



# Timing of eclosion affects diapause development, fat body consumption and longevity in *Osmia lignaria*, a univoltine, adult-wintering solitary bee

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## ARTICLE INFO

### Article history:

Received 4 June 2010

Received in revised form 16 August 2010

Accepted 23 August 2010

### Keywords:

Life cycle phenology  
Diapause ecophysiology  
Respirometry  
Winter survival  
Fat body  
Megachilidae  
Climate change  
Global warming

## ABSTRACT

Most insects from temperate areas enter diapause ahead of winter. Species diapausing in a feeding stage and accumulating metabolic reserves during permissive pre-wintering conditions are expected to enter diapause shortly before the onset of winter. In contrast, species diapausing in a non-feeding stage are expected to lower their metabolism as soon as possible to avoid excessive consumption of metabolic reserves. The solitary bee *Osmia lignaria* winters as a non-feeding adult within its cocoon, but previous studies show important weight losses and increased winter mortality in populations pre-wintered for extended periods. We measured respiration rates to assess diapause initiation and maintenance during pre-wintering, and tested whether timing of adult eclosion affected fitness by measuring fat body depletion, winter mortality and post-winter longevity. We worked with different cohorts of a population reared under natural conditions, and manipulated pre-wintering duration in a population reared under artificial conditions. In agreement with our expectation, *O. lignaria* lower their metabolic rates within a few days of adult eclosion, but nonetheless suffer strong weight loss during pre-wintering. Early developing individuals suffer greater weight loss and fat body depletion, and have short post-winter longevity. Although, we found no differences in winter mortality among treatments, our results indicate that increased mortality may occur in years with late winter arrivals. We discuss fundamental ecophysiological differences between adult and prepupal diapause within the Megachilidae, and hypothesize that species wintering as adults will be more negatively affected by a situation of extended summers under a scenario of global warming.

Published by Elsevier Ltd.

## 1. Introduction

In seasonal environments, animals need to time certain phases of their life cycle with a number of biotic and abiotic factors, such as availability of key resources (food, mates) or adequate environmental conditions (temperature, rainfall). The degree of phenological match may strongly affect individual fitness. For example, in Lepidoptera, larval performance is often critically dependent on the timing between egg hatching and budburst or senescence of the host plant (Cushman et al., 1994; Tammaru et al., 1999). In various copepod species, larval survival is strongly affected by fish predation. Females switch from producing offspring that hatch immediately and reproduce within the season

to producing eggs that enter diapause (and sink to the bottom and avoid predation) with the onset of predatory activity (Ellner et al., 1999; Varpe et al., 2007). Females with too late a switch suffer high offspring mortality, while females that switch too early miss a reproductive episode. In birds, clutch size is strongly related to lay date, with late-laying females producing smaller clutches (Winkler et al., 2002). In a scenario of climate change, increasing temperatures are expected to disrupt the timing between development and key seasonal events (Bale et al., 2002; Bale and Hayward, 2010), and reports of phenological mismatches are becoming increasingly frequent (Hassell et al., 1993; Visser et al., 1998; Visser and Holleman, 2001; Musolin, 2007; Post and Forchhammer, 2008; Taylor, 2008).

In insects, growth and development are critically dependent on temperature regimes. In years with unusual climatic conditions entire populations may collapse as a result of mismatches between the timing of development and environmental conditions. In the ladybird beetle *Coccinella septempunctata* extreme mortality was reported when anomalous weather

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delayed egg-laying by first-generation adults, leaving insufficient time for second-generation offspring to reach the adult (wintering) stage before the onset of winter temperatures (Hodek and Honek, 1996). In the Swiss Alps, an early temperature rise in March 1990 triggered development of diapausing eggs of the larch bud moth, *Zeiraphera diniana*. Subsequent cold temperatures prevented feeding, thus leading to extensive mortality (Baltensweiler, 1993). In 1992, the collapse of spruce budworm, *Choristoneura fumiferana*, populations in Québec coincided with extended cool-spring conditions (Han and Bauce, 1998). In the temperate zone, the onset of winter is a key seasonal event characterized by low temperatures and lack of food resources. To survive these adverse conditions some insect species migrate. Others, the majority, sequester metabolic reserves, synthesize cryoprotectants, and enter diapause ahead of the arrival of cold temperatures (Danks, 1996; Košťál, 2006; Hahn and Denlinger, 2007). In most species, winter diapause takes place at a specific developmental stage, and individuals not reaching the appropriate stage by the onset of adverse conditions are doomed. On the other hand, individuals reaching the appropriate stage ahead of adverse conditions may miss opportunities for reproduction or somatic growth. Some studies have addressed the effects of diapause timing in arthropods, and found intermediate timings at which fitness is maximized (Musolin, 2007). In multivoltine species, reproducing females are faced with the “decision” of when to switch from production of developing offspring to production of diapausing offspring (Cohen, 1970; Taylor, 1986; Ellner et al., 1999; Fiksen, 2000). In species wintering in a feeding stage, individuals must decide when to give up feeding and enter diapause. Extended feeding may result in increased body size (which is often correlated with fecundity) (Tammeru et al., 1996; Bradshaw et al., 1998; Ellers and van Alphen, 2002; Musolin et al., 2010), and accumulation of metabolic reserves and/or cryoprotectants enhancing winter survival (Pullin, 1987; Ohtsu et al., 1995), but may also entail risks of predation or being caught by an abrupt onset of unsuitable conditions (Taylor, 1980; Ellner et al., 1999). Thus, in any given population, the optimal time to enter diapause is an adaptive compromise between building/conserving resources and avoiding untimely exposure to winter cold (Bradshaw and Holzapfel, 2010). The fitness function is expected to be asymmetrical around this optimum, because the cost of entering diapause too late may be death and a fitness of zero, whereas entering diapause too early may just result in reduced somatic growth or missing a reproductive episode (Taylor, 1986).

In this study, we analyze the timing of winter diapause in relation to the onset of wintering temperatures and its consequences on diapause development, winter survival and post-wintering longevity in the mason bee *Osmia lignaria* (Hymenoptera: Megachilidae). Conclusions reached in the above-mentioned studies may not apply to this species for two reasons. First, *O. lignaria* is strictly univoltine. Second, it winters as a fully eclosed adult within its cocoon with no access to food, and accumulation of metabolic reserves to overwinter is entirely dependent on the food allocated by the nesting female to each individual larva within its nest cell. That is, the larva develops on a fixed amount of provision over which it has no control. However, the timing of adult eclosion does not depend on the amount of provision allocated, but mostly to the duration of the non-feeding prepupal period (Sgolastra, 2007), during which the species undergoes a summer diapause that lasts 1–2 months (Kemp et al., 2004). Given these life cycle traits, it appears that the best strategy would be to develop as quickly as possible and then enter diapause as soon as possible. This strategy would both avoid the risk of not having reached the adult stage by the onset of winter, and minimize consumption of metabolic reserves while temperatures are still warm in late

summer and early autumn. However, studies on *O. lignaria* and *Osmia cornuta* have shown that pre-wintering duration (defined as the time between adult eclosion and the onset of wintering temperatures) has a strong impact on winter survival in artificially reared populations. Individuals pre-wintered for 45–75 days lose more weight and have higher winter mortality than bees pre-wintered for 10–30 days (Bosch et al., 2000; Bosch and Vicens, 2002; Bosch and Kemp, 2004). These results suggest that, contrary to our rationale above, it may be advantageous for *O. lignaria* to reach adulthood and enter diapause as late as possible, provided it is before the onset of winter. If results obtained on artificially reared populations apply to wild populations, winter survival in *Osmia* populations may be critically dependent on the timing between adult eclosion and winter initiation. Slowly developing individuals will be eliminated because individuals wintering as prepupae or pupae are not viable (Bosch, 1994; Bosch and Kemp, 2000; Bosch et al., 2006). However, individuals developing too quickly will be exposed as adults to long pre-wintering periods and consume valuable metabolic reserves. In addition to increasing winter mortality, the depletion of metabolic reserves during pre-wintering may exact a toll on post-winter performance, for example through slowed ovary maturation, reduced longevity or reduced fecundity. A trade-off between diapause and post-diapause performance has been reported in various insects (Bradshaw et al., 1998; Irwin and Lee, 2000; Ellers and van Alphen, 2002; Musolin and Numata, 2003).

Previous studies show that within-population variability in developmental rate in *O. lignaria* is high, and the period of adult eclosion in a given year may span over a month (Bosch and Kemp, 2000; Bosch et al., 2000). Given this variability, the first objective of our study is to establish whether the timing of adult eclosion in relation to the onset of winter temperature has an effect on fat body depletion, winter survival and post-emergence longevity in *O. lignaria* populations reared under natural conditions. The second objective is to understand the physiological mechanisms underlying this potential relationship. To accomplish this, we worked with populations reared under natural conditions, but also with artificially reared populations, which allowed us to experimentally manipulate pre-wintering regimes. To monitor diapause development, we conducted respirometry measurements and measured body weight loss and fat body depletion throughout pre-wintering and wintering. We addressed the following questions: (1) What is the timing of adult eclosion and diapause initiation in relation to the onset of winter temperatures in natural populations? (2) Does pre-wintering duration affect diapause initiation and development? (3) Are individuals whose adult eclosion is better timed with winter temperatures more fit (more likely to survive the winter and/or more vigorous at emergence)?

## 2. Materials and methods

### 2.1. Natural history of *O. lignaria*

The family Megachilidae contains some 3000 species worldwide, of which more than 300 belong to the genus *Osmia*, distributed mostly in the Holarctic (Michener, 2000). They typically fly in early spring and spend the winter as unemerged adults within their cocoons (Bosch et al., 2001). Most *Osmia* species are either univoltine (wintering as adults) or semivoltine (spending the first winter as prepupae and the second as adults). Females lay their eggs in multi-celled nests, usually built in pre-established cavities (beetle burrows in dead timber, abandoned bee or wasp nests), which they provision with individual pollen-nectar masses. Eggs hatch in approximately a week and development proceeds through five larval instars (Torchio, 1989). The larva consumes the entire provision allocated by the nesting female, and

then spins a cocoon in which pupation occurs. Pupae become adults by late summer or early autumn, and adult body weight is highly correlated with provision weight (Bosch and Vicens, 2002). Eclosed adults remain in the cocoon throughout the winter, and emerge in the spring as temperatures increase. Upon emergence, adults fly to nearby flowers and sip nectar. After a 2–3 day period during which ovary maturation takes place, females begin nesting activities, which last 20–30 days (Bosch, 2008).

## 2.2. Populations and rearing methods

On 1 May 2002 we released an *O. lignaria* population consisting of 2500 females and approximately twice as many males in an apple orchard in North Logan (Utah, USA). Wood blocks with drilled holes were provided as nesting materials in several nesting shelters (wooden boxes with one side open) scattered across the orchard. Each drilled hole had an inserted paper straw (15 cm long, 7.5 mm diameter). Nesting lasted from early to late May. On 20 June, when the nesting period was complete, nesting materials were brought to the laboratory and paper straws containing nests were extracted from the wooden blocks. Half of the nests were kept in a 22 °C temperature cabinet. The other half were re-inserted in wood blocks and kept in an open North-facing barn in the apple orchard. Temperature in the barn was recorded hourly with a temperature logger. Beginning on 5 August, when bees started to reach adulthood, we X-rayed all nests every 3 days, using a Hewlett-Packard Faxitron machine. We used exposure times of 30 s at 25 kV. X-ray plates were then used to sex individuals (females are usually larger than males and located in the innermost cells within a nest; Torchio, 1989) and to establish adult eclosion dates.

## 2.3. Bees reared at 22 °C

### 2.3.1. Fat body condition, survival and longevity

As bees became adults, we dissected paper straws with a razor blade and collected cocoons containing male bees. We distributed these cocoons among 5 pre-wintering treatments (5, 15, 30, 45 and 60 days at 22 °C), so that no treatment received more than one individual from the same nest. Sample sizes were 45 males per treatment. Upon pre-wintering completion, cocoons were again X-rayed to score fat body condition. On X-ray plates, the abdomen of bees with fully developed fat bodies appears white, whereas the abdomen of bees with partially depleted fat bodies shows black (empty) areas (Fig. 1). We established a semi-quantitative scale of fat body condition. Bees with no signs of fat body depletion (abdomen appearing all white) were scored as 0; bees with signs of fat body depletion (black “spots” visible in the anterior half of the abdomen) were scored as 1; bees with extensive fat body depletion (most or all of the anterior half of the abdomen appearing black) were scored as 2 (Fig. 1). All bees were scored by three independent observers, and the mean of the three scores was used as an index of fat body depletion. A subsequent study (Sgolastra, 2007) showed our fat body depletion index to be significantly correlated to total body lipid content ( $n = 29$ ,  $r = -0.797$ ;  $P < 0.0001$ ;  $y = 11.57 - 3.13x$ ). Upon individual pre-wintering completion, cocoons were cooled at 14 °C for 7 days and then wintered in a 4 °C cabinet for 200 days (Bosch and Kemp, 2003). After wintering, cocoons were individually placed in glass vials and incubated at 20 °C. We checked cocoons daily, and noted emergence and death dates. Longevity without feeding was used as a measure of remaining energy reserves (Bosch and Kemp, 2000).

We established three fat body depletion classes: low (fat body index from 0 to 0.66), intermediate (from 0.67 to 1.33) and major depletion (from 1.34 to 2). We used a contingency table to analyze

differences across pre-wintering treatments in the frequency of individuals in each fat body class. Differences in winter survival were also analyzed with a contingency table, and differences in longevity (log-transformed) with one-way ANOVA. The distribution of emergence time was highly skewed to the right, even after log-transformation. Thus, we used the Kruskal–Wallis test to analyze emergence time across treatments.

### 2.3.2. Respiration rates

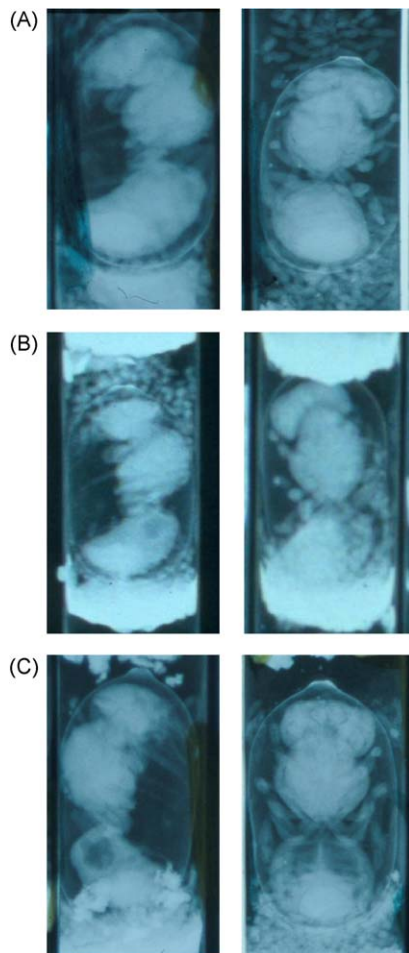
An earlier study showed that males and females followed similar patterns of respiration and weight loss (Kemp et al., 2004). We selected 7 females that reached adulthood on 11 August. These females were pre-wintered at 22 °C for 4 weeks, then transferred to 14 °C for 1 week, and finally wintered at 4 °C. We used these 7 females to measure O<sub>2</sub> consumption and CO<sub>2</sub> production twice a week (Monday and Friday) from adult eclosion until 4 October, and then once every 2 weeks until emergence. Because *O. lignaria* develop and winter inside a sealed nest, bees were kept in complete darkness throughout the study. We used a Sable Systems FC-1 O<sub>2</sub> Analyzer<sup>®</sup>, and a LI-COR 6251 CO<sub>2</sub> Analyzer<sup>®</sup> operating in differential mode with a 100 ml/min flow rate. This allowed accuracy of measurement that exceeded 0.001% in detecting departures from an undepleted air stream that had been scrubbed of CO<sub>2</sub> and water vapour with a Drierite<sup>®</sup> – Ascarite<sup>®</sup> column. Measurements lasted 2 h and were conducted in darkness in a 22 °C cabinet. The first 60 min served as a baseline equilibration interval during which bees were acclimatized and respiration chambers were filled with air scrubbed of CO<sub>2</sub> and locked down. Respiration was then allowed (O<sub>2</sub> depleted, CO<sub>2</sub> generated) for 15 min. The final 45 min were devoted to measuring the contents (O<sub>2</sub>/CO<sub>2</sub>) sequentially in each chamber, including the blank. Before each measurement, we weighed each female within her cocoon (after removing any attached fecal pellets). At the end of the winter, we dissected the cocoons, and weighed them. We subtracted cocoon weight to previous weight measures to obtain female body weight without the cocoon. We express respiration rates as µg of O<sub>2</sub> or CO<sub>2</sub> per 100 mg of body weight and hour. All computations were done with the Sable Systems software package Convol<sup>®</sup>. It is important to emphasize that all respiration measurements were conducted at 22 °C. Thus, we did not measure actual respiration rates at winter temperatures, but rather the respiration response of wintering bees when exposed to 22 °C. We call this measure “respiration response” and use its magnitude as a measure of diapause intensity, with low responses reflecting high diapause intensity (Sgolastra et al., 2010).

To test the effect of absence of wintering, a second group of 7 females reared at 22 °C was selected. These females were not transferred to 14 and 4 °C. Instead, they were left at 22 °C throughout the winter. Their weights and respiration rates were measured as above starting on 10 September, 4 weeks after adult eclosion.

## 2.4. Bees reared outdoors

### 2.4.1. Fat body condition, survival and longevity

As bees kept outdoors reached adulthood, we wrote individual adult eclosion dates on the outside of the paper straw, and established three cohorts. Individuals of the early cohort became adults between 3 and 5 August. Individuals of the mid cohort became adults between 15 and 17 August. Individuals of the late cohort became adults between 30 August and 1 September. On 1 October, in coincidence with a notable ambient temperature decline, we X-rayed nests and scored fat body condition of 45 males per cohort as indicated for bees kept at 22 °C. No cohort was assigned more than one individual from the same nest. Nests were wintered in the open barn in which they spent the summer.



**Fig. 1.** X-ray image of *Osmia lignaria* adults with various levels of fat body depletion. (A) No signs of depletion (score 0); (B) Partial depletion (score 1); (C): Extensive depletion (score 2).

On 15 March of the following year, as spring temperatures increased, nests were dissected and 65 cocoons per treatment were inserted individually in glass vials. Glass vials with cocoons were kept in the same barn where nests had been wintered, and checked daily for emergence. Upon emergence we transferred vials with emerged bees to 20 °C and recorded longevity as above. Results were analyzed as for the 22 °C bees.

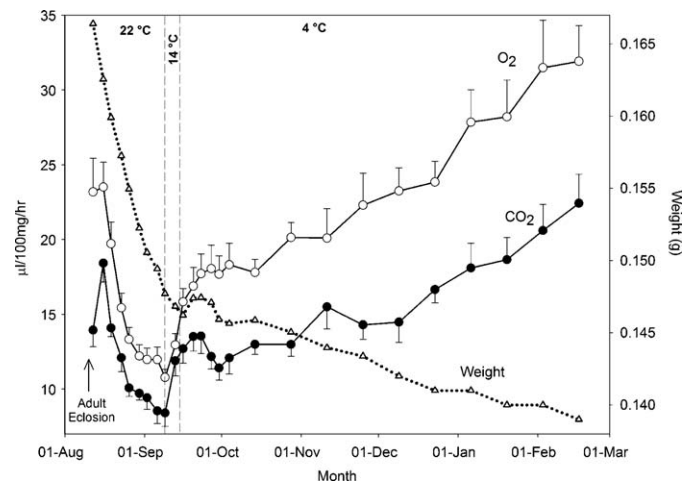
#### 2.4.2. Respiration rates

We selected 7 females that became adults on 11 August 2002 and measured their respiration rates as indicated above. As with 22 °C bees, measurements were taken twice a week from adult eclosion until 4 October, and then once every 2 weeks until emergence. Body and cocoon weights were obtained at the end of the winter as indicated above.

### 3. Results

#### 3.1. Respiration rates and body weight

Respiration rates of females reared at 22 °C remained high for the first 4–6 days after eclosion, and then declined sharply (Fig. 2). Then, respiration response rose sharply as soon as bees were transferred to 14 °C on 10 September. Following transfer to 4 °C, respiration response continued to increase, though at a much slower pace. Body weight dropped dramatically during pre-wintering, and then slowly when bees were transferred to 4 °C (Fig. 2).



**Fig. 2.** Mean (+SE) O<sub>2</sub> consumption and CO<sub>2</sub> production (measured at 22 °C), and body weight (SE omitted for clarity) in *Osmia lignaria* females ( $n = 7$ ) reared at 22 °C, pre-wintered at 22 °C for 4 weeks, chilled at 14 °C for 1 week and wintered at 4 °C. The first measurement was taken 1–3 days after adult eclosion.

Respiration response of females kept outdoors followed a very similar pattern, with a precipitous drop shortly after adult eclosion, and then a sharp increase in coincidence with a strong decline in ambient temperatures in the second week of September (Fig. 3). From then on, respiration response continued to increase slowly but steadily throughout the winter. As in bees kept at 22 °C, body weight plummeted during pre-wintering, and then weight loss was attenuated during wintering (Fig. 3).

Respirometry measurements of bees not wintered (kept at 22 °C) started 4 weeks after adult eclosion, at the time when respiration rates are at their lowest (see Figs. 2–3). Respiration levels of these bees were then 8–11 µl/100 mg/h, and never reached levels above 12–14 µl/100 mg/h (Fig. 4). Instead, CO<sub>2</sub> production and O<sub>2</sub> consumption dropped dramatically in early December and never recovered. Body weight declined rapidly in these bees (Fig. 4), none of which survived the winter.

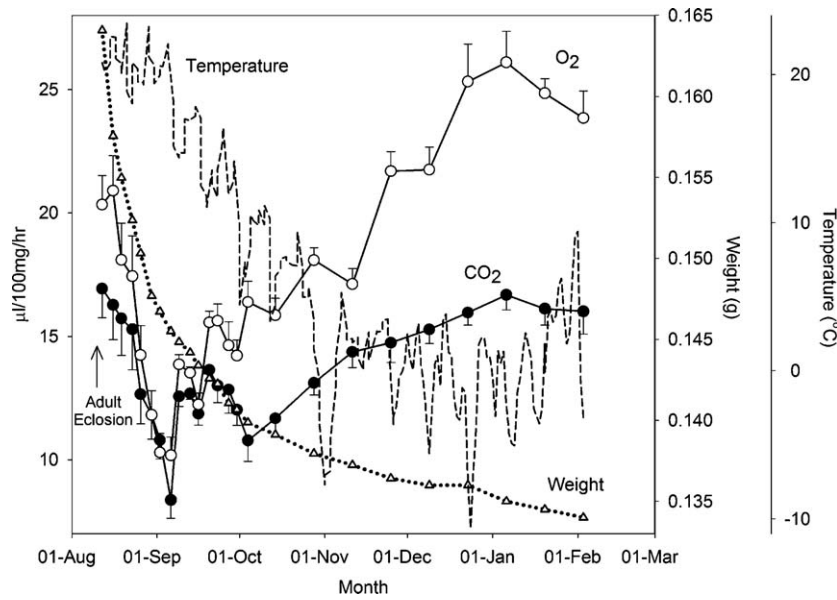
#### 3.2. Timing of adult eclosion

The period of adult eclosion of bees reared outdoors started in late July and lasted ~7 weeks (Fig. 5). There was a pronounced peak of eclosion in late August, just ahead of an important temperature decline in early September. However, all bees sampled had become adults by mid September. The period of eclosion of bees reared at 22 °C was slightly shorter (Fig. 5).

#### 3.3. Fat body condition, survival and longevity

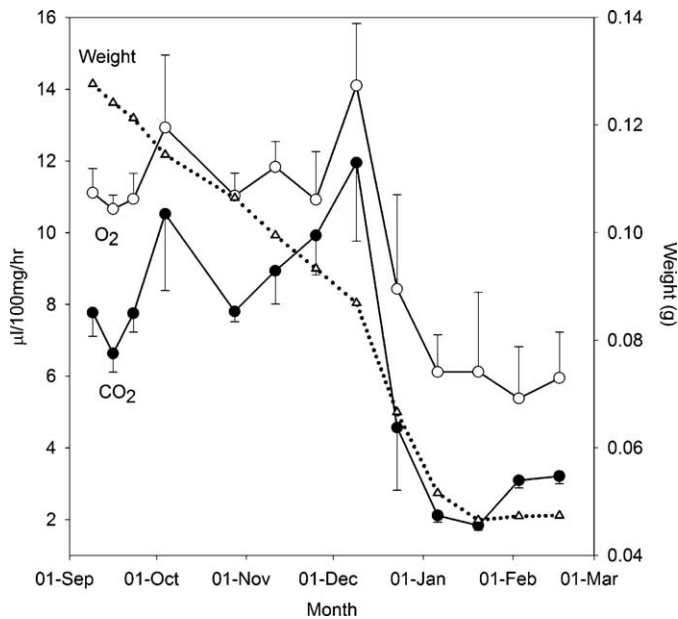
Pre-wintering duration had a significant effect ( $\chi^2 = 106.70$ ;  $df = 8$ ;  $P < 0.0001$ ) on fat body depletion in bees reared at 22 °C, but differences in mortality across pre-wintering treatments were not significant (Table 1) ( $\chi^2 = 2.35$ ;  $df = 4$ ;  $P > 0.6$ ). One bee pre-wintered for 45 days and two bees pre-wintered for 60 days died in the process of chewing their way out of the cocoon. Although these bees survived the winter, they were not vigorous enough to complete emergence and were scored as dead in the winter mortality counts. Emergence time (days to emerge following incubation at 20 °C) was lowest (indicating faster diapause development) at intermediate pre-wintering durations (Table 1) ( $H = 24.25$ ;  $df = 4$ ;  $P < 0.0001$ ), and longevity decreased with increasing pre-wintering duration (Table 1) ( $F = 40.13$ ;  $df = 4, 178$ ;  $P < 0.0001$ ).

In bees reared outdoors, fat body depletion again increased with pre-wintering duration, with individuals of the early cohort

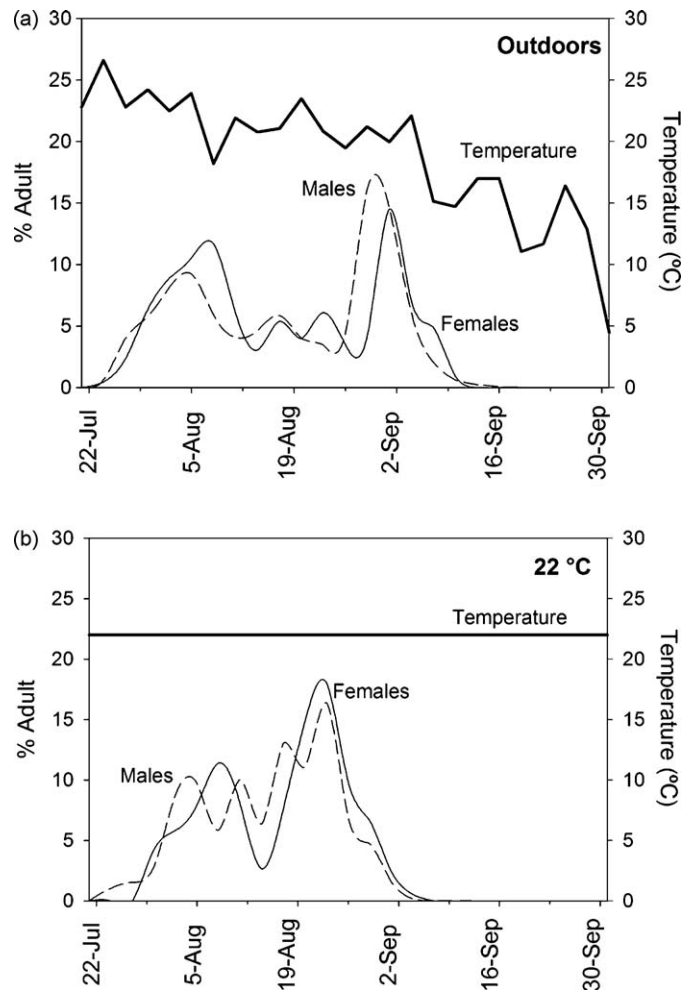


**Fig. 3.** Mean daily temperature, mean (+SE) O<sub>2</sub> consumption and CO<sub>2</sub> production (measured at 22 °C), and body weight (SE omitted for clarity) in *Osmia lignaria* females (n = 7) reared outdoors. The first measurement was taken 1–3 days after adult eclosion.

showing the most extensive fat body depletion (Table 2) ( $\chi^2 = 10.46$ ; df = 4;  $P = 0.03$ ). Mortality was 0% in all three cohorts (Table 2). Due to ambient temperature variability, bees reared outdoors emerged over two distinct, non-overlapping periods. Nonetheless, emergence results paralleled those obtained on bees reared at 22 °C. That is, individuals of the mid cohort tended to emerge earlier (Table 2;  $H = 7.26$ ; df = 2;  $P < 0.03$ ). Longevity again decreased with increasing pre-wintering duration (Table 2) ( $F = 41.69$ ; df = 2,180;  $P < 0.0001$ ). Bees of the late cohort lived 1.7 times longer than those of the early cohort.



**Fig. 4.** Mean (+SE) O<sub>2</sub> and CO<sub>2</sub> production (measured at 22 °C), and body weight (SE omitted for clarity) in non-wintered (kept at 22 °C) *Osmia lignaria* females (n = 7). Note that scales on both y-axes are different from those of Figs. 2 and 3, and that the first measurement was not taken 1–3 days after adult eclosion (as in Figs. 2 and 3), but 28–30 days after adult eclosion, when respiration rates were at their lowest.



**Fig. 5.** Mean daily ambient temperature and timing of adult eclosion of *Osmia lignaria* reared outdoors (1300 males + 427 females) and at 22 °C (1164 males + 377 females) in 2002.

**Table 1**

Fat body depletion, winter mortality, emergence time and post-winter longevity in *Osmia lignaria* males reared at 22 °C, pre-wintered at 22 °C for different periods, wintered at 4 °C for 200 days, and incubated at 20 °C. All means followed by SE.

Pre-wintering	n	Fat body depletion <sup>a</sup>	% Mortality	Emergence time <sup>b</sup>	Longevity <sup>c</sup>
5 days	44	0.2 ± 0.04	20.5	2.3 ± 0.4	6.7 ± 0.4
15 days	41	0.8 ± 0.07	17.1	0.9 ± 0.2	5.2 ± 0.3
30 days	43	1.0 ± 0.06	9.3	0.7 ± 0.2	4.2 ± 0.3
45 days	45	1.0 ± 0.06	13.3	1.0 ± 0.2	3.1 ± 0.2
60 days	44	1.2 ± 0.06	15.9	1.5 ± 0.2	2.3 ± 0.2

<sup>a</sup> Fat body depletion index from 0 to 2.

<sup>b</sup> Days to emergence following incubation at 20 °C.

<sup>c</sup> Days from emergence to death at 20 °C.

**Table 2**

Fat body depletion, winter mortality, emergence time and post-winter longevity in *Osmia lignaria* males with different adult eclosion times reared under natural conditions. All means followed by SE.

Adult eclosion	n	Fat body depletion <sup>a</sup>	Mortality	Emergence time <sup>b</sup>	Longevity <sup>c</sup>
Late	63	0.5 ± 0.06	0%	6.4 ± 0.6	7.6 ± 0.5
Mid	57	0.7 ± 0.07	0%	4.7 ± 0.6	5.1 ± 0.2
Early	64	0.8 ± 0.08	0%	5.4 ± 0.6	4.4 ± 0.1

<sup>a</sup> Fat body depletion index from 0 to 2.

<sup>b</sup> Days to emergence after 31 March 2003.

<sup>c</sup> Days from emergence to death at 20 °C.

#### 4. Discussion

*O. lignaria* enter diapause a few days after adult eclosion (Figs. 2 and 3). Diapause initiation does not appear to require a temperature or photoperiod cue, since 22 °C-reared bees were kept at a constant temperature in complete darkness throughout their development, indicating that winter diapause in *O. lignaria* is a fixed component of the ontogenic program (Košťál, 2006). This result is in agreement with our initial expectation of an early diapause initiation in an insect unable to feed in the months prior to wintering. However, even while maintaining low metabolic rates, non-chilled *O. lignaria* adults lose weight dramatically and quickly consume their fat bodies. This is surprising, because weight loss is typically low in diapausing insects, even at warm temperatures (Masaki, 1980; Liu et al., 2006). Prior to pupation, *O. lignaria* undergoes a summer diapause in another non-feeding stage (prepupa) (Kemp et al., 2004; Kemp and Bosch, unpublished). During this prepupal diapause, respiration rates are similar to respiration rates of pre-wintering adults, but weight loss is minimal (Kemp et al., 2004; Bosch and Vicens, 2002). In addition, and unlike *Osmia* adults (Fig. 4), *Osmia* prepupae can be kept at warm temperatures for many months and even years without dying (Bosch, 1994; Bosch and Kemp, 2000; Maeta et al., 2006; Sgolastra, 2007). Another Megachilid, the alfalfa leafcutting bee, *Megachile rotundata*, undergoes a summer–winter diapause in the prepupal stage. Respiration rates of *M. rotundata* prepupae are again similar to those of *Osmia* pre-wintering adults (Kemp et al., 2004). Yet, *M. rotundata* prepupae do not experience pronounced weight and lipid losses during pre-wintering, nor suffer fitness reductions in terms of winter mortality or post-emergence performance when exposed to extended pre-wintering durations (Kemp and Bosch, 2001; Pitts-Singer and James, 2009). In addition, *M. rotundata* appear to be much more tolerant than *Osmia* to warm wintering periods (Richards et al., 1987; Bosch and Kemp, 2003, 2004). These results suggest fundamental ecophysiological differences in the mechanisms underlying prepupal and adult diapause in solitary

bees. Wintering in the adult stage is a derived trait within the Megachilidae, most of which, including the ancestral clades, winter as prepupae, and is associated with the early flying period of *Osmia* (Bosch et al., 2001).

When exposed to cold temperatures, pre-wintering adults respond by quickly easing off diapause intensity. Both bees reared artificially and outdoors raise their respiration response ~1.5-fold within 2–3 days of a temperature drop from ~22 °C to ~14 °C. Respiration response then increases slowly throughout the winter and onto emergence the following spring. During this period weight loss is kept low by the cold winter temperatures, and although we did not measure respiration rates at winter temperatures, we assume those to be low throughout the winter. Bees not exposed to cold temperatures maintain low respiration rates, but, as mentioned, at a high metabolic cost, and eventually die. Thus, *O. lignaria* requires exposure to cold temperatures to complete diapause, a result which is in agreement with previous findings of low survival in populations wintered for short (<90 days) periods (Bosch and Kemp, 2003, 2004). Pre-wintering duration has an additional effect on diapause development in *O. lignaria*. Both in bees reared artificially and outdoors, faster diapause development (resulting in faster emergence), was obtained in bees exposed to intermediate pre-wintering durations. Although small, these differences have potential consequences on post-wintering performance, because body weight loss during the pre-emergence period (time between the beginning of incubation and emergence) is high in this species (Sgolastra et al., 2010), and females with long pre-emergence periods are less likely to establish and initiate nesting activities (Sgolastra, 2007).

As predicted, fat body depletion increased and longevity decreased with pre-wintering duration both in bees reared at 22 °C and outdoors. The ratio between CO<sub>2</sub> production and O<sub>2</sub> consumption (respiratory quotient, RQ) during pre-wintering was 0.6–0.8, indicating the likely use of triglycerides as the main energy substrate, and a follow-up study found a negative relationship between fat body depletion and lipid content (Sgolastra, 2007). However, fat body depletion did not correlate with survival, which, contrary to our expectation, did not differ among treatments (Tables 1 and 2). The weight loss pattern of non-wintered females (Fig. 4) indicates that we could have obtained significant mortality levels had we increased pre-wintering duration beyond 60 days in the 22 °C population. However, we did not want to use overly unrealistic treatments eliciting physiological responses unlikely to occur in nature. In fact, all bees from the outdoors population survived, and the levels of fat body depletion and longevity obtained in the early eclosing cohort were comparable to levels obtained in intermediate treatments of the 22 °C population. These results suggest that the effect of pre-wintering duration on natural populations of *O. lignaria* may be small or restricted to occasional years with extended summer-like ambient conditions. However, it is important to emphasize that adult eclosion of our outdoors population in 2002 was particularly well synchronized with the onset of wintering temperatures. Other populations reared under natural conditions have shown poorer synchronization and greater (up to 14%) winter mortality (Bosch and Kemp, 2000; Sgolastra, 2007). In addition, a follow-up study (Sgolastra, 2007) found increased mortality in bees subjected to simulated late winter arrivals. In the Tephritid fly *Rhagoletis pomonella*, which also diapauses in a non-feeding stage (pupa), increasing pre-wintering duration also resulted in increased weight loss and winter mortality (Feder et al., 1997). In the Pentatomid bug *Nezara viridula*, which diapauses as a feeding adult, females induced to emerge in September had higher mortality than those induced to emerge in October, and these differences were

related to warm temperatures prevailing in September (Musolin and Numata, 2003; Musolin, 2007).

In addition to lowering metabolic rates, other physiological processes related to accumulation of energy reserves and cold-tolerance contribute decisively to winter survival in insects. Although enhanced in response to exposure to low temperatures, these processes are considered an integrative component of the diapause syndrome and are potentiated during pre-wintering, while temperatures are still warm (Adedokun and Denlinger, 1984; Pullin and Bale, 1989; Danks, 1996; Košťál, 2006). In species that, unlike *Osmia*, winter as feeding stages, individuals eclosing in late autumn and thus experiencing short feeding periods have been found to have lower triacylglycerol content and lower over-wintering capability than individuals eclosing in early and mid autumn (Pullin, 1987; Ohtsu et al., 1995). In *Osmia cornifrons*, warm temperatures are necessary for the synthesis of polysaccharides following adult eclosion (Maeta et al., 2006). Polysaccharides have been related to over-wintering capacity (Danks, 1996), and *O. cornifrons* individuals transferred to cold temperatures upon adult eclosion fail to survive the winter (Maeta et al., 2006). Thus, overly short pre-wintering treatments may disrupt the build up of cryoprotectants in *Osmia*. In addition, bees of our treatments 5 and 15 days had no time to reach the low respiration levels ( $10 \mu\text{l}/100 \text{mg h}$ ) achieved by bees pre-wintered for 30 days and beyond, again possibly disrupting the pattern of diapause development characteristic of the species. It is therefore possible that the need to build up cryoprotectants and the need to conserve metabolic reserves exert opposing selective pressures on eclosion time in *Osmia*, resulting in an intermediate optimal timing, as suggested by the high mortality results at both ends of the pre-wintering range in the 22 °C-reared population (Table 1).

Besides winter mortality, pre-wintering duration could have an effect on post-emergence fitness via increased fat body depletion. Vitellogenin is manufactured in the fat body and transported through the haemolymph to the developing ovarioles (Maeta and Kurihara, 1971; Wheeler, 1996). Females emerging with depleted fat bodies could take longer to mature their first oocyte, prolonging pre-nesting time (time between emergence and the beginning of nesting activities). In two out of three years, pre-nesting time was negatively related to realized fecundity in *O. cornuta* (Bosch and Vicens, 2006). Pre-wintering duration could also affect fitness via reduced longevity. It remains to be tested whether longevity without feeding correlates to longevity in nature, which is strongly related to fecundity (Bosch and Vicens, 2006). Emerging bees may be able to rebuild their metabolic reserves and effectively “reset the clock” through ingestion of nectar and pollen. However, several observations indicate that post-winter longevity may be related to post-emergence performance in *O. lignaria* and *O. cornuta*. Some individuals pre-wintered for long periods die in the process of chewing their way out of the cocoon (Bosch, 1994; this study), indicating they survived the winter but were not strong enough to emerge. Other individuals emerge, but are not strong enough to fly to the nearest flowers to sip nectar. Small females have shorter post-winter longevity than large females (Bosch and Kemp, 2004) and are less likely to establish at nesting sites and start a nest (Tepedino and Torchio, 1989; Bosch and Vicens, 2006). A trade-off between diapause and post-diapause performance has been described for several insects. In the pitcher-plant mosquito, *Wyeomyia smithii*, larvae exposed to wintering conditions attained levels of pupal mass, adult longevity and fecundity comparable to larvae exposed to stressful conditions (increased temperature, decreased humidity, and reduced food), but lower than larvae reared under optimal conditions (Bradshaw et al., 1998). Increased diapause

duration in the Hymenopteran parasitoid *Asobara tabida* resulted in reduced fat levels and body weight, ultimately leading to low egg production (Ellers and van Alphen, 2002). *Nezara viridula* females entering diapause in October lived longer and were more fecund than females entering diapause in September (Musolin and Numata, 2003; Musolin, 2007). In other studies, however, costs associated with prolonged diapause have been shown to be off-set by post-emergence feeding (Peferoen et al., 1981; Jansson et al., 1989; Eijs and van Alphen, 1999).

Adult eclosion in the population reared outdoors spanned 1.5 months, evidencing high variability in the timing of adult eclosion in relation to the onset of winter temperatures. With a delayed peak of eclosion, the bulk of the population experienced a short pre-wintering period and thus avoided extensive fat body depletion. This strategy is not without risk, because individuals wintering as pupae are not viable (Bosch, 1994; Bosch and Kemp, 2000; Bosch et al., 2006). In *Rhagoletis pomonella*, individuals pupating too early are exposed to warm temperatures in the autumn, fail to enter pupal diapause, and die as adults with the onset of winter (Feder et al., 1997). However, all individuals reared under natural conditions in this and in a subsequent study (Sgolastra, 2007) reached adulthood in time. Adult eclosion date in *O. lignaria* is not correlated to egg-laying date (Sgolastra, 2007). Instead, it is correlated with the duration of the prepupal stage (Sgolastra, 2007), during which, as mentioned, the species undergoes a summer diapause. This diapause is short (one month) in our population and in other late-flying (April) populations from cold areas, and long (two months) in early flying (March) populations from warmer areas, such as Central California (Sgolastra, 2007). By comparison, differences between late- and early-flying populations in the duration of other developmental stages (larva, pupa) are much smaller or non-significant (Sgolastra, 2007). Thus, the duration of summer diapause appears to be the main mechanism through which *O. lignaria* populations synchronize adult eclosion with the onset of wintering temperatures. Prepupae from late-flying populations developing at a slow rate, will not be adults by the onset of winter temperatures and will perish. On the other hand, prepupae from early flying populations developing at a fast rate, will become adults well before the arrival of winter temperatures and thus be exposed to a long and warm pre-wintering period. When an April-flying population from northern Utah was forced to fly in February in warmer central California, the resulting progeny expressed short prepupal diapause typical of its geographic origin, reached adulthood by mid summer, and suffered heavy mortality (Bosch et al., 2000).

We have demonstrated that pre-wintering duration has important consequences on diapause development, fat body depletion and post-wintering longevity in *O. lignaria*, and provided an ecophysiological framework to study the potential effect of extended summers on the fitness of this species, in terms of winter mortality and/or reduced post-winter performance. The fitness consequences of developing too early were not apparent in this study, but may be strong in years with late winters (Sgolastra, 2007), which are expected to increase in the current scenario of global warming (Bale and Hayward, 2010; Bradshaw and Holzapfel, 2010). Future studies should investigate yearly variability in the timing of adult eclosion in relation to the onset of winter temperatures, as well as the effects of late summers on winter survival, post-winter longevity, ovary maturation, and fecundity under natural conditions. Our study also unveils important ecophysiological differences between adult and prepupal diapause. These differences lead us to hypothesize that, in the face of a global temperature increase, adult-diapausing Megachilidae are more likely to be negatively affected than prepupa-diapausing species.

## Acknowledgements

We are very grateful to G.E. Trostle and S. Kalaskar (USDA- ARS, Logan, Utah) for their outstanding assistance throughout the study. J.M. Espelta (CREAF, Bellaterra, Spain) and G.D. Yocum (USDA- ARS, Fargo, North Dakota) reviewed a draft of the manuscript and made suggestions for its improvement. This study was partially supported by a Ph.D. scholarship from the University of Bologna to F.S., and the project Montes CSD2008-00040 of the Consolider-Ingenio program (Spanish Ministry of Science and Innovation) to J.B.

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