tents (6.1×6.1×2.4 m), totaling nine tents in a blooming alfalfa field. Tents were placed 6 m from each other in a repeating pattern to ensure field uniformity. The experiment ran from 15 June until 6 July 2019 in Fargo, ND, USA (46.9183038, –96.8536053). In each tent, a nest box was attached to a pole in the center, facing southeast. Nest boxes were constructed from commercial Styrofoam blocks (Beaver Plastics, Acheson, Alberta, Canada) with 7 mm holes, each lined with a paper straw in a 12×12 grid (144 total holes).

Twenty female and 10 male bees were haphazardly assigned to each tent to ensure that resource availability would not be limiting (Pitts-Singer and Bosch, 2010). The ratio of 2 females to 1 male was used to allow the females to optimize their fitness without male harassment (Rossi et al., 2010). Prior to release in the field, females were marked on the thorax with a unique color pattern (Sharpie® Oil-Based Paint Markers, Atlanta, GA, USA). A preliminary test was performed in the greenhouse to determine which combinations would be possible in the field. Seven different colors were distinguishable under field conditions. Out of the 20 released bees, the first seven had a single dot on their thorax, while the remaining bees had two dots that were discernable from each other.

Female *M. rotundata* are known to return to the same hole each time after foraging until the nest is completed (Klostermeyer et al., 1973; McCorquodale and Owen, 1997). Therefore, a completed nest can be attributed to a known female. Bees were observed daily in the field for 3 weeks to determine when straws were completed and which female created each nest. Straws were identified as complete when they were capped with visible leaf pieces protruding out of the straw (Pitts-Singer and Cane, 2011). Once completed, we removed each straw from the cavity using forceps and placed it into a 29°C incubator to continue offspring development under the controlled conditions to minimize any effect of environmental variation that occurred in the field.

Offspring measurements

To determine the number of brood cells in each straw and their contents, each straw was X-rayed using a Faxitron Bioptics, LLC X-Ray (Tucson, AZ, USA) for 4 s at 28 kV and visualized using AllPro Imaging by Air Techniques (Melville, NY, USA). From the X-ray images, we determined whether brood cells contained offspring, parasites or pollen balls (brood cells without offspring). Leaves were allowed to dry for 1 week before individual brood cells were removed from straws and placed in a 24-well plate according to their cell position within the straw. Brood cells were X-rayed again after bees stopped emerging to determine which had overwintering offspring and offspring that had died during development. If the brood cells were destined for diapause, indicated by the presence of a prepupa, they were moved to the 6°C incubator for 5 months for overwintering. Non-diapausing bees were then moved to the 29°C incubator to continue development into adults.

For offspring that continued direct development, upon adult emergence, bees were placed in new 24-well plates without leaf matter, transported to a 50°C oven (Techne Hybridiser HB-1D, Princeton, NJ, USA) and dried to a constant weight. After 1 week, offspring were weighed to the nearest 0.01 mg on an analytical balance (Mettler AE100, Hightstown, NJ, USA). Brood cells containing dead offspring were dissected to determine at which life stage the offspring died. The reproductive fitness of each female was measured by the total number of brood cells from each individual and the total number of viable offspring.

Flight test

Upon releasing treated adult bees into the field cages, we discovered that some individuals were unable to fly. To quantify the prevalence of this defect, 50 male and 50 female bees per treatment were randomly selected for a drop test. Bees were individually placed in small petri dishes (60 mm diameter) and released at the top of a wide acrylic tube (height: 1 m, diameter: 0.25 m). Bees were categorized by their response to being dropped as: (1) flight (bees flew without hitting the bottom of the tube), (2) recovery (bees flew out after hitting the bottom of the tube) and (3) no flight and no recovery (bees fell to the bottom and did not fly out).

Statistical analysis

Data were analyzed using JMP Pro (version 14, SAS Institute, Cary, NC, USA), IBM SPSS (Statistics 24, Armonk, NY, USA) and R (version 1.1.423; https://www.r-project.org/). All data were assessed for normality and equal variance. ANOVA was used to determine differences in the number of brood cells using temperature treatment as a factor. A binomial logistic regression was used for a multitude of tests, including flight performance of treated adults (likelihood of flight, offspring viability, sex ratios and diapause incidence). For dry mass comparisons, Kruskal–Wallis tests were performed owing to unequal variances in non-diapausing offspring; therefore, not meeting the requirements of an ANOVA. An ANCOVA was used to determine the relationship between the number and mass of offspring produced by individual females. Then, a regression was used to compare the response across treatments. All means are presented ±s.e.m., and significance is determined by $P<0.05$.

RESULTS

Treated bees fell into three categories: (1) those having obvious morphological deformities (Fig. 1A,C), (2) those with subtle deformities (Fig. 1B) and (3) those with no deformities. In bees that had no obvious morphological defects, flight ability was significantly affected by temperature treatment ($\chi^2=46.1$, $P<0.0001$; Fig. 1D). Less than half of bees treated with constant low temperature stress flew in the flight test (40% of females and 44% of males flew), a lower percent than observed for those exposed to fluctuating low temperature stress (54% females, 76% males) and control bees (88% females, 98% males).

Of bees released in the field, females exposed to constant low temperature stress were significantly less likely to nest (10.0±7.64%; $\chi^2=11.3$, $P<0.004$) compared with those exposed to fluctuating low temperature stress (32.7±6.00%). Control females (25.0±5.77%) nested with a similar likelihood as constant females. Females exposed to constant low temperatures failed to establish in one tent, while another tent had one nest with two brood cells. The third tent with constant-treated bees produced five nests containing a total of 36 brood cells. Despite low establishment, the average number of brood cells produced per tent was not statistically different among treatments (control, 57.0±16.9; fluctuating, 87.7±30.9; constant, 12.7±11.7; $F_{2,35}=1.40$, $P=0.261$; Fig. 2B). In addition, constant low temperature females were more likely to have brood cells with viable offspring (97.2±2.78%) compared with controls (69.4±2.37%; $\chi^2=6.20$, $P<0.05$; Fig. 3A).

Diapause incidence of offspring was significantly affected by temperature treatment of parents ($\chi^2=46.7$, $P<0.0001$; Fig. 3B). Offspring of females treated with fluctuating temperatures were more likely to enter diapause (61.1±7.08%) than both the controls (11.5±6.80%) and constant offspring (19.1±19.1%). Offspring sex varied with diapause incidence ($\chi^2=13.5$, $P=0.0003$; Fig. 4). Non-diapausing bees were predominately female (control, 60.3%; fluctuating, 53.7%; constant, 69.6%) for all three treatments whereas diapausing bees were mostly males (control, 72.7%; fluctuating, 63.4%; constant, 75%). Dry masses were analyzed between sexes and development path (diapausing or continuous development; Fig. 5). Non-diapausing female offspring were at least 2 mg heavier than males across all treatments ($F_{1,206}=80.4$, $P<0.0001$). For diapausing offspring, offspring dry mass differed between sexes ($\chi^2=35.39$, d.f.=2, $P<0.0001$). When analyzing sexes separately, there was no difference in dry mass among treatments of non-diapausing males ($\chi^2=5.36$, d.f.=2, $P>0.069$; Fig. 5B). For non-diapausing females, treatment affected dry mass ($\chi^2=-32.9$, d.f.=2, $P<0.008$; Fig. 5A), with females produced by constant low temperature parents being larger than controls but not offspring of fluctuating temperature parents. Diapausing male offspring produced by both constant and fluctuating low temperature treated parents were at least 1 mg heavier than controls across treatments ($\chi^2=7.84$, d.f.=2, $P=0.020$; Fig. 5B). There was no significant difference among diapausing female offspring across treatments ($\chi^2=4.02$, d.f.=2, $P=0.134$; Fig. 5A).

Temperature treatment significantly affected the relationship between quantity and quality of offspring ($F_{2,37}=3.36$, $P=0.047$;

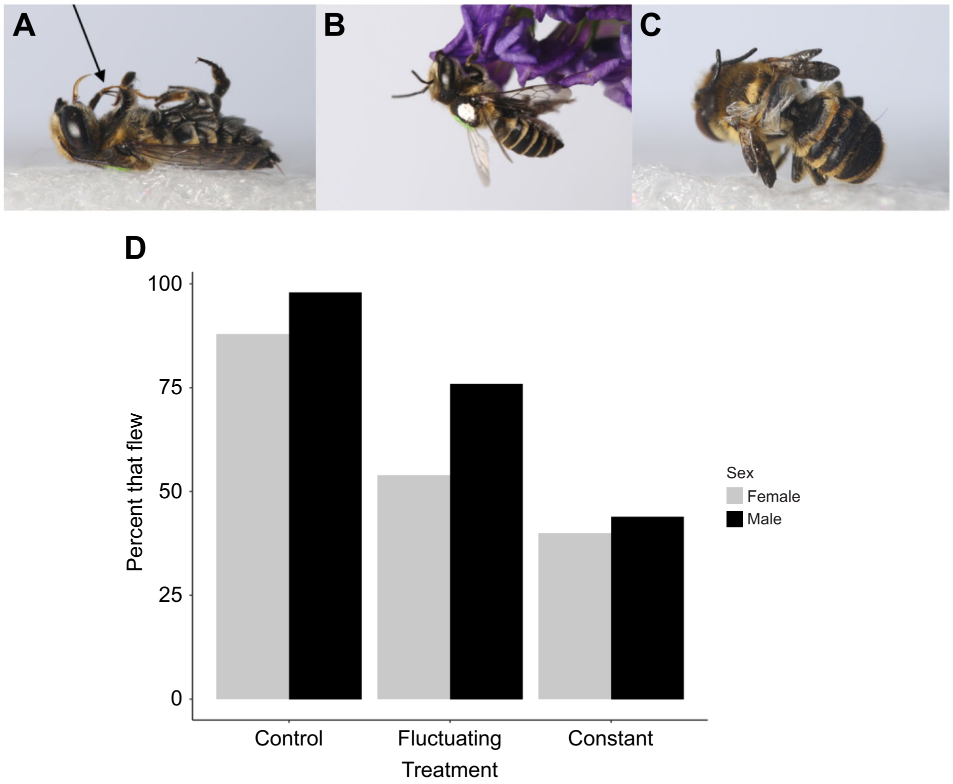

Fig. 1. Observed deformities in low temperature treatments. (A) Proboscis permanently extended in females treated with fluctuating temperatures. (B) Inability to fly and limited forewing control in constant temperature stress females. (C) Unexpanded wings in constant temperature treated males. (D) The likelihood of flight for females (*n*=50) and males (*n*=50) that experienced a low temperature stress during development.

Fig. 6). For fluctuating and control bees, there was no relationship between offspring average mass and the number of brood cells (P=0.403 and 0.386, respectively). However, for females exposed to constant low temperatures during development, there was a negative correlation between offspring average mass and number of brood cells ($F_{1,5}$=7.72, P=0.05; y=14.1x–0.473, r^2=0.659; Fig. 6).

DISCUSSION

Sudden bouts of low temperature during the spring pose a substantial risk to developing insects. Insects that survive this type of stressor may experience long-term effects on behavior, reproduction and offspring characteristics. Exposure to low temperature stress during development had mixed effects on the reproductive fitness of *M. rotundata*. Bees exposed to constant low

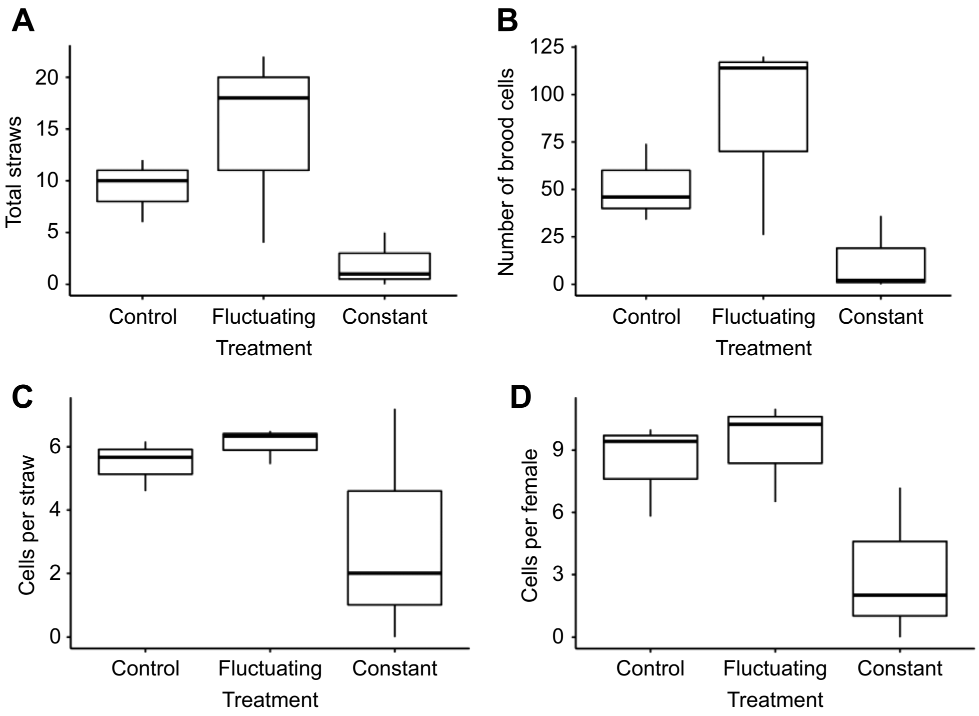

Fig. 2. Reproductive fitness of *M.* rotundata that were exposed to low temperature stress during metamorphosis. (A) Average number of straws, (B) average number of brood cells, (C) average brood cells per nest and (D) average number of brood cells produced per female in response to low temperature stress during pupal development. Boxes represent the 1st to 3rd quartile, while the lines extending from the box show the 25th and 75th percentiles of the data. The median is indicated by the line in the center of the box. There were no statistical differences detected by ANOVA among treatment groups (*n*=20 females per tent, 3 tents per treatment).

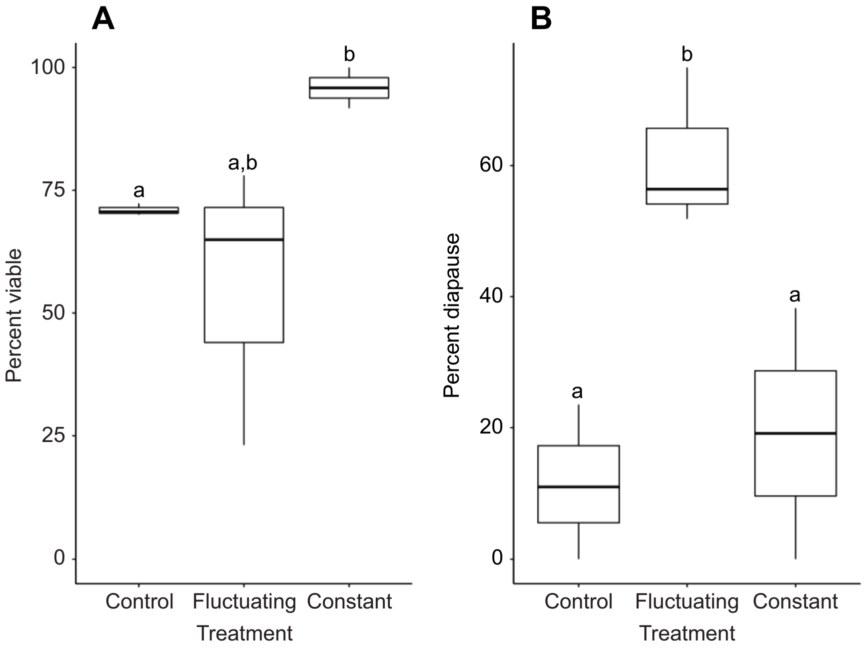

Fig. 3. Characteristics of offspring of bees exposed to low temperature stress during metamorphosis. (A) The percent of viable offspring that successfully emerged as adults. (B) The percent of offspring that entered diapause. Boxes represent the 1st to 3rd quartile, while the lines extending from the box show the 25th and 75th percentiles of the data. The median is indicated by the line in the center of the box. Boxes with different letters are significantly different, $P<0.05$ ($n=20$ females per tent, 3 tents per treatment).

temperature stress were less likely to nest and showed a trend toward fewer brood cells per female, even though the number of offspring produced among treatments did not differ significantly. The number of brood cells produced is an imprecise metric because two of the tents did not produce any offspring. For instance, females exposed to constant temperatures during development failed to establish nests in one tent and in another tent had only two offspring. In the third tent, more females established nests and produced numbers of brood cells comparable to those in other treatments. The low nesting rate is most likely attributed to sublethal effects that prevented females from flying, as observed in this and previous studies (Bennett et al., 2015). Interestingly, females that were exposed to the fluctuating temperature treatment were more likely to nest, which may be due to a hormetic response. The use of the constant and fluctuating temperature treatments in this study are essential to creating a framework that can be built upon to understand how insects are responding in the field. Future experiments will include exposing bees to a more rigid and complex temperature profile in the laboratory, with another group experiencing natural fluctuating temperatures in the field.

Our hypothesis that exposure to low temperature stress during development alters offspring characteristics was supported. This is the first study to show that the thermal history experienced by the parental generation during metamorphosis, and not adulthood, affects diapause incidence of offspring in *M. rotundata*. Also, this study is the first to show that thermal stress during spring development in the parental generation can alter offspring diapause incidence. *Megachile rotundata* undergoes facultative diapause and the number of summer generations varies with latitude (Kemp and Bosch, 2001; Krunic, 1972; Rank and Rank, 1989). Typically, early season offspring in temperate regions, such as the location of this study, are more likely to continue development and

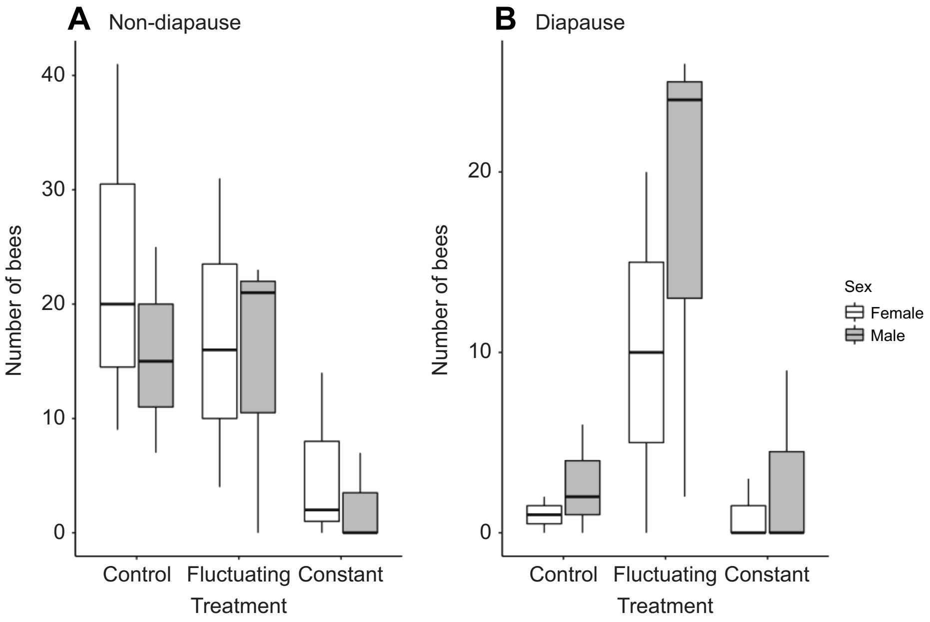

Fig. 4. The number of female and male offspring produced by temperature-treated bees ($n=20$ females per tent, 3 tents per treatment) that continued direct development or underwent diapause. (A) Direct development; (B) diapause. Boxes represent the 1st to 3rd quartile, while the lines extending from the box show the 25th and 75th percentiles of the data. The median is indicated by the line in the center of the box.

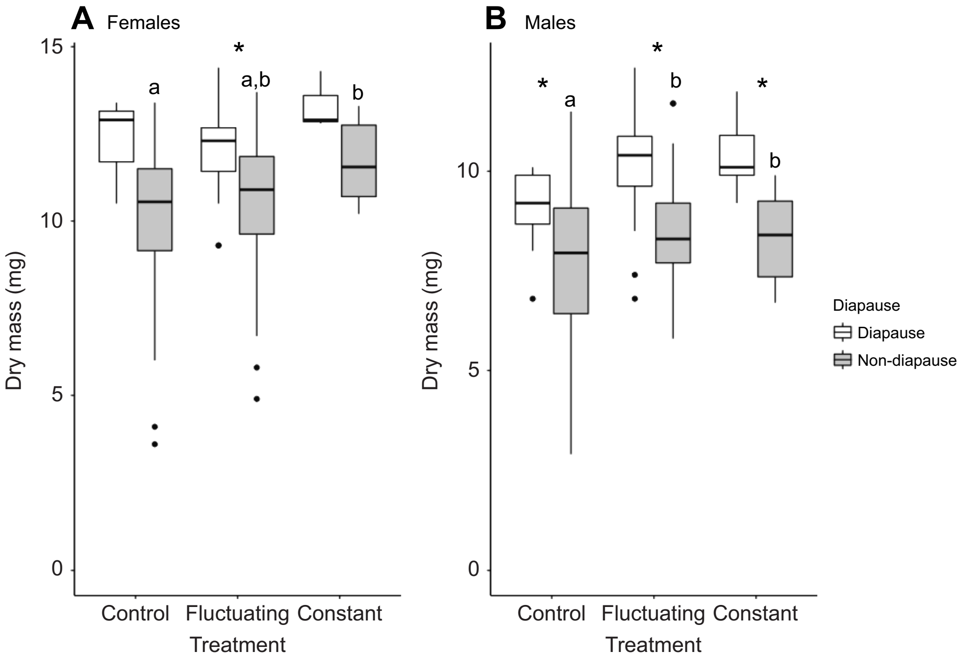

Fig. 5. Dry mass (mg) of diapausing (open boxes) and non-diapausing (gray boxes) offspring. (A) Females; (B) males. Boxes represent the 1st to 3rd quartile, while the lines extending from the box show the 25th and 75th percentiles of the data. The median is indicated by the line in the center of the box. Boxplots that share at least one letter above them are not significantly different by treatment. *Significant differences within a treatment between diapausing and non-diapausing bees (*n*=20 females per tent, 3 tents per treatment).

emerge as adults than late season bees, which are more likely to enter diapause and overwinter as prepupae (Hobbs and Richards, 1976; Krunic, 1972). As expected, control and constant treatments had low percentages of offspring that entered diapause, because the experiment occurred early in the season. Surprisingly, offspring produced by parents exposed to fluctuating low temperature stress were five times more likely to enter diapause, suggesting that offspring diapause is determined by the female parent and her thermal history. Similarly, in *Bombyx mori* (silkworm), temperature and light cues experienced during stages of parental development determine diapause incidence in offspring (Shiomi et al., 2015). The mechanisms involved in diapause determination in *M. rotundata* are unknown; however, previous research has suggested a maternal contribution (Rank and Rank, 1989). More studies are needed to determine the exact mechanism by which females control the diapause destiny of their offspring.

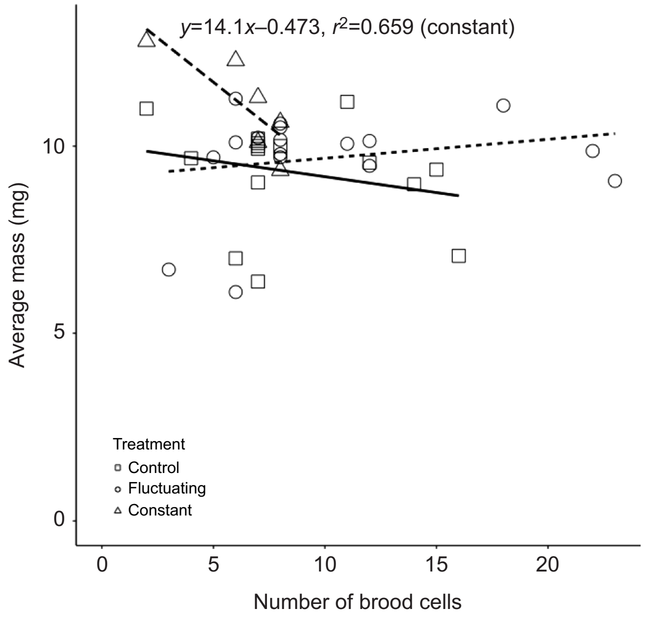

Fig. 6. Reproductive trade-offs caused by low temperature stress. Each dot represents one female's reproductive effort, plotted as the average mass of the offspring (mg) versus the total number of brood cells produced by that female (*n*=20 females per tent, 3 tents per treatment). The slope of the best fit line shows the change in offspring investment as females produced more offspring. A negative slope indicates decreased investment as the number of offspring increase, while a positive slope indicates increased investment as offspring number increases. There was a significant correlation between the number of brood cells and the average mass of offspring only in the constant temperature treatment (regression shown; long dashed line), but the other lines are shown for comparison. The slope of the control treatment is depicted by the solid line and fluctuating by the shorter dashed line.

Entering diapause at a suboptimal time may have an impact on survival and future reproduction. *Megachile rotundata* that enter diapause early in the season may be exposed to a different suite of stressors that could contribute to prepupal mortality. Much like developing bees subjected to low temperatures in the spring, immobile diapausing bees during the hottest part of summer must endure the increasing temperatures to which they are exposed. *Osmia ribifloris* offspring exposed to increased high temperature within their nesting cavity were less likely to survive (CaraDonna et al., 2018). Also, if offspring in one nest do not all enter diapause at the same time, those that have already entered diapause risk being killed by the emerging siblings as they exit the nest cavity. Female *M. rotundata* are more likely to chew through their siblings than males, which are more likely to circumnavigate (Tepedino and Frohlich, 1984a). Fratricide has been documented in other cavity nesting bees closely related to *M. rotundata*, (*Osmia taxana*, *O. montana*, *O. californica* and *O. iridis*) (Tepedino and Frohlich, 1984b; Torchio and Tepedino, 1982). In our experiment, nests were returned to the laboratory under the same conditions to prevent environmental effects on offspring and potential fratricide. Future studies are needed to determine the impact of mistimed diapause on offspring that remain in the field.

Low establishment rates of females that were exposed to constant low temperature stress may be attributed to morphological deformities. Noticeable deformities included bees with a permanently extended proboscis (Fig. 1A) and unexpanded wings upon adult emergence (Fig. 1B). Although bees with obvious deformities were excluded, those with more subtle morphological deformities may have been released in the field (Fig. 1C). Females exposed to constant temperatures during development were observed in the field crawling on alfalfa rather than flying and, when provoked, were unable to fly or control their forewings. A further test to examine flight capabilities showed that constant-temperature treated bees, both male and female, were less likely to be able to fly compared with fluctuating and control bees. Similar observations were noted for bees in tethered flight, where half of males and 10% of females without obvious morphological defects were unable to fly after exposure to the same constant treatment as in this study (Bennett et al., 2015). We likely found a greater percentage of affected bees in this study because of our larger sample size. The previous study used only 16 total constant-treated bees for the tethered flight. Female bees that are unable to fly cannot nest, provide parental care or visit flowers to feed. Male bees that cannot fly would likely be unable to mate, which could also reduce fitness in the constant-treated bees. Over time, population growth would be limited to the few females that were able to nest. One mechanism that could contribute to morphological deformities and decreased flight performance is cold injury.

Exposure to low temperatures during diapause as pupae is known to cause cold injuries, such as unexpanded and malformed wings, seen in the adult stage of the bertha armyworm (*Mamestra configurata*) (Turnock et al., 1983). Low temperatures chosen for this study could be low enough to cause cold injury in *M. rotundata*. Sublethal effects, such as wing deformities and degradation of muscles and the nervous system, attributed to cold stress have been observed in other insects (Hegdekar, 1971; Kelty et al., 1996; Rinehart et al., 2000; Turnock et al., 1983). In flesh flies, cold injury can result in neuromuscular degradation and changes in grooming behavior and proboscis extension reflex (Kelty et al., 1996; Yocum et al., 1994). Future experiments will be conducted to explore other possible physiological consequences of thermal stress in *M. rotundata*, including immune function and metabolic rate during a low temperature stress.

Exposure to low temperatures also affected parental care in treated females. Our results showed that those constant-treated females that were able to nest invested more resources in fewer, higher quality offspring, suggesting that there is a trade-off between the number and size of offspring produced (Stearns, 1989). This suggests that females receiving a constant low temperature stress during development alter their parental care to increase the quality of the offspring they produce as opposed to increasing the quantity. Competition could have been lower in tents with constant-temperature treated bees owing to decreased nesting; however, the number of released bees in this study was chosen to reduce competition and sexual harassment by males (Pitts-Singer and Bosch, 2010; Rossi et al., 2010). No trade-offs were detected in bees that were exposed to fluctuating temperatures, which could be explained by those individuals being better at acquiring or having more overall resources than the constant-temperature treated bees (King et al., 2011; van Noordwijk and de Jong, 1986).

Trade-offs in response to low temperatures have been seen in other insect species. For instance, when exposed to repeated cold exposures, *Drosophila melanogaster* invests in current survival over future reproduction, resulting in fewer offspring being produced (Marshall and Sinclair, 2009). The terminal investment hypothesis (Clutton-Brock, 1984) may explain why the constant temperature stress females invested more in their offspring compared with fluctuating and control females. According to the terminal investment hypothesis, individuals invest more into current reproduction when experiencing stress owing to aging, infection and physiological impairment (Clutton-Brock, 1984). Increased investment may better prepare offspring for future stressors and has been well documented in mammals, reptiles, amphibians and insects, including European earwigs, burying beetles, giant water bugs and decorated crickets (Brannelly et al., 2016; Cotter et al., 2010; Creighton et al., 2009; Duffield et al., 2015; Kight et al., 2000; Ratz et al., 2016; 2020; Velando et al., 2006). Changes in offspring investment may indicate that the response of females exposed to sudden low temperature stress aligns with the terminal investment hypothesis.

This study is the first to show that temperature stress during development invokes terminal investment of treated *M. rotundata* and provides strong evidence for maternal control of offspring diapause incidence. Results show that experiencing stress during development has cascading effects that may include more physiological systems than reproduction. For instance, susceptibility to viruses and pathogens may increase along with morphological, neurological and behavioral deformities. Additionally, *M. rotundata* are used in agriculture for pollination services. Farmers regularly use low temperature treatments, similar to our constant low temperature stress treatment, to delay adult emergence to align with alfalfa bloom (Pitts-Singer and Cane, 2011). The long-term consequences of this practice on reproduction and offspring, until now, have not been studied. Within the United States, prepupae destined for diapause are more likely to have increased mortality in commercial alfalfa production (Pitts-Singer and James, 2005). Seasonal bee loss has financial consequences for farmers that need to purchase new bees every year to supplement decreased populations (Pitts-Singer and Cane, 2011), which may be exacerbated by changes in diapause incidence.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.N.E., J.P.R., K.J.G.; Methodology: K.N.E., J.P.R., K.J.G.; Validation: M.S.P., K.J.G.; Formal analysis: K.N.E., K.J.G.; Investigation: K.N.E., M.S.P.; Resources: J.P.R., K.J.G.; Data curation: K.N.E., M.S.P., K.J.G.; Writing - original draft: K.N.E., M.S.P.; Writing - review & editing: K.N.E., M.S.P., J.P.R., K.J.G.; Visualization: K.N.E.; Supervision: J.P.R., K.J.G.; Project administration: J.P.R., K.J.G.; Funding acquisition: J.P.R., K.J.G.

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Data availability

The full dataset is available from the Dryad digital repository (Earls et al., 2021): https://doi.org/10.5061/dryad.vdncjsxvq.

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