Original article

Overwintering strategies in the red mason solitary bee—physiological correlates of midgut metabolic activity and turnover of nutrient reserves in females of *Osmia bicornis*

Oskar Wasielewski[1], Tatiana Wojciechowicz[2], Karol Giejdasz[1]*,
Natraj Krishnan[3]

[1]Institute of Zoology, Poznań University of Life Sciences, Wojska Polskiego 71C, 60-625 Poznań, Poland
[2]Department of Animal Physiology and Biochemistry, Poznań University of Life Sciences, Wojska Polskiego 35, 60-637 Poznań, Poland
[3]Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS 39762, USA

Abstract – The catabolic activity of midgut and the turnover of metabolic reserves in fat body and hemolymph was studied during 7 months of overwintering period (September–March) in the European solitary red mason bee *Osmia bicornis* L. (Hymenoptera: Megachilidae). Assays of lipids, protein, and carbohydrates in midgut, fat body, and hemolymph as well as carbohydrates in crop, were conducted during overwintering period, including the 3 months of diapause (November–January). In general, in midgut, lipids were used mainly during pre-wintering period (September–October); whereas in fat body and hemolymph, the stores of lipids as well as carbohydrates were mobilized. In midgut and crop, carbohydrate mobilization occurred during both beginning and end of the overwintering period. The activities of midgut enzymes (proteases and amylases) changed substantially during the overwintering period. While the midgut protease activity was relatively low at the beginning of the overwintering period (September), its activity increased significantly in the final stages of overwintering, during post-diapause quiescence (February–March). On the other hand, amylase activity showed high activity during pre-wintering and post-diapause quiescence, but its activity was low during the deep diapause period (December–January). During winter months, the fat body and hemolymph lipids and free hemolymph carbohydrate concentration slowly decreased. On the other hand, glycogen concentration dramatically dropped down after November and was at a very low level till February and then increased with the rise of ambient temperature. This reflects the dynamic physiological strategies adopted by the red mason bee to successfully overwinter.

digestion / midgut / nutrient reserves / *Osmia bicornis* / overwintering

1. INTRODUCTION

In temperate and cold climates, insect life cycles are correlated closely with seasonal changes. This ensures that insect development and reproduction are restricted to periods of the year when climatic conditions are favorable and there are suitable sources of food (Hondelmann and Poehling 2007). Diapause is a strategy to survive seasons with environmental conditions that are inadequate for sustaining continuous development or maintenance of the organism

Corresponding author: K. Giejdasz,
kgiejdasz@gmail.com
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(Denlinger 2002). In insects, an adult diapause is one of the most common types of quiescence observed which involves the arrest of reproductive development. The shortening of the day length in autumn or the change from long- to short-day photoperiod coupled with the lowering of temperature is sufficient to induce adult diapause in many insects (De Wilde et al. 1968; Krafsur et al. 1999; Herman and Tatar 2001), including some *Osmia* species (Bosch et al. 2010; Sgolastra et al. 2010).

The red mason bee, *Osmia bicornis* L., (also referred to as *Osmia rufa* in previous literature) is a native European solitary bee showing promise as a manageable pollinator of fruit, especially apples, pears, plums, raspberries, and strawberries (Giejdasz and Wilkaniec 2002). This univoltine species completes its development from egg to adult (imago) stage in the spring and summer season (Wilkaniec and Giejdasz 2003). To our knowledge, photoperiod has not been implicated in diapause of this *Osmia* species since development from egg to adult in this species takes place inside a sealed nest in complete darkness. Moreover, *O. bicornis* as most species of *Osmia* genus, is an adult-wintering species with obligate diapause. In the genus *Osmia*, some species can facultatively overwinter as prepupae and adults. However, overwintering in adult stage is a derived trait within the Megachilidae, most of which winter as prepupae, and it is probably in relation with the early flying period of *Osmia* (Bosch et al. 2001; Sgolastra et al. 2012). In *O. bicornis*, adults overwinter as imago inside a cocoon through the winter months (Giejdasz and Wilkaniec 2002). In natural conditions, as in other species of *Osmia*, the bees eclose the subsequent spring as the temperature rises (Bosch and Kemp 2000). Most information about the precise timing of diapause initiation, maintenance, and termination is available on a spring-flying solitary bee from North America *Osmia lignaria* (Sgolastra et al. 2010; Bosch et al. 2010). However, the precise timing of entering diapause, its maintenance and diapause termination is different in *O. bicornis* (Wasielewski et al. 2011a). Based on the classification of the eco-physiological phases of insect diapause (Kostál 2006), we determined that *O. bicornis* enters diapause in November and diapause termination occurs at the end of January. Then, the unemerged adult remains in post-diapause quiescence till April (Wasielewski et al. 2011b). For this study, the entire overwintering period of *O. bicornis* has been classified as: pre-wintering (September–October), diapause (November–January), and post-diapause quiescence (February–March).

Both diapausing and direct-developing insects store metabolic reserves of the same three macronutrients groups: lipids, carbohydrates, and amino acids as well as essential micronutrients, such as vitamins and minerals (Hahn and Denlinger 2011). In insects that diapause as adults, nutrient reserves accumulated in fat body prior to diapause are critical for restoring post-diapause functions, including the rebuilding of tissues atrophied as part of the diapause program and for providing energy for post-diapause activities, such as dispersal and reproduction (Hahn and Denlinger 2007). The gut may also act as a novel nutrient storage reserve in solitary bees, in addition to the fat body. The midgut is predominantly involved in the secretion of digestive enzymes and nutrient absorption. Macromolecular components present in the food are catalyzed by three major digestive enzymes in the gut: amylases, proteases, and lipases. Amylases catalyze the hydrolysis of α-D-(1,4)-glucan linkage in starch, glycogen, and other carbohydrates; gut proteases further release amino acids from peptides and lipases, which catalyze the hydrolysis of triacylglycerol. The products of digestion are absorbed by the midgut epithelium (Chapman 2011).

Managing metabolic resources is critical for insects during diapause when food is limited or unavailable and adequate nutrient budgeting and use enables survival of overwintering period and ensures post-diapause development. The present study aims to extend our knowledge on the catabolic activity of some midgut enzymes (proteases and amylases) and the energy substrates sequestered and deposited in the midgut, fat body, and hemolymph and their dynamic changes

during the use of these reserves during the entire overwintering period, including the period of diapause in the Megachilid bee, *O. bicornis*.

2. MATERIAL AND METHODS

2.1. Experimental animals

Experiments were conducted in the years 2011–2012 during the overwintering period of *O. bicornis*. The experimental material consisted of bees reared in artificial nests made of reed stalks following the method of Wójtowski and Wilkaniec (1978) and originated from nests kept in natural conditions in the botanical garden near the Institute of Zoology (Poznań, Poland). The experiments started after bees reached adulthood (beginning of September) and were completed in March before the emergence period. The experimental material was collected once in the middle of the appropriate month. Prior to conducting all experiments, reed stalks from the nests were brought and dismantled in the laboratory. Cocoons were transferred to Petri dishes, dissected, and imago's were sexed according to morphological characters. For all biochemical assays, we only used females: females accumulate far more fat body reserves with a huge amount of energy substrates necessary not only for successful wintering, including diapause but also for completing their ovarian development during post-diapause period (Wilkaniec et al. 2004). From each female, the fat body (for glycogen and lipid content), hemolymph (for carbohydrate, lipid, and protein content), midgut (for carbohydrate, lipid, protein content, and protease and amylase activity), and crop (for carbohydrate content) were collected, and appropriate analysis were conducted according to the methods described below. For analysis of nutrient reserves and their utilization the fat body, hemolymph, midgets, and crops were sampled from approximately from 70 to 109 individuals (varying from 6 to 24/per month) during the entire period of study, depending on availability of specimens for analyses.

2.2. Lipids

The lipids from the *O. bicornis* fat body and midgut were extracted using chloroform: methanol (ratio, 2:1) solution following the method of Folch et al. (1957). The tissue samples were homogenized by sonication on ice (Sonic Ruptor 250, Omni International Homogenizer Company), then the homogenates were centrifuged at 4 °C for 4 min at $10,000 \times g$ and dried in vacuo (Eppendorf Concentrator 5301). After drying, 100 μL of sulfuric acid was added and lipid content was determined by the classic sulfo-phospho-vanillin test (Zöllner and Kirsch 1962).

Lipid concentration in hemolymph was also determined according to Zöllner and Kirsch (1962) with oleic acid as a standard. Briefly, the hemolymph (0.5 μL) was mixed with 100 μL sulfuric acid, heated for 10 min at 100 °C and cooled in room temperature. After cooling, 1 mL of vanillin solution was added. The optical densities at 546 nm in a spectrophotometer were converted to micrograms lipids per milligram of tissue (fat body and midgut) or microliters of hemolymph with the aid of a calibration graph based on known amounts of oleic acid.

2.3. Proteins

Hemolymph samples (1 μL) were collected with microcapillaries (Drummond), diluted in 400 μL of ice-cold physiological saline (130 mM NaCl, 1.3 mM KCl, 0.9 mM CaCl$_2$·2H$_2$O, 1.9 mM MgCl$_2$·6H$_2$O, and 2 mM NaHCO$_3$) and centrifuged at $10,000 \times g$ for 3 min. The supernatants were analyzed for protein content. The proteins from the midgut were isolated with 0.1 M Tris–HCL buffer, pH 7.8, containing 1 % Triton X-100. Each midgut was homogenized (as described above) in this solution and extracts were analyzed. Protein concentration was assayed using a bicinchoninic acid Protein Assay Kit (Pierce, Rockford, IL) with bovine serum albumin as a standard (Smith et al. 1985), and the absorbance was measured at 562 nm using a Synergy2 Multi-Mode Microplate Reader (BioTek Instruments).

2.4. Carbohydrates

The carbohydrates from the hemolymph samples (2 μL), midgut and crop were extracted with 600 mL of 70 % ethanol. Each midgut and crop was homogenized (as described above) in the extraction mixture. Hemolymph was shaken several minutes to obtain full extraction of free sugar. The extracts were centrifuged for 4 min at $10,000 \times g$, and appropriate

aliquots were used for the determination of carbohydrates using the classical phenol-sulfuric chemical assay (Dubois et al. 1956). Glucose-D was used as the reference standard to convert the optical densities of the samples measured at 490 nm to micrograms of carbohydrates per milligram of tissue (midgut and crop) or microliters of hemolymph.

2.5. Glycogen estimation

The anthrone method (Seifter et al. 1950) was used to determine the fat body glycogen. A known quantity of glycogen was homogenized in 30 % KOH and boiled for 20 min with occasional agitation. Glycogen was precipitated with ethanol, taken up in distilled water and determined with anthrone method. Glucose-D was used as the reference standard and the intensity of the color was read on the UV spectrophotometer at 620 nm.

2.6. Protease activity

Protease activity was measured using azocoll (Sigma), a red dye-labeled standard protease substrate (Chavira et al. 1984). The assay was modified and adapted for measurements with a microtiter plate photometer (Kopáček et al. 2000). Azocoll was treated as described (Nirmala et al. 2001) and used for the assay (100 µL of 50 mM Tris–HCl, pH 7.5 containing 15 mg/mL of azocoll/well of a flat-bottomed microtiter plate). Before the assay, midguts were isolated and homogenized on ice for 20 s with Tris–HCL buffer (50 mM, pH 7.5). The homogenates were centrifuged at $15,000 \times g$ for 3 min at 4 °C, and the supernatants were used for subsequent analyses. Midgut homogenate samples were pipetted into a 96-well microplate (100 µL/well) The microtiter plates were incubated with shaking at 37 °C for 2 h. Azocoll was allowed to settle for 15 min and 100 µL aliquots of clear supernatants of the reaction mixtures were transferred onto another plate for optical density measurements. The absorbance of the azo-dye liberated by the proteolytic activity of midgut homogenates was measured at 490 nm in a Synergy 2 Multi-Mode Microplate Reader (Bio-Tek Instruments). Serial dilutions of trypsin in Tris–HCl buffer (50 mM, pH 7.5) were made to give the following range of concentrations from 2,500 to 1.22 ng/mL. A standard curve of absorbance against the amount of peptide fragments was constructed to enable calculation of the amount of peptide fragments released during trypsin activity. Protease activity was expressed as percent of initial activity (September) per milligrams of midgut tissue attained after 2 h of incubation. Appropriate negative controls without trypsin and positive controls containing trypsin were assayed simultaneously with the reaction mixture.

2.7. Amylase activity

The midgut from *O. bicornis* females was separated and homogenized by sonication on ice for 20 s with Tris–HCl buffer (0.1 M, pH 7.2). The homogenates were centrifuged at $15,000 \times g$ for 20 min at 4 °C, and the supernatants were used for subsequent analyses. The α-amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure (Bernfeld 1955), using 2.5 % soluble starch as substrate; 10 µL of the enzyme was incubated for 30 min at 30 °C with 500 µL Tris–HCl buffer (0.1 M, pH 7.2) and 40 µL soluble starch. The reaction was stopped by addition of 100 µL DNS and heated in boiling water for 10 min. 3,5-DNS is a colored reagent that the reducing groups released from starch by α-amylase action are measured by the reduction of 3,5-DNS. The absorbance was read at 540 nm after cooling in ice for 5 min. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 30 °C. A standard curve of absorbance against the amount of maltose released was constructed to enable calculation of the amount of maltose released during α-amylase assay. Serial dilutions of maltose in Tris–HCl buffer (0.1 M, pH 7.2) were made to give the following range concentrations of 2, 1, 0.75, 0.5, 0.25, 0.125 mg/mL. A blank without the substrate but with α-amylase extract and a control containing no α-amylase extract but with substrate were run simultaneously with the reaction mixture.

2.8. Data analysis

The results were plotted using the graphic program Prism (GraphPad Software, version 5.03, San Diego, CA). Statistical analysis was conducted using the software package Statistica (Statsoft, version.

9.0). To test for assumptions of normality and homogeneity of variances in the samples obtained, the Kolmogorov–Smirnov D test was used. Following this, data were analyzed by ANOVA with Tukey's post hoc tests. In the figures, parameters are presented as means±standard deviations.

3. RESULTS

3.1. Lipid content in fat body, hemolymph, and midgut during overwintering

During pre-wintering time, we observed a dynamic decline of lipid concentration, both in fat body and hemolymph (Figure 1a, b). In successive months of pre-wintering time, gradual changes in lipids concentration were noticed (one-way ANOVA, $F_{6, 66}=166.5$, $P<0.0001$; Figure 1a). During diapause period, we observed drastic reduction of total fat body lipid concentration (Tukey's post hoc test, $P<0.0001$). After diapause termination, when the imago remains in post-diapause quiescence, fat body lipids decreased slightly but nonsignificantly (Tukey's post hoc test, $P>0.05$). At the end of overwintering and the lipid concentration was reduced compared with the beginning of overwintering by approximately 81 %.

Similarly to fat body lipids concentration, a significant decrease in hemolymph lipid concentration was recorded at the end of pre-wintering (55.2 ± 4.3 μg/μL; Tukey's post hoc test, $P<0.0001$). It was the largest decline in lipid content among all months of overwintering. During diapauses, the decline in lipid concentration was lower compared with previous months despite minor differences between diapause months. In comparison with pre-wintering, the lipid level in hemolymph finally reached 6.7 ± 2.3 μg/μL at the end of the post-diapause quiescence and decreased by 92.4 %. The gradual reduction of the lipid concentration was closely linked to the outdoor temperature changes.

The concentration of lipids in midgut at the beginning of pre-wintering was the highest at 3.1 ± 0.2 μg/mg. In successive months, gradual but significant changes in lipid concentration were

noticed (one-way ANOVA, $F_{6, 69}=149.57$, $P<0.0001$; Figure 1c). In diapausing adults, the decline in lipid concentration was lower compared with pre-wintering months and no statistically significant differences were detected ($P>0.05$; Figure 1c). Following diapause termination, the adults of *O. bicornis* remain in post-diapause quiescence because of unfavorable outside conditions. Despite the fact that the overwintering period was over, the lipids concentration still remained almost at the same level and reached 0.8 ± 0.2 μg/mg, a decrease of 75 % compared with the levels at the start of overwintering.

### 3.2. Protein content during overwintering

At the initial month of pre-wintering, the total protein concentration in hemolymph was the lowest at $1,569.4\pm452.6$ μg/mL. During all subsequent months of overwintering, the protein concentration gradually increased (ANOVA $F_{6, 103}=116.93$, $P<0.001$; Figure 2a). At the end of post-diapause quiescence, we recorded a significant increase in protein concentration (Figure 2a) which reached $4,504.3\pm580.8$ μg/mL, the highest level recorded, during the entire overwintering period.

The amount of proteins in midgut extract at the beginning of overwintering (in September) in *O. bicornis* females was the lowest at 65.7 ± 2.4 μg/mg. During all subsequent months of overwintering, the protein concentration gradually but significantly changed (one-way ANOVA, $F_{6, 69}=260.98$, $P<0.0001$; Figure 2b). With the rise of outdoor temperature at the end of the post-diapause quiescence period, we recorded a significant increase in protein concentration (Figure 2b). The quantity of midgut protein reached 248.0 ± 19.0 μg/mg, which was the highest level recorded during the entire overwintering period.

3.3. Carbohydrate content in hemolymph, midgut, and crop during overwintering

Hemolymph free sugar concentration was the highest during the first part of pre-wintering period and amounted to 82.6 ± 4.8 μg/μL (Figure 3a). In consecutive months of overwintering time, the carbohydrate concentra-
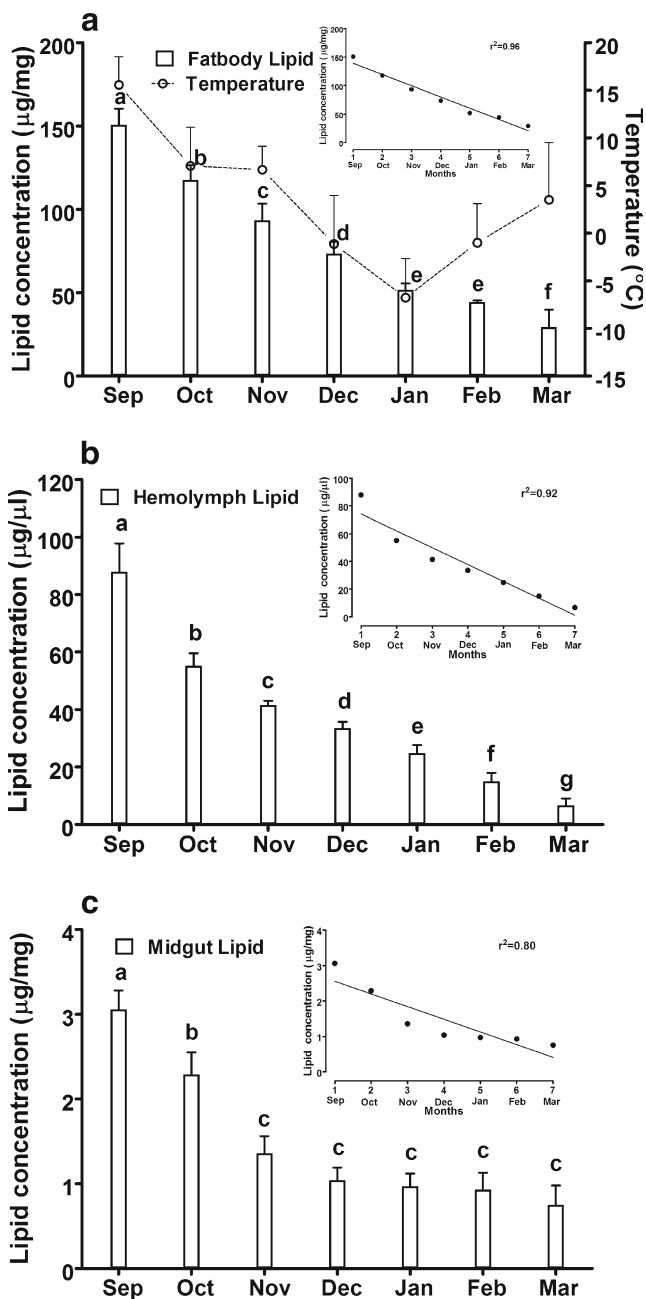
Figure 1. Profile of total lipid concentration in fat body (**a**), hemolymph (**b**) and midgut (**c**) of *O. bicornis* females during overwintering months. Data for each month are mean±SD of 6–24 individuals sampled. The mean outdoor temperature±SD has been depicted for each month in (**a**). *Bars with different superscripts* denote significant difference ($P<0.05$) using one-way ANOVA followed by Tukey's multiple comparison test. *Inset* shows a regression analysis of lipid profiles in fat body, hemolymph, and midgut over months of overwintering. Each data point is the mean of 6–24 individuals/month.
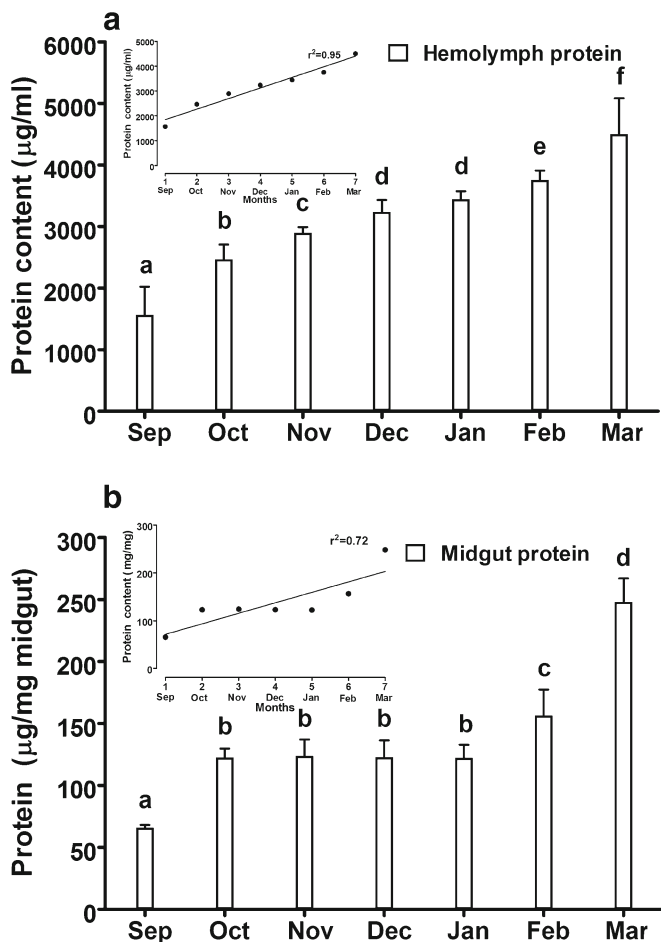
Figure 2. Profile of total protein concentration in hemolymph (**a**) and midgut (**b**) of *O. bicornis* females during overwintering months. Data for each month are mean±SD of 9–20 individuals sampled. *Bars with different superscripts* denote significant difference ($P<0.05$) using one-way ANOVA followed by Tukey's multiple comparison test. *Inset* shows a regression analysis of total protein content profiles over months of overwintering. Each data point is the mean of 9–20 individuals/month.

tion in hemolymph decreased (one-way ANOVA, $F_{6, 100}=137.9$, $P<0.001$; Figure 3a). Following diapause initiation, carbohydrate content declined ($P<0.01$) and till the end of diapause carbohydrate concentration varied only slightly ($P>0.05$). Following the termination of diapause when adults started their post-diapause quiescence, the carbohydrate level in hemolymph once again dropped down significantly ($P<0.01$).

During pre-wintering time, we observed a dynamic decline in the concentration of carbo-

hydrates, both in midgut and crop (Figure 3b, c). A drastic reduction of carbohydrates concentration was noted during mid-diapause. With the rise of outside temperature during post-diapause quiescence, a significant increase in carbohydrates concentration (2.6±0.3 μg/mg) was recorded ($P<0.05$; Figure 3b).

Similarly to midgut carbohydrates concentration, the amount of carbohydrates in crop was the highest at 3.2±0.24 μg/mg during the pre-wintering months (Figure 3c). In successive months, gradual but significant changes in

carbohydrates concentration were noticed (one-way ANOVA, $F_{6, 63} = 185.10$, $P < 0.0001$; Figure 3c). A statistically significant decrease of the carbohydrates during the first 3 months of pre-wintering (September–November; $P < 0.001$) was also recorded. During diapause, the changes in carbohydrates concentration were nonsignificant ($P > 0.05$) and fluctuated between 0.9 and 1.4 μg/mg. As in midgut tissue, also in the crop, the carbohydrates concentration was strongly elevated again at the end of the period of post-diapause quiescence.

3.4. Fat body glycogen concentration

The concentration of glycogen extracted from fat body of overwintering females was the highest at the beginning of pre-wintering and amounted to 3.9±0. 6 μg/mL. In the following months, glycogen concentration strongly fluctuated and the amount varied depending on the period of overwintering (ANOVA $F_{6, 67} = 47.3$, $P < 0.001$; Figure 4). During the second part of pre-wintering, glycogen concentration dropped down and amounted to 1.3±0.4 μg/mL (Tukey's post hoc test, $P < 0.0001$). We noted that the subsequent decline of glycogen concentration was associated with low winter temperatures ($P < 0.01$; Figure 4). The glycogen dropped to its lowest level (0.2±0.1 μg/mL) during diapause and then increased towards the end of post-diapause quiescence. This change was strongly correlated with a rise of outdoor temperature.

3.5. Activity of protease and amylase in midgut during overwintering

Our investigation of protease and amylase activity showed a different profile of these enzymes during the entire overwintering period of *O. bicornis* females (Figures 5 and 6). In consecutive months of overwintering period, the activity of both midgut enzymes changed significantly (one-way ANOVA protease $F_{6, 62} = 266.50$, $P < 0.0001$; amylase $F_{6, 69} = 196.83$, $P < 0.0001$; Figures 5 and 6). Protease was

characterized by relatively low activity at the beginning of pre-wintering (represented as 100 %). Thereafter, the protease activity increased at the end of pre-wintering (67 % more than in the beginning), following this, the protease activity remained at the same level during the subsequent months, including the diapause period ($P > 0.05$; Figure 5). Upon diapause termination, we recorded a statistically significant increase in protease activity (Tukey's post hoc test, $P < 0.001$). This was correlated with the start of metabolic activity and a gradual increase of outdoor temperature. Just before emergence, the highest protease activity was measured. In contrast to the protease activity in midgut lumen, we noted that amylase activity showed a totally different pattern, probably correlated with the type of nutrient reserves present and utilized. We observed three different phases of amylase activity: the first phase during pre-wintering phase, the second during diapause phase, and the third during post-diapause quiescence. Two phases, the first and the third were characterized by high enzymatic activity. During the first month of pre-wintering, the activity was the highest and reached 0.086±0.006 U/mg (Figure 6). In subsequent months, the activity decreased and amounted to the lowest value at the end of diapause (0.004±0.002 U/mg; Figure 6). During post-diapause quiescence phase the amylase activity increased ($P < 0.05$; Figure 6). This increase of activity is probably connected with the start metabolic activity of imago and mobilization of carbohydrates which are the main fuel during flight in bees.

4. DISCUSSION

Osmia species overwinter on a fixed energetic budget. That is, pre-wintering *Osmia* adults are not able to take advantage of benign conditions during pre-wintering to feed and accumulate additional energy reserves. In these species, metabolic reserves for wintering are entirely dependent on the food allocated by the
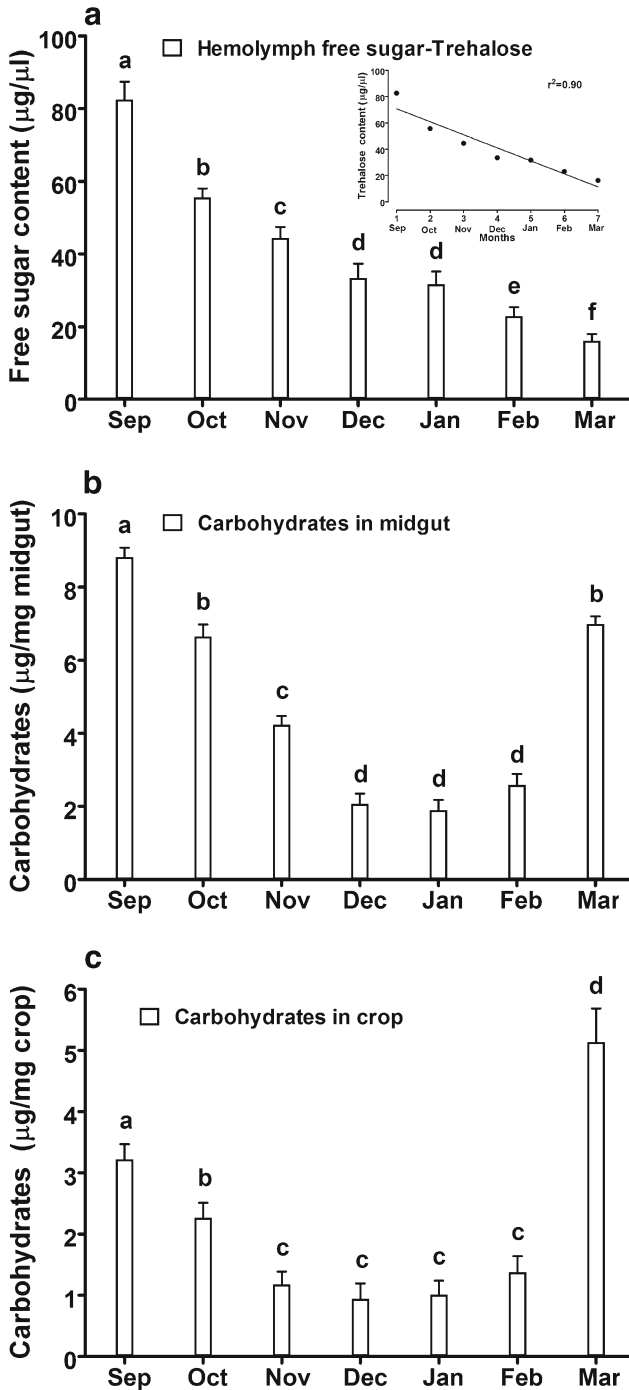
Figure 3. Profile of free carbohydrate concentration in hemolymph (**a**), midgut (**b**) and crop (**c**) of *O. bicornis* females during overwintering months. Data for each month are mean±SD of 9–19 individuals sampled. *Bars with different superscripts* denote significant difference ($P<0.05$) using one-way ANOVA followed by Tukey's multiple comparison test. *Inset* shows a regression analysis of free carbohydrate (trehalose) profiles over months of overwintering. Each data point is the mean of 9–19 individuals/month.
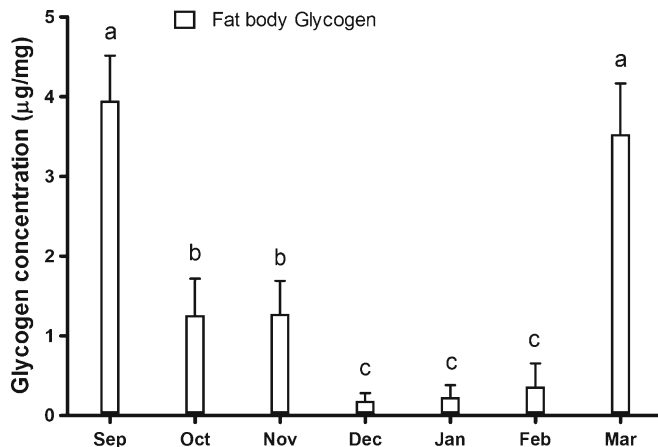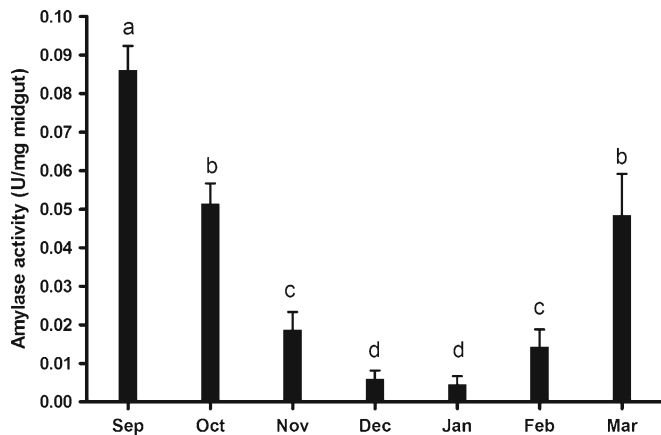
Figure 4. Profile of fat body glycogen concentration in *O. bicornis* females during overwintering months. Data for each month are mean±SD of 9–15 individuals sampled. *Bars with different superscripts* denote significant difference (*P*<0.05) using one-way ANOVA followed by Tukey's multiple comparison test.

nesting female to each offspring within its nest cell (Sgolastra et al. 2011). This implies that the economic utilization of reserves during overwintering is necessary. Several observations performed in *O. lignaria* and *Osmia cornuta* indicated that longer pre-wintering period or exposure to milder winter temperatures in-

creased fat body depletion and probably utilization of energy substrates (Bosch and Kemp 2004; Bosch et al. 2010; Sgolastra et al. 2010). An early consumption of reserves would disrupt emergence from the cocoon, flying to the nearest flowers to sip nectar or even nesting (Bosch and Vicens 2006).
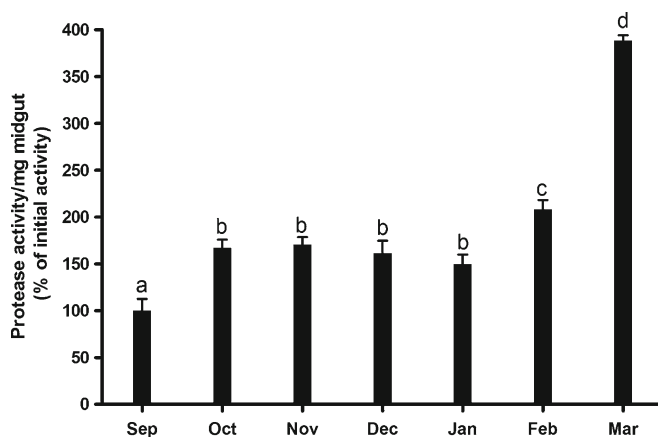
Figure 5. Protease activity in midgut of *O. bicornis* females during overwintering months. Data for each month are mean±SD of ten individuals sampled. *Bars with different superscripts* denote significant difference (*P*<0.05) using one-way ANOVA followed by Tukey's multiple comparison test. Data are expressed as percent initial activity (*Sep*) per milligram of fresh weight of the initial material (midgut) attained after 2 h of incubation.

Figure 6. Amylase activity in midgut of *O. bicornis* females during overwintering months. Data for each month are mean±SD of 10 individuals sampled. *Bars with different superscripts* denote significant difference ($P<0.05$) using one-way ANOVA followed by Tukey's multiple comparison test. Data are expressed in activity units per milligram midgut (1 unit, *U* release of 1 mg maltose equivalent/min from the starch substrate).

In the case of *O. bicornis*, diapause is initiated in November and diapause termination occurs at the end of January followed by a period of post-diapause quiescence till April (Wasielewski et al. 2011b). Numerous authors have shown that stored reserves decline during diapause and that individuals experiencing longer diapause periods contain fewer reserves after diapause (Danks 1987). We observed a similar situation in overwintering *O. bicornis* females. During pre-wintering time, we observed a dynamic decline of lipid concentration, both in fat body and hemolymph. The situation is reminiscent of the situation in the overwintering pistachio fruit hull borer, *Arimania comaroffi*, where the total body lipid decreased during overwintering and reached its lowest level at the end of March (Bemani et al. 2012). In another Megachilid species, a spring-flying solitary bee from North America, *O. lignaria*, the ratio between CO_2 production and O_2 consumption (respiratory quotient) during pre-wintering was 0.6–0.8, indicating the likely use of triglycerides as the main energy substrate (Bosch et al. 2010; Sgolastra et al. 2010; Sgolastra et al. 2011). As in the case of hemolymph and fat body, a dynamic utilization of lipids was observed in the case of

midgut in *O. bicornis*. The profiles of lipids in midgut correspond to the lipid concentration profiles in hemolymph and fat body.

During diapause, the profile of fat body and hemolymph protein can vary dynamically. In the fat body of a diapausing greater wax moth, *Galleria mellonella*, the amount of specific proteins was very low, while in hemolymph it increased continuously during several days of diapause when it reached very high levels. High concentrations of this group of proteins were observed until the end of diapause (Godlewski et al. 2001). Whether storage proteins produced during diapause are playing a role in amino acid storage and intermediary metabolism or some other function, such as humoral defense or cold tolerance, is not known, but their rapid disappearance following diapause termination suggests a role in post-diapause tissue remodeling (Hahn and Denlinger 2011). In *O. bicornis* females, similarly, during all months of overwintering time, the protein content in hemolymph increased. After diapause termination, the protein concentration continuously increased and was the highest in March, before emergence. In contrast, in fat body tissue, our previous work indicated that during overwintering

the protein content rapidly decreased, especially after winter months indicative of a rapid utilization and mobilization of these reserves (Wasielewski et al. 2011a). This could be due to the release of vitellogenins into the hemolymph, leading to a depletion of proteins in the former and an increase in the latter. This correlates with the significant increase in size of the ovaries and oocytes during overwintering (Wasielewski et al. 2011a). This pattern was strongly correlated with the pattern of protease activity in the midgut. Since the insect does not feed during the diapause stage, the causes behind the observed increase in protein content or protease activity are unclear.

Trehalose may play two major roles in diapausing insects: fuelling catabolism and cryo-protection (Hahn and Denlinger 2007). In *O. bicornis* at the beginning of pre-wintering, we noted the highest level of hemolymph carbohydrates. During pre-wintering, despite the closure of the cocoon, the imago inside is still very metabolically active. Therefore, in our opinion, a high concentration of carbohydrates, which are metabolized during pre-wintering, are a pre-requisite, till the bees enter diapause. The gradual and significant decline of trehalose levels in hemolymph indicates that it likely may not have any specific role as a cryoprotectant. The carbohydrate content in midgut changed between three different periods of overwintering: pre-wintering (gradual and significant decrease), diapause (low levels), and post-diapause quiescence (gradual and significant increase). In crop, we noted a similar pattern of carbohydrate content but additionally at the end of post-diapause quiescence, a huge increase of carbohydrate content was recorded. Since the insect is not feeding during these winter months, this raises the question of where such carbohydrates could have come from. One plausible explanation is that it could come from reabsorption of sugars from the hemolymph. It is unclear why such a process should exist and could probably be connected to resumption of metabolic activity of midgut following termination of diapause.

Diapause is associated with qualitative and quantitative shifts in glycogen metabolism. Glycogen is also mobilized for the production

of glucose or trehalose and a variety sugar alcohols or sugar-based cryoprotectant molecules under stressful conditions of temperature and drought (Arrese and Soulages 2010). Studies of several insects have shown that cold acclimation leads to an increase of trehalose and glucose supported by a rapid decrease in glycogen (Hahn and Denlinger 2011). Conversion of glycogen into cryoprotectants and reconversion of cryoprotectants back into glycogen or other metabolic substrates is a dynamic process that can occur multiple times during diapause. For example, glycogen stores increase as cryoprotectants are cleared and are reconverted back into glycogen at the end of the cold season in the goldenrod gall fly, *Eurosta solidaginis* (Storey and Storey 1986) or *Sarcophaga crassipalpis* (Adedokun and Denlinger 1985). Similarly, in *O. bicornis* females, the glycogen content in fat body tissue has recycled. Our measurements indicate that during overwintering, the profile of glycogen concentration has changed.

Regardless of their feeding habitats, most insects must digest proteins, carbohydrates, and lipids and so they have a similar array of enzymes in the midgut. Nevertheless, the enzymes produced do reflect the type of food eaten by each species and stage. Even though similar enzymes are present in different species, their activities may reflect the nature of their food (Chapman 2011). Overall, the activities of midgut enzymes (protease and amylase) in *O. bicornis* females changed substantially over the course of the overwintering season. In Colorado potato beetle, *Leptinotarsa decemlineata*, protease activity substantially differs between the initiation and maintenance phases of diapause. Analysis of serine protease transcripts showed low expression of gene encoding the protease during initiation phase of diapause and relatively high expression from the start of maintenance phase (Yocum et al. 2009a). Furthermore, it was demonstrated that genes encoding enzymes needed to digest a blood meal (trypsin and a chymotrypsin-like protease) are downregulated in diapause-destined *Culex pipiens* females (Robich and Denlinger 2005). As the end of diapause approaches, the accumulation of tryp-

sin and chymotrypsin-like mRNA indicates that females are preparing for blood feeding and subsequent egg production.

An overall metabolic profile of overwintering *O. bicornis* females exhibits a totally different pattern of amylase activity than protease, probably strongly correlated with the type of nutrient reserves. Two phases, first (pre-wintering) and the last (post-diapause quiescence) were characterized by high enzymatic activity. The high activity of midgut amylase was correlated with a high level of carbohydrates in the crop at the end of post-diapause quiescence. It is worth noting that bees are a particular group of insects, which exclusively utilize carbohydrates to power flight. These insects support flight by using sugars stored in the crop rather than reserves from the fat body (Suarez et al. 2005). This probably is the main reason that the amylase activity has fluctuated during the overwintering period. We also noted that in *O. bicornis* females, the glycogen content in fat body tissue is recycled during overwintering time. This is very significant since glycogen is also mobilized for the production of cryoprotectant molecules and an absence of change in the maximum activity of midgut amylase in *O. bicornis* during winter time can be associated with activity of enzymes mobilizing glycogen. A slightly different pattern of amylase activity was exhibited in Colorado potato beetle, *L. decemlineata* (Yocum et al. 2009a). Analysis of gene transcripts encoding particular types of amylases showed differences between the initial and maintenance phase of diapause. In contrast to *O. bicornis* females, in potato beetles the amylases are inactive at the beginning of diapause but the transcripts increased as beetles entered the maintenance stage (Yocum et al. 2009b). Many temperate zone insect species which diapause during winter time exhibit decreased digestive enzyme activity because of nonfeeding period and metabolism cost reduction (Socha et al. 1997; Socha et al. 1998; Bandani et al. 2009; Yocum et al. 2009a, b). Amylase activity pattern towards ingested carbohydrates varies slightly in *O bicornis* females compared with most other wintering species. We noted two peaks of amylase activity: at the beginning and at the end of overwintering. This indicates that during pre-wintering, despite the closure of the cocoon, the imago inside is still very metabolically active (first peak) and as the most of bees species need carbohydrates for fuel for active flight then the sugars are mobilized just before emergence (second peak).

The study thus indicates a dynamic profile of turnover and use of nutrient reserves as well as a metabolic role for midgut for physiologically preparing the red mason bee for a successful overwintering.

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OPEN ACCESS

Stratégies d'hivernage chez l'abeille maçonne solitaire—Corrélations physiologiques entre l'activité métabolique de l'intestin moyen et le renouvellement des réserves nutritives chez les femelles d'*Osmia bicornis*

Digestion / métabolisme / intestin moyen / *Osmia bicornis* / hivernage / Megachilidae

Überwinterungsstrategien der Roten Mauerbiene—physiologische Korrelate der metabolischen Aktivität im Mitteldarm und Umsatz von Nährstoffreserven in Weibchen von *Osmia bicornis*

Verdauung / Mitteldarm / Nährstoffreserven / *Osmia bicornis* / Überwinterung

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