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Thermoprofile Parameters Affect Survival of *Megachile rotundata* During Exposure to Low-Temperatures

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Synopsis Insects exposed to low temperature stress can experience chill injury, but incorporating fluctuating thermoprofiles increases survival and blocks the development of sub-lethal effects. The specific parameters required for a protective thermoprofile are poorly understood, because most studies test a limited range of thermoprofiles. For example, thermoprofiles with a wave profile may perform better than a square profile, but these two profiles are rarely compared. In this study, two developmental stages of the alfalfa leafcutting bee, *Megachile rotundata*, eye-pigmented pupae, and emergence-ready adults, were exposed to one of eight thermoprofiles for up to 8 weeks. All the thermoprofiles had a base of 6°C and a peak temperature of either 12°C or 18°C. The duration at peak temperature varied depending on the shape of the thermoprofile, either square or wave form. Two other treatments acted as controls, a constant 6°C and a fluctuating thermal regime (FTR) with a base temperature of 6°C that was interrupted daily by a single, 1-h pulse at 20°C. Compared with constant 6°C, all the test thermoprofiles significantly improved survival. Compared with the FTR control, the thermoprofiles with a peak temperature of 18°C outperformed the 12°C profiles. Bees in the eye-pigmented stage exposed to the 18°C profiles separated into two groups based on the shape of the profile, with higher survival in the square profiles compared with the wave profiles. Bees in the emergence-ready stage exposed to 18°C profiles all had significantly higher survival than bees in the FTR controls. Counter to expectations, the least ecologically relevant thermoprofiles (square) had the highest survival rates and blocked the development of sub-lethal effects (delayed emergence).

Introduction

As ectotherms, insects are susceptible to shifts in temperature. Insects use seasonal cues to predict the arrival of winter, but abrupt or non-seasonal temperature changes that cannot be predicted may have serious consequences. In spring, most insects are likely to encounter broad temperature fluctuations, which are expected to be stressful during times of active growth and development. Extensive research on chill injury has demonstrated that cold exposure increases mortality and causes sub-lethal effects in survivors (Yocum et al. 1994; Kostal et al. 2006; Lalouette et al. 2011; Teets and Denlinger 2013). The failure of multiple physiological processes contributes to chill injury, including altered phase transitions of cellular membranes (Lee 2010), disruption of ion homeostasis, metabolic imbalance (Kostal et al. 2004, 2006, 2016), and oxidative damage (Rojas and Leopold 1996; Joanisse and Storey 1998; Lalouette et al. 2011). These failures accumulate during cold exposure, eventually causing the sub-lethal effects and mortality associated with chill injury.

In an effort to better understand the effects of temperature on an insect's physiology, many studies

have used one or more constant temperatures. However, results from exposing insects to various constant temperatures suggest that the relationship between temperature and physiological state is non-linear, with non-additive effects occurring over time (Colinet et al. 2015). Temperatures above a physiological threshold may have disproportionate effects on metabolic processes compared with those below the threshold (Martin and Huey 2008). This non-linearity is referred to as Jensen's inequality (Jensen 1906; Rhel and Ayres 1999) and indicates that the effect of temperature is rarely a simple summation of degree exposure. Therefore, determining the effect of exposure to a particular temperature exposure can be complicated.

The use of fluctuating thermal treatments has challenged an underlying assumption of constant temperature studies, that of a golden mean. In other words, constant temperature studies are often set up to identify a particular optimal temperature, meaning that any temperatures higher or lower would be considered "stressful," non-optimal temperatures. On the contrary, temperature regimes that fluctuate, whether by varying around a mean or by providing brief pulses of other temperatures, increase survival and longevity in such a broad range of contexts that their benefit cannot be ignored (Chen and Denlinger 1992; Nedvěd et al. 1998; Hanč and Nedvěd 1999; Renault et al. 2004; Colinet et al. 2006, 2018; Colinet and Hance 2010; Kostál et al. 2007; Rinehart et al. 2011, 2013, 2016; Bennett et al. 2013; Prasifka et al. 2015). Although the mechanism by which fluctuating temperatures improve survival remains unclear, several studies point to the warm period as a time of repair (reviewed in Colinet et al. 2018). In support of this idea, a warm pulse during sub-optimal temperature exposure up-regulates expression of genes related to the repair of cold injury (Torson et al. 2015, 2017). Particular physiological parameters that are thought to be repaired include ion transport (Kostal et al. 2016), oxidative damage (Rojas and Leopold 1996; Lalouette et al. 2011), and water balance (Kostal et al. 2004).

A challenge to our understanding of how fluctuating temperatures contribute to the physiological benefits is that studies employ a wide range of thermal profile parameters, but rarely compare parameters within a single study. Colinet et al. (2015) identified eight different classes of thermal regime, representing a wide range of profiles. For example, thermal profiles can rapidly transition between temperatures (such as a square profile) or gradually shift between low and high temperatures (such as a sine wave). Studies typically test either a square or sine profile and rarely compare the two. In addition, the amplitude of the profile may differ between but not within studies, such that large fluctuations are never directly compared with small fluctuations, making it difficult to pinpoint which parameter provides the benefit.

One form of square thermoprofile, known as a fluctuating thermal regime (FTR), is characterized by short durations at a higher temperature (e.g., 1 h at 20°C). FTRs have been studied for their role in the cold storage of many insect species (Colinet et al. 2018). Although FTRs are unnatural thermoprofiles, most laboratory studies are conducted at constant low temperatures, an even more unnatural condition. Exposing insects to FTRs abrogates many of the deleterious effects of exposure to constant low temperatures (Colinet et al. 2018). However, it remains unclear what part of the FTR improves outcomes for insects.

Because of its economic importance, the alfalfa leafcutting bee, *Megachile rotundata* has been extensively used as a model system to study the effects of FTR (Rinehart et al. 2011, 2013, 2016; Yocum et al. 2012; Adbelrahman et al. 2014; Bennett et al. 2015). As the primary pollinator for alfalfa, *Medicago sativa* L. (F.) in North America (Pitts-Singer and Cane 2011), the alfalfa leafcutting bee may be subjected to two periods of low-temperature storage during its life cycle. The longest period of exposure to low temperatures, which may be up to 10 months in duration in managed bees, is during the overwintering storage of the diapausing prepupa (Pitts-Singer and Cane 2011). During this time, bees are assumed to have protection against low temperatures, because they are in diapause and post-diapause quiescence. The second period of low temperature exposure may occur during the spring. In the wild, bees may be exposed to rapid decreases in ambient temperature due to extreme weather events. In agricultural systems, bees may be placed back into low temperature storage to delay development if there is a change in the weather that could cause a delay in crop bloom or expose the newly emerged bees to deleterious temperatures (Undurraga and Stephen 1980; Stephen 1981; Rank and Goerzen 1982, Yocum et al. 2010).

Although placing developing bees in low temperatures is a common practice in agriculture, the risk of mortality increases (Rinehart et al. 2011). Furthermore, bees that survive replacement in low temperature storage exhibit sublethal effects. Just 1 week of exposure to 6°C caused developing *M. rotundata* pupae to exhibit a suite of harmful effects. Adult males had decreased flight performance and

decreased longevity, and bees of both sexes had delayed emergence and shorter wings (Bennett et al. 2015). Those sub-lethal effects were ameliorated using FTRs (Bennett et al. 2015). However, use of FTR does not block the accumulation of all sub-lethal effects. Storing diapausing *M. rotundata* prepupae under hypoxic conditions and FTR significantly increased their cold tolerance and had no observable morphological effects, but the same prepupae exhibited decreased feeding and adult longevity (Abdelrahman et al. 2014). Therefore, identifying a more optimal thermal regime would improve survival and decrease the presence of sub-lethal effects.

The rapid rise in temperature and the short duration of exposure to the peak temperature normally employed in FTR are not ecologically relevant and therefore may not reflect the true ability of *M. rotundata* to tolerate sub-optimal temperatures. The objectives of this investigation are two-fold. The first is to determine if a wave form of thermoprofiles or thermoprofiles with exposure to the peak temperature for 6 or 12 h will improve *M. rotundata* survival when compared with FTR and constant 6°C. The second objective is to determine if the various thermoprofiles induce sub-lethal effects impacting time-to-emergence.

Materials and methods

Insects

Megachile rotundata prepupae were purchased from JWM Leafcutters, Inc. (Nampa, ID) as loose brood cells in the spring of 2013. Upon receipt, the prepupae were transferred to 6°C and darkness until initiation of the experiment. Each individual cell was inspected to ensure that the cell contained a complete cocoon, indicating that the larva had reached the prepupal stage. Cells were placed individually in wells of 24-well culture plates (Falcon, Corning, NY). Plates were assigned to developmental group and treatment using a random number table. The experimental design was a 10×8×2 arrangement consisting of 10 thermoprofiles (experimental and control thermoprofiles; Fig. 1), exposure durations to the thermoprofiles (1–8 weeks), and initiation of the exposure at developmental stages (eye-pigmented pupae and emergence-ready adults). Once a week, three plates from each temperature treatment were transferred to constant 29°C to resume development. Plates were checked weekly on Monday, Wednesday, and Friday, and emergence date and sex were recorded.

Temperature treatments

The profiles for the temperature treatments were chosen based on 1) literature showing that *M. rotundata* pupae do not progress in development below 16–18°C (O'Neill et al. 2011), 2) literature indicating insects respond differently to wave versus square thermal profiles (Yoshii et al. 2009; Miyazaki et al. 2011), and 3) our prior observations that warm pulses between 15 and 25°C are beneficial to *M. rotundata* pupae exposed to temperatures low enough to cause chill injury (Rinehart et al. 2016). In the experimental treatments, 4 square thermoperiods were used with a base temperature of 6°C and upper temperature of 12°C or 18°C, with a cryophase (CP) of 12 or 18 h and a thermophase (TP) of 6 or 12 h (Fig. 1). These square thermoprofiles are referred to as "12°C pulse; 12CP:12TP," "12°C pulse; 18CP:6TP," "18°C pulse; 12CP:12TP," and "18°C pulse; 18CP:6TP." Four pseudo-sinusoidal (wave) thermoprofiles with a base temperature of 6°C and a peak temperature of 12°C or 18°C, with either a symmetrical shape (symm) with an approximate ramp rate of 10 h up and down or an asymmetrical shape (asymm) with an approximate ramp rate of 8 h up and 10 h down. These wave thermoprofiles are referred to as "12°C pulse; symm," "12°C pulse; asymm," "18°C pulse; symm," and "18°C pulse; asymm." In addition to the eight experimental regimes, we included two control thermoprofiles, constant 6°C and a FTR control with a base temperature of 6°C and daily 1 h pulse at 20°C (Rinehart et al. 2013). We chose constant 6°C, because it represented the base temperature without any warm pulse and is already known to cause chill injury in this context (Rinehart et al. 2013, 2016). The thermoprofile with a daily 1 h pulse at 20°C was chosen, because it has already been demonstrated to improve survival compared with constant 6 degrees C (Rinehart et al. 2011, 2013, 2016). We exposed bees to these 10 regimes for 1–8 weeks at one of two developmental stages (see the section "Description of life stages"). In addition, 72 prepupae were exposed to constant 29°C until they emerged as adults. The constant 29°C served as a no-storage control and represented a baseline survival if there were no cold exposure during pupal development.

All temperature regimes were carried out using environmental chambers, Conviron model I25L (Winnipeg, Manitoba, Canada) or Percival models I30BLL and PGC-105 (Percival Scientific, Perry, IA). Temperatures were maintained within ±0.5°C. All thermoprofile chambers used in this investigation were calibrated at 6°C (base temperature) and their respective upper pulse temperature (12, 18, or 20°C) with an Omega HH506R data logger (Omega Engineering, Stamford, CT) with a type-T 30-gauge

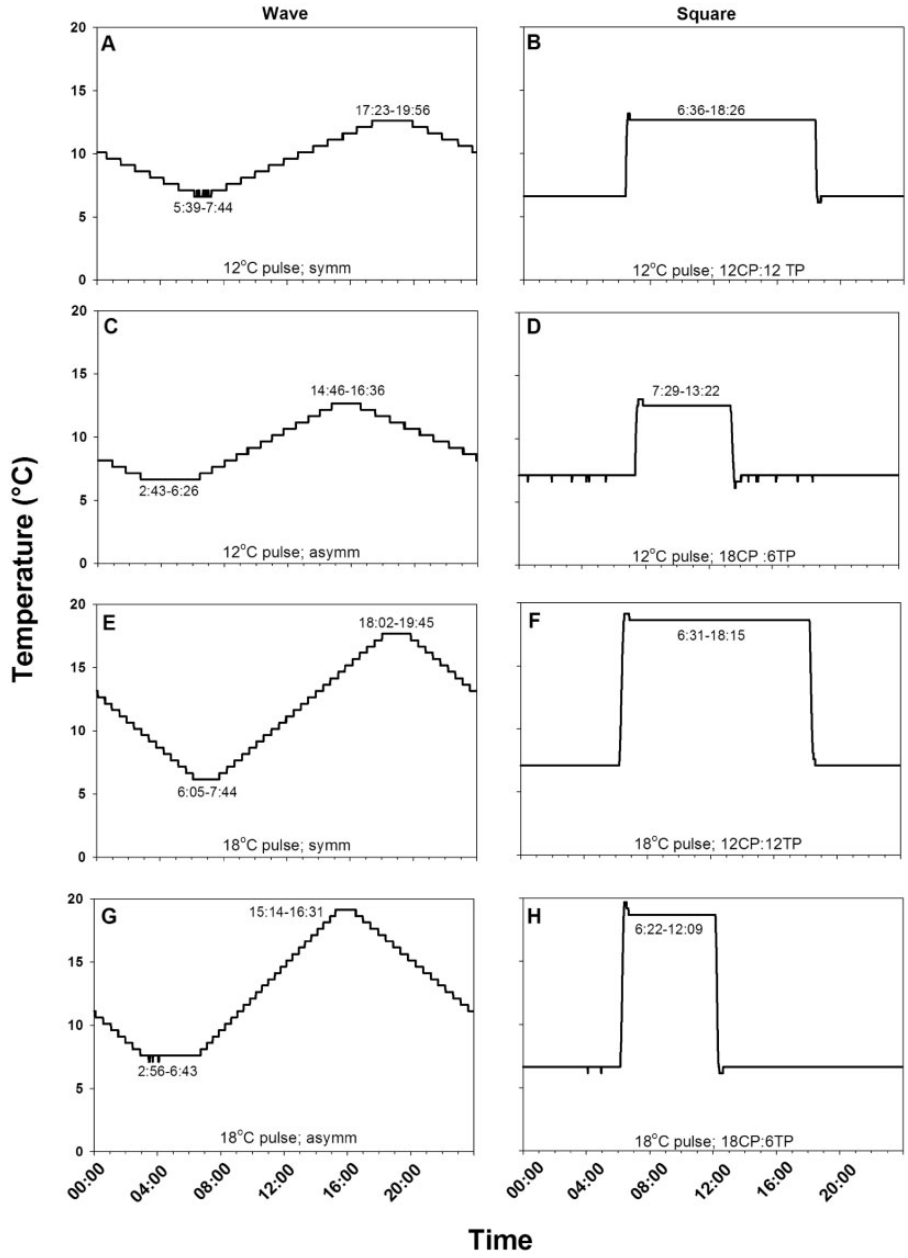

Fig. 1 Thermoprofiles of the low temperature storage regimes. The base temperature was 6°C (cryophase, CP) for all temperature regimes, and upper temperature was either 12°C or 18°C (thermophase, TP). The wave form thermoprofiles had an overall symmetrical (symm) shape with an approximate temperature ramp time of 10 h up and 10 h down or an asymmetrical (asymm) shape with an approximate ramp time of 8 h up and 10 h down. The square shape thermoperiods had ramped speeds between the base temperature and upper temperature of 30 min or less. The curves shown were constructed from iButton recordings.

copper-constantan thermocouple prior to running the experiment. The constant 6°C and 29°C control chambers were calibrated only at their set temperatures. The thermocouple was placed in the same location as the plates of bees. To ensure that the chambers functioned correctly throughout the experiment, iButtons (Maxim Integrated, San Jose, CA) were placed in the chambers during the last week of the experiment to record the chambers' temperatures.

Description of life stages

Developing *M. rotundata* were exposed to the thermoprofiles at two developmental stages: eye-pigmented pupae and emergence-ready adults. These two stages were chosen, because each is easily identified by external characteristics (color of the eyes and whether bees are emerging). Emergence-ready bees are more sensitive to cold stress than eye-pigmented pupae (Rinehart et al. 2016), making a comparison of these two stages necessary for

understanding chill injury in *M. rotundata*. To determine when pupae were in the correct stage to place into the experimental treatments, "guide bees" were used. Guide bees were comprised of 72 prepupae that were dissected out of their cocoons and placed into 24-well culture plates. Plates were placed into a 25.4 cm diameter×8.9 cm plastic container (Pioneer Plastic, Newark, NJ), along with three cups (118 mL, Sweetheart Plastics, Wilmington, MA) of saturated sodium chloride (Morton, Chicago, IL) to maintain relative humidity at 74% (Winston and Bates 1960). All bees were transferred to 29°C to initiate development. Once 50% of the guide bees reached the eye-pigmented stage, the eye-pigment group was transferred to their designated thermoprofile. The emergence-ready group was transferred to their designated thermoprofile once the first guide bees started to emerge. Emergence of the guide bees was defined as legs and wings expanded and the bee standing upright within the well. Those experimental emergence-ready bees that emerged before storage were noted and not used in the statistical analysis.

Statistical analysis

Logistic regression analysis of survival data

To determine the effect of the different thermoprofiles upon survival, logistic regression was carried out using the FTR treatment as the reference treatment (JMP, Version 13.2.1, SAS Institute Inc., Cary, NC). The thermoprofiles were treated as categorical variables and week as a continuous variable. Following the logistic regression, individual least square mean contrast analysis was carried out for each treatment compared with the constant 6°C and FTR treatment.

Analysis of exponential emergence generalized linear mixed model for censored data

This statistical approach was achieved via the GLIMMIX Procedure in SAS/STAT 14.3 Version 9.4 (TS1M5) (SAS Institute Inc.) following the implementation described in Stroup (2013). The two response variables were the censoring variable (C), and the time-to-emergence (T). An observation was censored if emergence did not occur prior to the defined completion of the study, which indicated death or emergence of parasitic wasp species. An uncensored observation means that the bee emerged while at 29°C, prior to the conclusion of the study. Based on the high survival rate of the 29°C control group (84.5 ± 1.2%, mean ± SEM), all mortality observed in the bees exposed to the low-temperature treatments was attributed to the treatments. Therefore, the observed mortality occurred before the observation period resulting in the bees being left-censored in the statistical analysis. The censored event was coded in the data to have occurred on day 0 of the post-storage observation period at 29°C. The censoring variable is the response variable with a Poisson distribution. The log of time-to-emergence (T) is an *offset* in the model. Fixed effects were the interaction of the factors thermoprofile and week, and the random effect identifies the design structure, where each observational subject is the individual plate within a treatment by week combination.

The option statement LSMEANS provided the estimates of log of the rate parameter of emergence, λ, and the ILINK option provides the actual values of the estimated hazard function, the inverse link of the mean emergence day. The LSMESTIMATE option provided estimates of the mean emergence time, and comparisons of the cell means of the thermoprofiles and week interactions.

Results

Overview

The impact of the various thermoprofiles on survival was dependent on the developmental stage examined (Table 1). Although the 12°C pulse thermoprofiles improved survival of the bees relative to the constant 6°C control in both the eye-pigmented and emergence-ready stages, there was a clear developmental effect in the bees exposed to the 18°C pulse thermoprofiles. The eye-pigmented bees had survival rates equivalent (18°C pulse; asymm and 18°C pulse; symm) or greater (18°C pulse; 12CP:12TP and 18°C pulse; 18CP:6TP) than the FTR control, whereas all of the 18°C thermoprofiles significantly improved survival in the emergence-ready adult bees. By week 8, the 18°C thermoprofiles can be further subdivided based on the profiles' shapes, with the square profiles outperforming the wave profiles.

The effect of the peak temperature on survival is even more delineated in the emergence-ready stage (Table 1). As in the eye-pigmented stage, all the thermoprofiles improved survival compared with constant 6°C. The 12°C thermoprofiles only achieved survival rates equivalent to the FTR control (12 pulse; 12CP:12TP and 12 pulse; symm) or lower, whereas the 18°C pulse thermoprofiles improved survival over that of the FTR bees.

Survival of eye-pigmented pupae

Interrupting the development of eye-pigmented pupae by exposure to various low-temperature

Table 1 Percent survival (mean±SEM) of *Megachile rotundata* whose spring incubation was interrupted by various low-temperature treatments

		Treatments				
Developmental stage	**Week**	**12°C pulse; 12CP:12TP**	**12°C pulse; symm**	**12°C pulse; 18CT:6TP**	**12°C pulse; asymm**	**6°C constant**
Eye-pigmented	1	84.5±1.2	87.3±2.4	90.2±5	86.1±1.3	91.6±2.4
	2	91.7±0	88.6±3	88.6±5.6	79.1±6.3	80.5±1.3
	3	86.1±3.7	84.7±6	80.5±3.6	72.2±5.5	65.2±1.3
	4	73.6±2.8	73.1±7.1	68±6	61.1±7.7	34.7±11.1
	5	56.3±3.2	58.3±7.2	40.2±2.7	40.2±9.1	16.7±4.7
	6	40.3±11.4	34.7±9.1	30.5±9.1	22.2±3.6	5.5±1.3
	7	30.6±3.7	36.1±8.4	13.8±1.3	8.6±6.6	4.1±2.4
	8	31.9±8.4	29.6±10.1	18±3.6	4.2±0	1.3±1.3
		Treatments				
Developmental stage	**Week**	**18°C pulse; 12CP:12TP**	**18°C pulse; symm**	**18°C pulse; 18CT:6TP**	**18°C pulse; asymm**	**FTR**
Eye-pigmented	1	95.8±4.1	86.1±3.6	91.6±0	84.7±3.6	91.6±2.4
	2	84.6±3.5	88.8±3.6	88.8±1.3	84.7±3.6	83±4.8
	3	87.5±4.1	77.7±2.7	91.6±4.1	93±5	88.8±3.6
	4	89.9±6.3	87.5±4.1	84.7±3.6	88.7±2.7	70.8±4.1
	5	81.9±2.7	87.5±4.1	86.1±2.7	83.3±2.4	77.7±3.6
	6	86.1±3.6	71.8±7.7	81.9±2.7	69.4±2.7	65.2±9.1
	7	80.1±3.9	49.3±1.8	66.1±4.8	42.8±7.5	50.6±1.8
	8	73.1±1.8	38.8±2.7	72.2±2.7	55.5±3.6	27.7±5
		Treatments				
Developmental stage	Week	**12°C pulse; 12CP:12TP**	**12°C pulse; symm**	**12°C pulse; 18CT:6TP**	**12°C pulse; asymm**	**6°C constant**
Emergence-ready	1	74.2±15.4	83.9±3.3	95.8±0	84.7±7.7	91.6±4.1
	2	84.7±5	83.3±4.8	88.6±5.6	86.1±1.3	56.9±5
	3	79.1±8.6	79.1±2.4	68±3.6	70.8±8.6	31.9±10
	4	58.3±6.3	50±8.3	56.2±2	36.1±5.5	4.1±2.4
	5	23.6±3.6	22.2±2.7	9.7±2.7	11.1±5	1.3±1.3
	6	16.6±8.3	6.1±1.3	2.7±1.3	0±0	0±0
	7	1.3±1.3	2.7±1.3	1.3±1.3	0±0	0±0
	8	1.3±1.3	12.7±6.4	0±0	1.3±1.3	0±0
		Treatments				
Developmental stage	**Week**	**18°C pulse; 12CP:12TP**	**18°C pulse; symm**	**18°C pulse; 18CT:6TP**	**18°C pulse; asymm**	**FTR**
Emergence-ready	1	93±5	84.7±6	91.4±0.1	86.1±2.7	93±2.7
	2	79.6±10.4	91.6±2.4	78.3±9.4	90.2±2.7	91.6±4.1
	3	80.5±3.6	81.9±6	91.6±2.4	87.5±2.4	80.1±3.9
	4	86.1±5	77.7±2.7	86.1±5	87.1±5	63.7±12.4
	5	84.4±6	58.3±2.4	67.6±2.5	69.9±4.8	32.5±6.5
	6	69.4±7.7	41.6±2.4	54.9±0.7	51.5±3.1	15.2±2.7
	7	73.6±6.9	33.3±6.3	57.1±7.5	51.3±1.3	5.5±1.3
	8	77.7±6	20.8±2.4	29.4±3.8	37.9±4.4	1.3±1.3

thermoprofiles had a significant impact on survival ($\chi^2 = 1867.7$, df = 19, $P < 0.0001$; Fig. 2A). The effects of treatment ($\chi^2 = 543.7$, df = 9, $P < 0.0001$), week ($\chi^2 = 1044.8$, df = 1, $P < 0.0001$), and their interaction ($\chi^2 = 151.8$, df = 9, $P < 0.0001$) were all significant.

Compared with the constant 6°C control, all the individual low-temperature thermoprofiles significantly increased the survival of the bees ($P < 0.0001$; Table 2). Comparing the survival of the bees exposed to the various thermoprofiles to the daily 1 h pulse at 20°C (FTR) control group yielded three distinct groups (Table 2). The first group consisted of thermoprofiles that were not significantly different from the FTR control: 18°C pulse; symm ($\chi^2 = 1.39$, $P = 0.239$), and 18°C pulse; asymm ($\chi^2 = 3.04$, $P = 0.081$). The second group was formed by the thermoprofiles that had significantly lower rates of survival than the FTR control: 12°C pulse; 12CP:12TP ($\chi^2 = 6.61$, $P = 0.010$), 12°C pulse; 18CP:6TP ($\chi^2 = 28.14$, $P < 0.0001$), 12°C pulse; symm ($\chi^2 = 7.15$, $P = 0.007$), 12°C pulse; asymm ($\chi^2 = 69.60$, $P < 0.0001$), and 6°C ($\chi^2 = 166.85$, $P < 0.0001$). The final group consisted of two thermoprofiles, 18°C pulse; 12CP:12TP ($\chi^2 = 23.59$, $P < 0.0001$) and 18°C pulse; 18CP:6TP ($\chi^2 = 17.79$, $P < 0.0001$) that had significantly higher rates of survival compared with FTR control.

Survival of emergence-ready adults

The survival of emergence-ready adults exposed to various low-temperature thermoprofiles was significantly impacted ($\chi^2 = 3038.131$, df = 19, $P < 0.0001$; Fig. 2B). The effects of treatment ($\chi^2 = 980.856$, df = 9, $P < 0.0001$), week ($\chi^2 = 2030.196$, df = 1, $P < 0.0001$), and their interaction ($\chi^2 = 316.385$, df = 9, $P < 0.0001$) were all significant. All the thermoprofiles significantly improved survival when compared with the constant 6°C control.

Comparing the survival of emergence-ready adults exposed to the various thermoprofiles to the FTR control produced groupings similar to those seen in the eye-pigmented pupae (Table 3). Two of the 12°C thermoprofiles, 12°C pulse; 18CP:6TP ($\chi^2 = 19.21$, $P < 0.0001$) and 12°C pulse; asymm ($\chi^2 = 35.83$, $P < 0.0001$), as well as the 6°C constant ($\chi^2 = 141.86$, $P < 0.0001$) negatively impacted *M. rotundata* survival. Counter to the results for eye-pigment pupae, all the 18°C thermoprofiles significantly improved survival of emergence-ready adults, 18°C pulse; 12CP:12TP ($\chi^2 = 103.83$, $P < 0.0001$), 18°C pulse; symm ($\chi^2 = 21.82$, $P < 0.0001$), 18°C pulse; 18CP:6TP ($\chi^2 = 63.35$, $P < 0.0001$), and 18°C pulse; asymm ($\chi^2 = 57.77$, $P < 0.0001$). Two thermoprofiles, 12°C pulse; 12CP:12TP ($\chi^2 = 4.44$, $P = 0.035$) and 12°C pulse; symm ($\chi^2 = 2.59$, $P = 0.18$), did not significantly impact survival when compared with the FTR control.

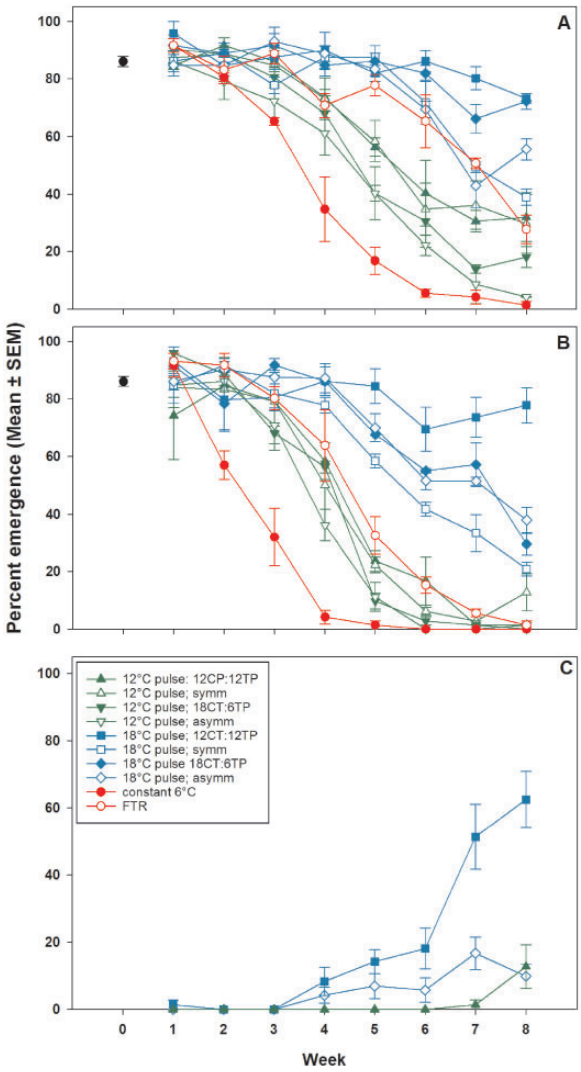

Fig. 2 Percent (mean±SEM) emergence of *M. rotundata* following exposure to low-temperature thermoprofiles for 1–8 weeks: (**A**) eye-pigmented pupae, (**B**) emergence-ready adults, and (**C**) emergence-ready adults that emerged during treatment. For a description of the thermoprofiles see caption of Fig. 1. The FTR treatment consisted of a base temperature of 6°C and a daily 1 h pulse at 20°C. Each time-point consisted of three groups of 24 bees each.

Premature emergence during treatment

Some emergence-ready bees emerged during the long-term exposure to the thermoprofiles, a response not observed at the eye-pigment stage (Fig. 2C). Bees emerged early from the following thermoprofiles: 12°C pulse; 12CP:12TP, 18°C pulse; 12CP:12TP and 18°C pulse; asymm. Early emergence started

Table 2 Least square mean comparisons of survival of eye-pigmented *Megachile rotundata* stored under various low temperatures[a]

Reference	Treatment	Value[b]	Std error	χ^2	Prob > χ^2
6°C constant	12°C pulse; 12CP:12TP	1.66	0.17	110.7	0.000
6°C constant	12°C pulse; symm	1.65	0.17	108.64	0.000
6°C constant	12°C pulse; 18CP:6TP	1.26	0.17	58.72	0.000
6°C constant	12°C pulse; asymm	0.82	0.17	23.45	0.000
6°C constant	18°C pulse; 12CP:12TP	2.83	0.18	306.84	0.000
6°C constant	18°C pulse; symm	2.22	0.18	5.72	0.000
6°C constant	18°C pulse; 18CP:6TP	2.72	0.18	286.94	0.000
6°C constant	18°C pulse; asymm	2.31	0.18	213.79	0.000
FTR	12°C pulse; 12CP:12TP	−0.38	0.15	6.61	0.010
FTR	12°C pulse; symm	−0.39	0.15	7.15	0.007
FTR	12°C pulse; 18CP:6TP	−0.78	0.15	28.14	0.000
FTR	12°C pulse; asymm	−1.22	0.15	69.60	0.000
FTR	18°C pulse; 12CP:12TP	0.79	0.16	23.59	0.000
FTR	18°C pulse; symm	0.18	0.15	1.39	0.239
FTR	18°C pulse; 18CP:6TP	0.68	0.16	17.96	0.000
FTR	18°C pulse; asymm	0.27	0.15	3.04	0.081
FTR	6°C constant	−2.04	0.18	166.85	0.000

[a]The covariance value for week was set to the default value of 4.5.
[b]The variable "value" is the difference between the log odds between the two treatments.

Table 3 Least square mean of survival of emergence-ready *Megachile rotundata* stored under various low temperatures[a]

Reference	Treatment	Value[b]	Std error	χ^2	Prob > χ^2
6°C constant	12°C pulse; 12CP:12TP	2.62	0.33	107.35	0.000
6°C constant	12°C pulse; symm	2.71	0.32	118.93	0.000
6°C constant	12°C pulse; 18CP:6TP	2.14	0.34	51.76	0.000
6°C constant	12°C pulse; asymm	1.84	0.34	35.78	0.000
6°C constant	18°C pulse; 12CP:12TP	4.57	0.32	540.00	0.000
6°C constant	18°C pulse; symm	3.69	0.32	301.84	0.000
6°C constant	18°C pulse; 18CP:6TP	4.23	0.33	427.61	0.000
6°C constant	18°C pulse; asymm	4.15	0.32	416.74	0.000
FTR	12°C pulse; 12CP:12TP	−0.34	0.16	4.44	0.035
FTR	12°C pulse; symm	−0.25	0.16	2.59	0.18
FTR	12°C pulse; 18CP:6TP	−0.82	0.19	19.21	0.000
FTR	12°C pulse; asymm	−1.12	0.19	35.83	0.000
FTR	18°C pulse; 12CP:12TP	1.60	0.16	103.83	0.000
FTR	18°C pulse; symm	0.72	0.16	21.83	0.000
FTR	18°C pulse; 18CP:6TP	1.26	0.16	63.35	0.000
FTR	18°C pulse; asymm	1.9	0.16	57.77	0.000
FTR	6°C constant	−2.97	0.32	141.86	0.000

[a]The covariance value for week was set to the default value of 4.5.
[b]The variable "value" is the difference between the log odds between the two treatments.

between weeks 3 and 4 in the thermoprofiles 18°C pulse; 12CP:12TP and 18°C pulse; asymm, with the rate of emergence increasing in the following weeks. Early emergence began between weeks 6 and 7 in the 12°C pulse; 12CP:12TP thermoprofile. By week 8, emergence during low-temperature exposure reached

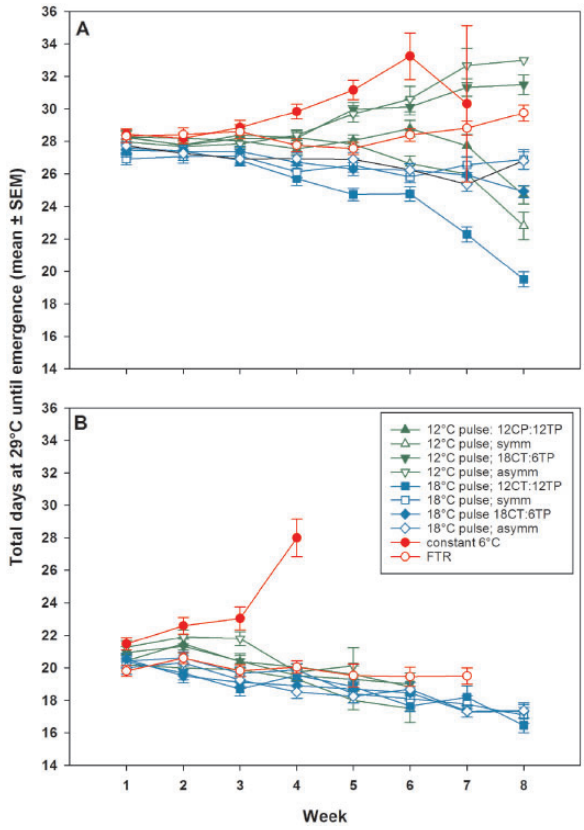

Fig. 3 Total number of days at 29°C until emergence (time-to-emergence) for *M. rotundata* following exposure to low-temperature thermoprofiles for 1–8 weeks: (**A**) eye-pigmented pupae and (**B**) emergence-ready adults. For a description of the thermoprofiles, see caption of Fig. 1. Each time-point consisted of three groups of 24 bees each.

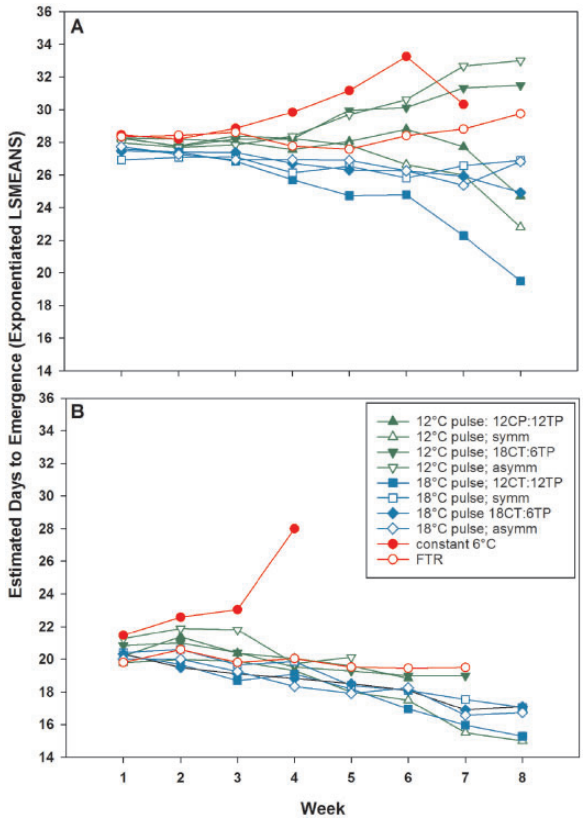

Fig. 4 A plot of LSMEANS values of the estimated days to emergence for all treatment and time (week) exposures in eye-pigmented pupae (**A**) and emergence-ready adults (**B**). The LSMEANS estimates account for all terms in the statistical model, which includes the random factors due to experimental blocking and the correlated aspects of the censored (dead) observations and the emerged observations. Treatment combinations that experienced large proportions of censored observations resulting in sample sizes of 2 or less are not represented in the plot.

62 ± 8.5%, 12.7 ± 6.5%, and 9.8 ± 3.6% for the 18°C pulse; 12CP:12TP, 12°C pulse; 12CP:12TP, and 18°C pulse; asymm thermoprofiles, respectively.

Effect of exposure to low-temperature thermoprofiles on time-to-emergence

Compared with FTR week 1 bees, the various thermoperiods had little effect on the time at 29°C required for emergence (Figs. 3 and 4, Table 4). The only statistically significant impact on time-to-emergence at 29°C was observed in the eye-pigmented pupae exposed to the pulse 18°C; 12CP:12TP for 8 weeks ($P = 0.0465$). These bees took significantly fewer days to emerge (19.5 ± 0.5, mean ± SEM) compared with the week 1 FTR bees (23.3 ± 0.4) days.

Discussion

Insects have complex responses to temperature that are only beginning to be understood (Colinet et al. 2015, 2018). We exposed *M. rotundata* to different low-temperature regimes during two developmental stages with the goal of determining if more ecologically relevant (wave form) thermoprofiles or thermoprofiles with extended times at peak temperatures would improve survival or protect against delays in time-to-emergence when compared with constant temperature or the abrupt temperature changes and short pulse employed in FTR treatments. In contrast to our predictions, thermoprofiles with abrupt changes in temperature resulted in better outcomes for bees. The other major finding, the positive correlation of survival with increasing peak temperature and duration at peak temperature observed in this investigation, is consistent with previous reports (Leopold et al. 1998; Hanč and Nedvěd 1999; Colinet et al. 2015; Yocum et al. 2010; Rinehart et al. 2016). As the temperature increases, the duration needed to achieve a specific level of survival became shorter.

Table 4 Mean (±SEM) number of days at 29°C required for emergence of *Megachile rotundata* whose spring incubation was interrupted by various low-temperature treatments

		Treatments				
Developmental stage	**Week**	**12°C pulse; 12CP:12TP**	**12°C pulse; symm**	**12°C pulse; 18CT:6TP**	**12°C pulse; asymm**	**6°C constant**
Eye-pigmented	1	28.3±0.4	28.2±0.4	28.3±0.3	28.0±0.4	28.5±0.3
	2	28.2±0.3	27.8±0.7	27.8±0.4	27.7±0.4	28.2±0.3
	3	28.0±0.4	28.4±0.4	28.2±0.4	27.9±0.3	28.9±0.5
	4	27.6±0.4	28.2±0.4	28.2±0.4	28.4±0.3	29.8±0.5
	5	28.1±0.4	27.8±0.3	30.0±0.4	29.7±0.5	31.2±0.6
	6	28.8±0.5	26.6±0.5	30.1±0.5	30.6±0.8	33.3±1.4
	7	27.7±0.7	26.0±0.5	31.3±0.5	32.7±1.1	30.3±4.8
	8	24.7±0.6	22.8±0.9	31.5±0.6	33.0±0	0±0
		Treatments				
Developmental stage	**Week**	**18°C pulse; 12CP:12TP**	**18°C pulse; symm**	**18°C pulse; 18CT:6TP**	**18°C pulse; asymm**	**FTR**
Eye-pigmented	1	27.6±0.4	26.9±0.3	27.5±0.4	27.7±0.4	28.3±0.4
	2	27.4±0.4	27.1±0.4	27.4±0.4	27.3±0.4	28.4±0.4
	3	26.8±0.4	27.1±0.5	27.4±0.3	26.9±0.4	28.6±0.4
	4	25.7±0.4	26.1±0.4	26.7±0.4	26.9±0.4	27.8±0.4
	5	24.7±0.4	26.5±0.4	26.3±0.4	26.9±0.4	27.6±0.4
	6	24.8±0.5	25.8±0.4	26.3±0.4	26.3±0.4	28.4±0.4
	7	22.3±0.5	26.6±0.5	25.9±0.5	25.4±0.4	28.8±0.4
	8	19.5±0.5	26.9±0.6	24.9±0.4	26.8±0.5	29.8±0.5
		Treatments				
Developmental stage	**Week**	**12°C pulse; 12CP:12TP**	**12°C pulse; symm**	**12°C pulse; 18CT:6TP**	**12°C pulse; asymm**	**6°C constant**
Emergence-ready	1	20.4±0.4	20.2±0.3	20.9±0.4	21.3±0.4	21.5±0.4
	2	21.5±0.4	20.0±0.3	21.3±0.4	21.9±0.4	22.6±0.5
	3	20.4±0.4	19.9±0.4	20.4±0.5	21.8±0.4	23.0±0.7
	4	20.1±0.4	19.3±0.5	19.5±0.4	19.7±0.5	28±1.2
	5	19.6±0.5	18.0±0.6	19.3±0.3	20.1±1.1	0±0
	6	18.8±0.9	17.5±0.9	19.0±0	0±0	0±0
	7	0±0	0±0	0±0	0±0	0±0
	8	0±0	0±0	0±0	0±0	0±0
		Treatments				
Developmental stage	**Week**	**18°C pulse; 12CP:12TP**	**18°C pulse; symm**	**18°C pulse; 18CT:6TP**	**18°C pulse; asymm**	**FTR**
Emergence-ready	1	20.4±0.4	20.4±0.3	20.5±0.4	20.0±0.4	19.8±0.3
	2	19.7±0.4	20.6±0.4	19.5±0.4	20.3±0.4	20.6±0.3
	3	18.7±0.4	19.7±0.4	19.1±0.4	19.3±0.4	19.8±0.34
	4	19.5±0.4	19.9±0.4	18.9±0.4	18.5±0.4	20.0±0.4
	5	18.8±0.5	18.4±0.6	18.7±0.5	18.3±0.4	19.5±0.7
	6	17.7±0.3	18.1±0.5	18.5±0.4	18.7±0.4	19.5±0.6
	7	18.2±0.7	17.8±0.5	17.3±0.4	17.3±0.4	19.5±0.5
	8	16.5±0.5	17.1±0.5	17.3±0.4	17.4±0.5	0±0

Time spent at peak temperature alone is not responsible for the early emergence observed in this study. Early emergence has been previously observed in emergence-ready *M. rotundata* (Yocum et al. 2012). In that investigation, emergence-ready bees were incubated under two different thermoperiods with equivalent time at the peak temperature (20°C). Twenty-five to thirty-three percent of bees had early emergence between weeks 6 and 8. In this study, thermoprofiles 18°C pulse; 12CP:12TP and 18°C pulse; asymm induced early emergence between weeks 3 and 4, and the 12°C pulse; 12CP:12TP induced early emergence after week 7. Therefore, one might predict that the other 18°C pulse thermoprofiles would also trigger early emergence, based on the time spent at the peak temperature. However, this was not the case. Although 18°C pulse; symm received equivalent time at the peak temperature as the thermoprofile 18°C pulse; asymm, and the thermoprofile 18°C pulse; 18CP:6TP received a little over 4 h more at the peak temperature than either of the two wave thermoprofiles, neither pulse 18°C; symm or 18°C pulse; 18CP:6TP induced early emergence. These intriguing results are consistent with previous literature showing that subtle changes in temperature treatments can result in a major and non-linear impact on an insect's physiology (Jensen 1906; Martin and Huey 2008; Sinclair et al. 2016).

For example, exposing diapausing Colorado potato beetles, *Leptinotarsa decemlineata*, to either constant 0°C or constant −2.5°C induced expression of different isoforms of the 70 kDA heat shock protein (HSP70) (Yocum 2001). Furthermore, the expression patterns of these HSP70 isoforms also varied depending on if the beetles were exposed to constant temperature (0°C or −2.5°C) or thermoperiods with mean temperatures of 0°C or −2.5°C. A temperature fluctuation as small as 0.4°C is sufficient to synchronize eclosion rhythms in the tsetse fly, *Glossina morsitans* (Zdarek and Denlinger 1995).

Brief exposure to stressful, high temperatures has been demonstrated to alter insects' sensitivity to future exposure to normally lethal low and high temperatures. Exposing the Antarctic midge, *Belgica antarctica* to −5°C for as little as 15 min (a process known as rapid cold hardening, RCH) induces tolerance to normally lethal exposure to −18°C (Kawarasaki et al. 2013). In addition, in the flesh fly, *Sarcophaga crassipalpis*, exposure to 45°C for 30 min increased tolerance to stressful, high temperatures, whereas a 60 min exposure decreased tolerance (Yocum and Denlinger 1993). This dynamic physiological responsiveness to environmental conditions is highly ecologically relevant in that it enables insects to adjust to current and future environmental conditions. Exposing *Drosophila melanogaster* to an ecologically based thermoperiod induced RCH during the cooling phase of the thermoperiod and the authors argued that this enabled the flies to remain active longer during the cooler part of the day (Kelty and Lee 2001). The onion fly, *Delia antique* uses the thermoperiod to time its adult eclosion and emergence from the soil (Tanaka and Watari 2003). Due to the insulating properties of soil, the thermoperiod is delayed and its amplitude is decreased as the soil depth increases. Tanaka and Watari (2003) demonstrated that *D. antique* is able to compensate for soil depth by advancing eclosion timing to the amplitude of the thermoperiod, ensuring synchronization of adult flies. The early emergence induced by only some of the thermoprofiles in this study and the results from previous studies highlight that the modeling of insects' responses to temperature is not a trivial undertaking.

Insects are not likely to repeatedly experience the abrupt temperature shifts induced by the square thermoperiods used in most FTR investigations. Only a few published studies have compared the physiological response of insects with square and wave thermoprofiles. Maintaining *D. melanogaster* under a natural wave thermocycle entrained flies' peak activity to morning and evening, whereas a square thermoperiod shifted peak activity to the middle of the day (Yoshii et al. 2009). Interactions between amplitude and the shape (square versus wave) of the thermoprofile are species-specific. Decreasing the amplitude of the thermoprofile shifts the adult emergence earlier in the day for the flesh fly, *S. crassipalpis* (Miyazaki et al. 2011) and the alfalfa leafcutting bee, *M. rotundata* (Yocum et al. 2016). Besides shifting the timing of emergence, the amplitude of the thermoprofile can alter the number of days over which emergence occurred in *M. rotundata* (Yocum et al. 2016). The amplitude-induced shift to earlier emergence only occurred under wave and not square profiles in *S. crassipalpis* (Miyazaki et al. 2011). In contrast, in *M. rotundata*, decreasing the amplitude of the square profile did shift the emergence to earlier in the day (Yocum et al. 2016). Another interesting observation related to square versus wave thermoprofiles is the "startle response" observed in *Drosophila* by Yoshii et al. (2009). Upon the rapid temperature increase in their square thermoprofiles, flies exhibited a sharp peak of activity, which was not observed under wave profiles and was interpreted as a startle response to the rapid increase in temperature. This period of peak activity could not be re-entrained but was always linked with

the rise in the temperature. Abrupt temperature changes have been demonstrated to induce stress responses (Lindquist 1986; Colinet et al. 2007). Indeed, stress genes have been demonstrated to be upregulated in *M. rotundata* in response to FTR (Torson et al. 2015, 2017). Therefore, the rapid changes in temperature experienced by bees under both the FTR and square thermoprofiles are likely to be inducing some form of stress response, explaining their increased survival. Although the number of studies comparing square and wave thermoprofiles is still small, clearly the physiological responses triggered by these two types of thermoprofiles are not the same.

Conclusion

Climate change is expected to increase not only the average temperature, but also the likelihood of extreme weather events, such as cold snaps in the spring. However, predicting the effect of temperature on insect physiology is not always a straightforward proposition. The early emergence of *M. rotundata* emergence-ready adults during low-temperature exposure does not appear to be dependent on the peak temperature, duration at peak temperature, nor the shape of the thermoprofile. As average temperatures increase, we may begin to see more early emergence of field-overwintering bees. The impact of a thermoprofile on survival can be also enigmatic. Although the 18°C pulse; 12CP:12TP in this study offered the greatest level of protection, under this same thermoprofile, the diapausing red seed sunflower weevil, *Smicronyx fulvus* LeConte, had a lower survival rate than the constant 6°C or FTR controls (Prasifka et al. 2015). This suggests that extrapolating one's findings to other species may be difficult. Also, counter to what would be expected, the results presented here demonstrate that the less ecologically-relevant profile (square) with an appropriate peak temperature protected the bees to the greatest extent from increased mortality, suggesting that quick changes in environmental temperature may provide hormetic protection against extreme weather events. We propose a working model for the beneficial effects of square form thermoprofiles composed of four interacting factors: developmental stage, peak temperature, duration at peak temperature, and a rapid increase in temperature of sufficient amplitude to trigger a stress response. Development of an accurate model will require data on metabolism, growth, and development across a range of constant and fluctuating temperatures. Our larger research group is currently tackling this challenge for *M. rotundata* and two other bee species, *Osmia lignaria*, the blue orchard bee, and *Bombus impatiens*, the Eastern bumble bee.

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Conflict of interest statement

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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