The successful management of the alfalfa leafcutting bee, *Megachile rotundata* (F.), is vital to the production of alfalfa seed in the United States and Canada (see Peterson et al. 1992, Free 1993, Delaplane and Mayer 2000, Stephen 2003, and Pitts–Singer 2008 for reviews). A crucial component of successful bee management includes winter storage of diapausing prepupae, with incubation the following spring to resume development to adulthood (Pitts–Singer and Cane 2011). While many aspects of *M. rotundata* management have been optimized for its use as a pollinator in alfalfa seed production, obstacles restricting more widespread use remain. For instance, resuming development earlier than normal during the overwintering period results in significantly slower development times (Richards et al. 1987, Yocum et al. 2006). This limits the use of *M. rotundata* in early blooming crops and is a potential hurdle when developing unique applications for this species, such as using a boreal population of *M. rotundata* for pollination in the southern hemisphere during the austral summer (Donovan et al. 1982, Bitner and Peterson 2003). Additionally, the price of *M. rotundata* is affected by yearly fluctuations in both supply and demand. Between 2007 and 2009, prices for a gallon (averaging 10,000 bees) ranged from US$20 to US$85 (Hodgson et al. 2011), and have continued to rise to as much as US$140 per gallon, creating financial uncertainty for those who rely on this alternative pollinator.

Previously, our group has addressed the optimization of a short-term storage protocol for developing *M. rotundata*, which can be used to add flexibility to spring bee management. While developing bees could be stored under a static thermal regime (STR) at 6°C for up to 1 wk without a significant increase in mortality (Yocum et al. 2010), implementing a fluctuating thermal regime (FTR) by which the 6°C storage temperature was interrupted by a daily 1 h pulse at 20°C extended successful storage up to 5 wk (Rinehart et al. 2011). Additional studies demonstrated that survival varied directly with both high temperature pulse incidence and duration, although premature emergence during the longer pulse durations and more frequent pulse incidence limited their practical application (Yocum et al. 2012). The benefits of FTR during cold storage for up to 23 mo, where bees were exposed are not limited to *M. rotundata*. A daily high temperature pulse has also been shown to increase survival of cold storage in the collembolan *Orchesella cincta* L. (Nedvěd et al. 1998), the fire bug *Pyrrhocoris apterus* L. (Háně and Nedvěd 1999), the tropical beetle *Alphitobius diaperinus* (Panzer) (Renault et al. 2004), and in the parasitic wasp *Alphitobius diaperinus* (Panzer) (Renault et al. 2004), and in the parasitic wasp
A subsequent series of experiments was conducted on bees from the 2009 field season to replicate the previously described experiment and to investigate the effects of quiescent stage (as described by Yocum et al. 2006) on the efficacy of FTR storage. In each instance, prepupae were stored at a 6°C constant darkness STR until the start of the experiment. The first experiment started with prepupae in diapause, which were placed into the STR and FTR treatment groups in November 2009, after 2 mo of storage at STR alone (Fig. 1a). The second experiment started with early postdiapause quiescent prepupae that were placed into the two treatment groups in April 2010, after 7 mo of storage at STR alone (Fig. 1b). To repeat the aforementioned experiment, FTR was initiated on a third set beginning in August 2010, after 11 mo of storage at STR (Fig. 1c). Finally, to test the effects of FTR on very late postdiapause quiescent prepupae, FTR was started on a final set in October 2011, after 13 mo of storage at STR (Fig. 1d). Prepupae were treated as described above, with the exception of 15 monthly timepoints (May 2010 through August 2011) used for the true diapause and early postdiapause groups, 12 monthly timepoints (September 2010 through August 2011) for the repeat experiment, and 10 monthly timepoints (November 2010 through August 2011) for the very late postdiapause prepupae. This resulted in 7,488 prepupae in 312 plates that were randomly assigned to a treatment group (STR or FTR) and a monthly timepoint. The diversity of starting times precluded random assignment of prepupae to the quiescent stage tested.

In all instances, storage regimes were conducted in Percival model I-30BLL reach-in incubators. As previously described (Rinehart et al. 2011), the STR consisted of a 6°C ±0.5°C constant temperature with a photoperiod of 15:9 (L:D) h. The FTR consisted of 21 h at 6°C, a 1 h ramp to 20°C (with a ramp speed of 0.23°C/min), 1 h incubation at 20°C, and a 1 h ramp down to 6°C, with the high temperature exposure occurring during the photophase of a 15:9 (L:D) h light cycle. At monthly intervals, three plates (containing 72 prepupae) were removed from both treatments, and transferred to 29°C and constant darkness, which is the standard protocol to initiate development and eventual adult emergence (Pitts-Singer 2008). Because emergence would be expected in ~3 wk under normal circumstances (Pitts-Singer and Cane 2011), storage survival was assessed 2 mo after transfer to 29°C, with survival being defined as the ability of a fully developed adult to emerge from the cell.

Adult Longevity. As a metric of adult quality after preupal storage, an additional study monitored adult longevity after emergence. During the late spring and early summer of 2011, bees from the 2010 field season stored under STR (as per current management practices or CMP) were compared with bees from the 2009 field season stored under FTR since August 2010 (extended storage or ES). Individual bee cells were placed in one pint wide mouth jars fitted with screened lids, along with a one ounce lidded cup that provided access to a 10% sugar solution via a dental
wick. These jars were placed in large sealable storage boxes containing 4 oz. cups filled with a saturated NaCl solution to maintain a humidity of 75% (as described in Winston and Bates 1960), while temperature was maintained at 29°C. Sugar solution was changed weekly, while NaCl was replenished as needed. Jars were checked daily for adult emergence and mortality, with the latter being defined as ataxia after the jar was tapped lightly. Bee gender was determined at the end of the experiment. The final number of insects in each of the four experimental groups (CMP males, CMP females, ES males, and ES females) was dependent on the sex ratio and emergence rate of each treatment group.

**Statistical Analysis.** All data sets were analyzed using SigmaPlot 11. The survival data from the stored 2008 bee cells was arcsine transformed and analyzed using Kruskal–Wallis one way analysis of variance (ANOVA) on ranks. To address the problem of unbalanced design because of months with no emergence in the 2009 bee cells long-term survival experiment, a χ² test was added to all values, and the data were then normalized by arcsine transformation. The month of transfer to FTR and month the bees were transferred to 29°C to initiate development were used as the independent variables. Holm–Sidak’s pairwise multiple comparison procedure was used to compare for a decrease in proportion surviving between treatment groups and month of transfer within groups. The adult longevity data were normalized by taking the square root of the days of adult survival. The 2009 field season bees long-term survival and adult longevity data sets were normally distributed with equal variance. The alpha value was set to 0.05 for all analysis. All data are presented as the mean ± SEM throughout the manuscript.

**Results**

**FTR and Long-Term Storage.** When a FTR protocol was implemented during late postdiapause quiescence, bees survived significantly better than their STR counterparts ($H = 22.47$; $df = 1$; $P < 0.001$; Fig. 2). Prepupae stored under STR beginning in 2008 retained high levels of survival through September and October of 2009 (totaling 12–13 mo of storage), but viability quickly decreased thereafter, with 43% viability in November 2009, 18% in December 2009, and nearly 0% survival by January 2010 (Fig. 2). In contrast, bees stored under FTR fared much better, with survival remaining above 75% through December 2009 (corresponding to 15 mo of storage) before starting a gradual decline, with survival not falling below 50% until April 2010 (19 mo of storage), and 26% remaining viable in August of 2010. This final time-point represented a full year of storage under FTR conditions, and nearly two total years of storage for these 2008 field season bees. The difference in survival between FTR and STR storage was statistically significant beginning in November 2009, and remained as such throughout the remainder of the experiment.

**Effect of Timing of FTR Initiation.** When 2009 field season bees were stored in FTR starting in November of 2009 (while in true diapause, protocol in Fig. 1a), viability throughout the course of the experiment was substantially better than their STR counterparts.
(Fig. 3a). For the FTR treated bees, there was no significant decrease in viability until March 2011 (18 mo of storage), with viability remaining above 50% until April 2011 (19 mo of storage), with a steady decrease until the final timepoint in August 2011 (23 mo of storage), with 29% viability.

Starting FTR while the bees were in postdiapause quiescence (protocol in Fig. 1b) also substantially improved bee viability when compared with STR (Fig. 3b). This delay in starting FTR resulted in no significant decrease in viability until May 2011 (20 mo of storage), viability remaining above 50% until July 2011 (22 mo of storage), and 40% of the stored bees remaining viable at the end of the experiment in August 2011 (23 mo of storage). Similar to the 2008 field season bee experiment, starting FTR during late postdiapause quiescence (protocol in Fig. 1c) substantially increased viability when compared with STR counterparts as well (Fig. 3c); FTR treated bees showed increased longevity, with 19% of the FTR stored bees remaining viable at the last timepoint (23 mo of storage). Finally, starting FTR in October 2010 (protocol in Fig. 1d) had a minimal effect on viability (Fig. 3d), with only 17% viability in April 2011, and 8% viability the following August.

As suggested by the above data, the efficacy of FTR storage with respect to viability was significantly affected ($P < 0.05$) when comparisons were made among the FTR treatments beginning during diapause, postdiapause quiescence, and late quiescence (with October start being omitted because of lack of substantial survival) (Fig. 3). With the exception of the November timepoint of the late quiescent start, there were no significant differences in percent survival among the treatments through January 2011. In February 2011, both the treatments starting during diapause and postdiapause quiescence were significantly higher than the treatment starting during late quiescence, while in March, April, and May 2011, the viability of those bees whose treatment started during postdiapause quiescence was significantly higher than the other treatment groups. In the final three timepoints, there was no significant difference in viability among the treatments.

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**Discussion**

Similar to our previous findings for the short-term storage of developing bees (Rinehart et al. 2011, Yocum et al. 2012), quiescent bees stored under FTR survived significantly better than their STR counterparts. Furthermore, this study found no differences in adult longevity between bees stored as prepupae for one season and those stored as prepupae for two seasons, and a companion study found no effects on adult metabolism as a result of extended storage (Bennett et al. 2013). Hence, it appears that based on these two studies, FTR can greatly extend quiescent prepupal longevity, effectively causing artificial semivoltinism in this species.

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**Adult Longevity.** Using the extended storage protocol had no significant effect on the longevity of emerged adults (Fig. 4). Bees from the 2009 field season that were stored under STR until August 2010, and then under FTR until the spring of 2011 (extended storage treatment) had similar longevity as adults as did bees from the 2010 field season that were stored under STR until the same spring (current management practice). There was no statistically significant difference by length of storage or by gender (Fig. 4).
of FTR can produce viable bees for two alfalfa seed field seasons, although at reduced survival in the second season. Additionally, it is likely that the protocol defined herein can be refined to improve these results.

The practical benefits of FTR with respect to alternative uses of *M. rotundata*, such as lowbush blueberry pollination, are much more immediate. Effective pollination of lowbush blueberries by alfalfa leafcutting bees has been well documented (Argall et al. 1996, Javorek et al. 2002), with spring incubation of *M. rotundata* usually beginning in early April (Stubbs 2007). Our results indicate that when FTR is initiated during early postdiapause quiescence, there is no significant decrease in survival when prepupae are removed from storage the following April. Hence, with the current protocol, bees could be stored throughout the first growing season with adult development initiated the following April, with no reduction in survival (Fig. 3b) or adult longevity (Fig. 4). Other earlier blooming crops in which *M. rotundata* has proven to be an effective pollinator, such as onion seed (Voss et al. 1999) and carrot seed production (Tepedino 1997) could also benefit from this new management strategy.

An alternative interpretation of the benefits of FTR for long-term *M. rotundata* storage is that it can be used to give an end-user essentially year-round access to viable bees, because once survival begins to decrease...
in the spring of the second year of storage, prepupae in the first year of storage are ready for use. This should not only help in the development of *M. rotundata* as a model organism for research, but could improve the use of this species in glasshouse pollination where, although is rarely used (Pitt-Singer and Cane 2011), it has been shown to be effective (Trostle and Torchio 1994, Cane 2002, Maeta and Adachi 2005). Regardless of the specific practical application, FTR storage of *M. rotundata* for more than one season should substantially ease yearly price fluctuations. Bee producers could store bees through a year with low prices, and users could stockpile prepupae as insurance against higher prices in the subsequent year. This could also lead to wider use of this alternative pollinator, because price fluctuations have been cited as an impediment to more widespread use of *M. rotundata* in cases such as the sizable seed production acreage in California (Mueller 2008). What insight does the extended longevity by FTR incubation gives us into the underlying mechanisms of diapause? Of primary interest is that the thermal history experienced during diapause can have profound effects on diapause physiology and survivorship. This is in agreement with past studies that have demonstrated that temperature fluctuations, such as those experienced under field conditions, result in vastly different diapause molecular profiles than those experienced in the laboratory, where diapause is usually induced and maintained under static temperatures (Yocum et al. 2009a,b, 2011). Hence, this study adds to a growing body of evidence demonstrating the physiological differences between insects that diapause under constant temperatures in the laboratory and those that diapause under fluctuating temperatures. Our findings of increased survival during FTR storage could have important implications in understanding the increased longevity (Herman and Tatar 2001) and increased stress tolerance (Denlinger 2002) inherent to diapause. For instance, stress responses such as elevated heat shock proteins are present during diapause in several species including *M. rotundata* (Yocum et al. 2005), and they have been shown to be essential to increased resistance to acute stress characteristic of diapause (Rinehart et al. 2007). Whether these mechanisms of stress resistance are enhanced by FTR during long-term storage, or whether other mechanisms are involved will be important in deciphering the molecular processes that protect diapausing insects from the injuries accrued during extended periods of cold exposure. Finally, our findings of no apparent decrease in adult mortality rates after extended FTR storage could have important implications in understanding the slowing of aging processes characteristic of diapause (Herman and Tatar 2001, Tatar and Yin 2001). Our adult mortality rates, which have been used in the past as a gauge of aging during diapause (Tatar and Yin 2001), suggest that aging is slowed substantially during the additional year of storage under FTR conditions. While additional studies are underway to decipher the molecular processes underlying our observations, as well as to optimize the FTR protocol for multi-year storage of *M. rotundata* for alfalfa seed production, this study firmly establishes the importance of a fluctuating thermal regime to diapause physiology as well as successful long-term cold storage of insects.

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