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Characterization of Triacylglycerols From Overwintering Prepupae of the Alfalfa Pollinator *Megachile rotundata* (Hymenoptera: Megachilidae)

James S. Buckner,^{1*} William P. Kemp,² and Jordi Bosch³

Alfalfa leafcutting bees, *Megachile rotundata* (F.), overwinter as prepupae. The internal lipids were extracted from prepupae that had been wintered at 4°C for 7 months. *Megachile rotundata* prepupae possessed copious quantities of internal lipids (20% of the fresh weight) that were extracted with CHCl₃/methanol (2:1). Transmission electron microscopy revealed that lipids were stored within very large intracellular vacuoles. Separation by silica chromatography revealed that 88% of the internal lipids were triacylglycerols. Ester derivatives of fatty acids from triacylglycerol components were analyzed by gas chromatography-mass spectrometry and 15 fatty acid constituents were identified. The majority (76%) of the triacylglycerol fatty acids were unsaturated fatty acids. The major triacylglycerol fatty acid constituent (30%) was the C₁₆ monounsaturated fatty acid, palmitoleic acid (16:1, hexadec-9-enoic acid), with substantial amounts of linolenic acid (18:3, octadec-9,12,15-trienoic acid, 15%), palmitic acid (16:0, hexadecanoic acid, 14%) and oleic acid (18:1, octadec-9-enoic acid, 13%). Palmitoleic acid as the major fatty acid of an insect is an unusual occurrence as well as the presence of the 16-carbon polyunsaturated fatty acids, 16:2 and 16:3. The major intact triacylglycerol components were separated and identified by high performance liquid chromatography-mass spectrometry. A complex mixture of approximately 40 triacylglycerol components were identified and major components included palmitoyl palmitoleoyl oleoyl glycerol, palmitoyl palmitoleoyl palmitoleoyl glycerol, myristoyl palmitoleoyl palmitoleoyl glycerol, myristoleoyl palmitoyl palmitoleoyl glycerol, and palmitoyl palmitoleoyl linolenoyl glycerol. The function of these internal lipids and their relevance to winter survival and post-wintering development of *M. rotundata* is discussed. Arch. Insect Biochem. Physiol. 57:1–14, 2004. Published 2004 Wiley-Liss, Inc.[†]

KEYWORDS: alfalfa leafcutting bee; storage lipids; unsaturated fatty acids; palmitoleic acid

INTRODUCTION

The alfalfa leafcutting bee, *Megachile rotundata* (F.) is a summer-flying, cavity-nesting, solitary bee. This species is the pollinator of choice for alfalfa,

Medicago sativa L., seed production throughout western North America (Peterson et al., 1992) and much emphasis has been placed on the development of wintering procedures and improved incubation schedules to synchronize the emergence of

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Abbreviations used: BSA = N,O-bis-(trimethylsilyl) acetamide; FAME = fatty acid methyl ester; L = linoleic acid; La = lauric acid; Ln = linolenic acid; M = myristic acid; Mo = myristoleic acid; O = oleic acid; P = palmitic acid; Po = palmitoleic acid; P₁ = palmitlinoleic acid; P_{1n} = palmitlinolenic acid; S = stearic acid; TAG = triacylglycerol; *t*-BME = *tert*-butylmethylether; TMS = trimethylsilyl.

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M. rotundata with alfalfa bloom (Johansen and Eves, 1973; Taséi and Masure, 1978; Richards et al., 1987; Richards and Whitfield, 1988; Murrell, 1991; Kemp and Bosch, 2000). At most North American latitudes, *M. rotundata* emerge and mate during June and July, and female bees build nests in abandoned beetle burrows in timber, in cavities associated with farm buildings, or artificial nesting materials provided by alfalfa seed producers (Stephen, 1981; Rank and Goerzen, 1982; Richards, 1984). Nests consist of a linear series of cells constructed from cut-leaf pieces. Each cell is provisioned with a mass of pollen and nectar, on top of which an egg is deposited. Completed nests are sealed with plugs of leaf cuttings. By mid-summer, fifth-instar *M. rotundata* completely consume their provision, defecate, and spin a cocoon. In this stage (prepupa), most individuals undergo a dormant period that lasts through the winter. Alternatively, some individuals may pupate within the summer and emerge as a partial second generation, whose progeny winter as prepupae (Parker and Tepedino, 1982; Rank and Rank, 1989; Kemp and Bosch, 2001).

A recent study has shown that respiration rates of *M. rotundata* significantly decline within 7 days after cocoon completion and remain low throughout late summer and winter (Kemp et al., 2004). Other evidence that prepupal dormancy in *M. rotundata* is diapause-mediated comes from empirical results showing that prepupae artificially wintered for insufficiently long periods take much longer to resume development after being exposed to incubation temperatures (Johansen and Eves, 1973; Taséi and Masure, 1978; Richards et al., 1987). Other studies have addressed glycerol production and supercooling points in *M. rotundata* prepupae (Gusta, 1982; Krunic et al., 1982). Beyond these studies, we know little about the overwintering physiology of *M. rotundata*. A better understanding of the physiological processes underlying the summer-winter dormancy of this species is important because most Megachilidae (Bosch et al., 2001), and most Hymenoptera (Danks, 1987; Gauld and Bolton, 1988) overwinter as prepupae. Ultimately, this information is also important to understand winter mortality in *M.*

rotundata and helps improve management protocols to optimize the development, health, and synchronized emergence of this important alfalfa pollinator (Bosch and Kemp, 2002).

For insects overwintering in temperate regions (frequently in a non-feeding diapause condition), energy required for winter survival, post-diapause development, and the emergence of adults comes from a variety of intermediary metabolites, including stored internal lipids (Downer, 1985; Lee and Denlinger, 1991). Accumulation of triacylglycerols (TAGs) as energy reservoirs and an increase in the ratio of unsaturated/saturated fatty acids has been reported for several overwintering temperate insects (Kurthiala and Chippendale, 1989; Ohtsu et al., 1993, 1995; Joannis and Storey, 1996). In this study, the first in a series of investigations elucidating relevant aspects of *M. rotundata* developmental physiology, internal lipids were extracted from diapausing *M. rotundata* prepupae that had been wintered at 4°C for 7 months. Internal lipids were quantified and characterized by thin-layer chromatography (TLC), silica column chromatography, gas chromatography-mass spectrometry (GC-MS), and high performance liquid chromatography-MS (HPLC-MS).

MATERIALS AND METHODS

Materials

Silica (Porasil 125 Å) was purchased from Waters Corp., Franklin, MA. High-performance thin-layer plates (10 × 10 cm, E. Merck, silica gel 60) were purchased from EM Science, Gibbstown, NJ. Authentic lipid standards were purchased from NuChek Prep, Inc., Elysian, MN. Tertiary-butylmethyl-ether and pyrrolidine were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. The 10% HCl in methanol reagent was prepared in-house by bubbling gaseous HCl into redistilled methanol. Reactivials®, N,O-bis-(trimethylsilyl) acetamide (BSA), and dimethylformamide were purchased from Pierce Chemical Co., Rockford, IL. Tousimis/Millonig's buffer (0.1M, pH 7.35), 2.5% glutaraldehyde in Millonig's buffer, and Spurr's epoxy resin were pur-

chased from Tousimis Research Corp., Rockville, MD. Other chemicals were of analytical grade.

Insects and Lipid Extraction

The diapausing *M. rotundata* prepupae used in this study were from a population maintained at the USDA-ARS Bee Biology & Systematics Laboratory, Logan, UT, at 4°C for approximately 210 days, starting in September 2001. Overwintering, co-cooned prepupae, within the nesting cells, were shipped in March 2002 at 4–10°C to the USDA-ARS Biosciences Research Laboratory, Fargo, ND, and held at 4°C until use. Prepupae were carefully removed from their cells and their fresh weight was determined. For determination of water content, prepupae were frozen at –80°C and lyophilized for 24 h. The freeze-dried prepupae were then reweighed and the free water content was determined.

Prior to extraction of internal lipids, the cuticular lipids were removed from pooled groups of *M. rotundata* prepupae by submersion in hexane for 60 sec, followed by a 30-sec rinse with chloroform (CHCl₃). The internal lipids were removed from either 5 or 10 *M. rotundata* prepupae by tissue homogenation in CHCl₃/MeOH (2:1) and extraction into CHCl₃ (Folch et al., 1957). Pre-weighed prepupae were placed in a glass Ten-Broeck tissue grinder. The ratio of volume of extracting solvent to the number of prepupae was 1 ml CHCl₃/MeOH per prepupa. After sonication in the water bath of an ultrasonic cleaner (Fisher Scientific, Model FS 60) for 45 min, the prepupae were homogenized with the hand-held tissue grinder. The homogenate was transferred into a glass separatory funnel and the lipids recovered in the CHCl₃ layer from partitioning the homogenate with water. The water layer was partitioned with additional CHCl₃. The combined CHCl₃ layers were backwashed once with water and, after evaporating the CHCl₃, the gravimetric weight of the lipid residue was obtained before storage under argon at –20°C.

Lipid Class Analysis

For TLC analysis of lipid extracts and lipid fractions, portions were dissolved in CHCl₃ and 5 µl

(usually 0.005–0.01 insect equivalent) spotted alongside wax ester, fatty aldehyde, TAG, and fatty alcohol standards onto a 10 × 10 cm (150 µm) silica gel plate. TLC plates were developed in hexane/ethyl ether/formic acid (80:20:1). For visualization of lipid classes, plates were charred by spraying with 5% H₂SO₄ in 95% ETOH and heating to 200°C.

The internal lipids of *M. rotundata* prepupae were separated into neutral lipids and free fatty acids using a modification of a silica Sep-Pak column procedure (Hamilton and Comai, 1988). For separation of internal lipids from a pooled sample of 5–10 prepupae, 0.183 g of silica was packed into a 5¾" glass Pasteur pipette (0.5 cm i.d.) fitted with a small plug of glass wool. The silica was washed and equilibrated by washing with 4 ml of hexane/*tert*-butylmethylether (*t*-BME) (96:4) followed by 12 ml of hexane. The internal lipids were dissolved in hexane (10 mg/ml), with heating if necessary, and 0.2 ml (2 mg) was applied to the column. An additional 8 ml of hexane, followed by 8 ml of hexane/*t*-BME (99.5:0.5) was added to remove any surface lipid contaminants (hydrocarbon, wax esters, aldehydes). Triacylglycerols, free fatty acids, and any fatty alcohols were then eluted from the column with successive 8-ml additions of hexane/*t*-BME (96:4), hexane/acetic acid (100:2), and *t*-BME/acetic acid (100:0.2), respectively. Column fractions were analyzed for lipid class content and purity by TLC as described above.

Hydrolysis and Formation of Fatty Acid Derivatives

The fatty acids of TAG fractions were obtained by alkaline hydrolysis with 5% KOH in MeOH. Reaction with 10% HCl in MeOH was used to convert fatty acids to their methyl ester derivatives. The methods for hydrolysis and esterification to yield fatty acid methyl esters (FAME) have been previously described (Buckner and Hagen, 2003). Fatty acids were converted to their trimethylsilyl derivatives by reaction with BSA. Fatty acids from the alkaline hydrolysis of approximately 50 µg of TAG were dissolved in 20 µl dry benzene, 20 µl dry dimethylformamide, and 60 µl BSA. The reaction

mixture was heated at 70°C for 30 min. After cooling, the sample was diluted 10-fold using BSA/DMF/benzene (20:20:60) and portions analyzed by CGC-MS. For determination of double bond location for the fatty acids, the FAMES from the TAG fraction were converted to their pyrrolidine derivatives. Methods for reaction of FAME with pyrrolidine and recovery of the fatty acid pyrrolidides were as previously described (Buckner and Hagen, 2003).

Gas Chromatography-Mass Spectrometry

The trimethylsilyl ester derivatives of fatty acids from the TAG fraction were analyzed by GC-MS using a 40-meter J&W DB-1 capillary column (0.18 mm i.d., 0.4 µm phase thickness). Operating conditions for the GC-MS analysis for lipid identification and methods for using authentic standards for determination of lipid quantities have been previously described (Buckner et al., 1999; Buckner and Hagen, 2003). Fatty acid pyrrolidides were analyzed using a Hewlett-Packard 5970 GC-MS equipped with a Supelco SP-2380 capillary column (30 m × 0.25 mm × 0.2 µm film thickness) and splitless injector at 260°C. The oven temperature was held at 50°C for 2 min, then ramped at 25°C per minute to 260°C and held 10 min. Helium flow rate was 1 ml/min. Mass spectra data were compared to the mass spectra of the fatty acid pyrrolidides of authentic standards as previously described (Buckner and Hagen, 2003).

High Performance Liquid Chromatography-Mass Spectrometry

Intact TAGs were separated and identified by HPLC-MS using a Waters Model 2695 Separations Module (Waters Corp., Milford, MA) equipped with a 250 × 4.6 mm Chrospher 5 Lipid column (Chrompack, The Netherlands). The column was equilibrated at 1 ml/min and 30°C with hexane/toluene/ethyl acetate (90:5:5), then a linear gradient: to 15% acetone in 15 min; to 100% acetone in 10 min; and to 10% acetonitrile in 5 min. For increased resolution of closely eluting peaks and single peaks containing multiple TAG compo-

nents, portions of TAG samples were passed through two Chrospher 5 Lipid columns connected in series. All solvents were dried over molecular sieves before use. Mass spectra of TAG components were obtained using a Waters Integrity System Therma-beam Mass Detector.

Electron Microscopy

Prepupae were removed from nesting cells and tissues were prepared for transmission electron microscopy by dissection and fixation in ice-chilled 2.5% glutaraldehyde in Tousimis/Millonig's buffer. The samples were post-fixed in 2% osmium tetroxide in the same buffer. Following fixation, samples were dehydrated, embedded in Spurr's epoxy resin, and prepared for examination using a JEOL JEM 100CX transmission electron microscope as previously described (Buckner et al., 2002).

RESULTS

Quantities and Localization of Internal Lipids

The mean weight per *M. rotundata* prepupa was 48.1 ± 8.0 mg (mixed sex; $n = 20$) and the quantity of extractable internal lipid from a pooled group of 20 *M. rotundata* prepupae was 9.9 mg per prepupa (lipid as % of fresh weight = 20.6%/prepupae). TLC analysis of a portion of the internal lipid extract showed only one major band that co-migrated with authentic TAG standard (data not shown) and a minor band at the origin (likely phospholipid). Portions of the internal lipids were fractionated by silica column chromatography, and 88% of the lipid applied to the column was recovered in the TAG fraction following elution with hexane/*t*-BME (96:4). The remainder of the internal lipid fraction was likely polar lipids (phospholipids) that were not eluted by silica column chromatography.

Megachile rotundata prepupae were fixed in a buffered glutaraldehyde solution, and internal tissues were excised and prepared for examination by transmission electron microscopy. Staining of ultra-thin tissue sections revealed the presence of

lipid storage vacuoles within presumably fat body cells (Fig. 1). Within each cell, storage vacuoles were numerous and many were very large (20–25 μm in diameter).

Characterization of Fatty Acids From Triacylglycerols

A typical GC-MS chromatogram of TMS derivatives of fatty acids from the TAG fraction is shown in Figure 2 and 15 fatty acids were identified. Percentage composition values were determined from integrated peak areas and comparisons to standard curves for TMS derivatives of authentic fatty acids. The majority of the TAG fatty acids were unsaturated fatty acids (76%) and the major constituent (29.5%) was the monounsaturated fatty acid, palmitoleic acid (16:1), with lesser amounts of

other monounsaturated acids, i.e., oleic acid (12.9%) and myristoleic acid (8.2%) (Table 1). Also present were the C_{18} polyunsaturated fatty acids, linoleic acid (5.0%) and linolenic acid (14.8%), and small amounts of the C_{16} counterparts, 16:2 and 16:3. TMS derivatives of fatty acids from the TAG fraction also included the saturated fatty acids, lauric (1.2%), myristic (7.8%), palmitic (13.9%), and stearic (0.6%).

The position of double bonds in the unsaturated fatty acids from TAG was determined by GC-MS analysis of their pyrrolidine derivatives as previously described (Buckner and Hagen, 2003). For the mass spectrum of an unsaturated component, if an interval of 12 atomic mass units is observed between the most intense peaks of clusters of fragments containing n and $n-1$ carbon atoms

Fig. 1. Transmission electron micrograph of fat body cells from *M. rotundata* prepupae. Cells contained numerous

and very large (20–25 μm diameter) lipid storage vacuoles (L).

Fig. 2. A typical CGC-MS chromatogram of fatty acid trimethylsilyl esters from a portion of the TAG fraction of 10 *M. rotundata* prepupae (insect equivalent = 6.25×10^{-5}) on a J&W DB-1 capillary column. Fatty acids are identified according to Table 1.

TABLE 1. Identification and Percentage Composition of the Constituent Fatty Acids (as Their Trimethylsilyl Ester Derivatives) for the Triacylglycerols From *M. rotundata* prepupae

Peak Number ^a	Fatty acid component			% Composition ^c
	Short-hand notation ^b	Common name of acid	Scientific name	
1	12:0	Lauric (La)	Dodecanoic acid	1.2
2	14:1 Δ7		Tetradec-7-enoic acid	0.6
3	14:1 Δ9	Myristoleic (Mo)	Tetradec-9-enoic acid	8.2
4	14:0	Myristic (M)	Tetradecanoic acid	7.8
5	16:2	Palmitoleic (Pl)	Hexadecadienoic acid	0.3
6	16:3	Palmitolinolenic (Pln)	Hexadecatrienoic acid	1.2
7	16:1 Δ7		Hexadec-7-enoic acid	0.3
8	16:1 Δ9	Palmitoleic (Po)	Hexadec-9-enoic acid	29.5
9	16:1 Δ11		Hexadec-11-enoic acid	1.5
10	16:0	Palmitic (P)	Hexadecanoic acid	13.9
11	18:2 Δ9,12	Linoleic (L)	Octadec-9,12,15-dienoic acid	5.0
12	18:3 Δ9,12,15	Linolenic (Ln)	Octadec-9,12,15-trienoic acid	14.8
13	18:1 Δ9	Oleic (O)	Octadec-9-enoic acid	12.9
14	18:1 Δ11	Vaccenic	Octadec-11-enoic acid	2.3
15	18:0	Stearic (S)	Octadecanoic acid	0.6

^aNumbered peaks from the 19–34-min region of a GC-MS chromatogram (see Fig. 2) of individual fatty acid trimethylsilyl esters of TAG fatty acids.

^bThe numbers to the left and the right of the colon are the number of carbons and the number of double bonds, respectively, and the Δ numbers indicate the carbon positions for the double bond(s), counting from the carboxyl end.

^cPercentage composition values were calculated from the quantities of fatty acid TMS derivatives as determined from the GC-MS integrated peak area for each component and standard curves for authentic standards as described in Materials and Methods.

of the acid moiety, the double bond occurs between carbons n and $n+1$ in the molecule (Andersson and Holman, 1974). For GC-MS analysis of the *M. rotundata* TAG fatty acids, the mass spectrum of the major component (16:1) was consistent for the pyrrolidine derivative of hexadec-9-enoic acid (palmitoleic acid) with a molecular ion at m/z 307, a base peak at m/z 113 for the McLafferty rearrangement of all fatty acid pyrrolidides, and diagnostic fragment ions 12 amu apart at m/z 196 and m/z 208 for a double bond at the Δ^9 position. The other major monounsaturated components, 14:1 and 18:1, with mass spectra showing molecular ions at m/z 279 and m/z 335, respectively, had the same diagnostic fragments ions 12 amu apart for a Δ^9 double bond position. For the GC-MS peak of 18:2, a molecular ion at m/z 333 and characteristic series of fragment ions at m/z 196, 208 and m/z 250, 262 for double bond positions at Δ^9 and Δ^{12} , respectively, are consistent for the pyrrolidine derivative of octadec-9,12-dienoic acid (linoleic acid). A molecular ion at m/z 331 and an overall mass spectral fragmentation pattern that was identical to the mass spectrum for the pyrrolidide of authentic octadec-9,12,15-trienoic acid strongly suggested that the 18:3 peak from *M. rotundata* TAG fatty acids was the $\Delta^{9,12,15}$ fatty acid, linolenic acid. For the three monounsaturated fatty acids, 14:1, 16:1, and 18:1, small quantities of other double bond positional components were identified: Δ^7 positions for 14:1 and 16:1, and Δ^{11} positions for 16:1 and 18:1 (see Table 1 for % compositions). Molecular ions at m/z 305 and 303 confirmed the presence of 16:2 and 16:3 for the small peaks that eluted before the 16:1 Δ^7 peak (Fig. 2), but double bond positions could not be clearly established from the mass spectral fragmentation patterns of their pyrrolidine derivatives.

HPLC-MS Identification of Triacylglycerols

Intact TAG were separated using an HPLC column packed with silica material derivatized with a cation exchange resin in the Ag^+ ionic form. The mixture of TAG components from *M. rotundata* prepupae TAG fraction were eluted from the col-

umn as groups (nine) of peaks according to degree of unsaturation, and within each group according to fatty acid chain length (Fig. 3). TAG components were structurally identified by interpretation of their electron ionization mass spectra. Diagnostic ions included the molecular ion, the diacylglyceride (DAG) ions (with the most intense ion corresponding to the loss of the most unsaturated fatty acid), and the monoglyceride (MAG) ions and acyl ions for each fatty acid constituent. Many of the peaks in the TAG fraction, as shown in Figure 3, contained one or more TAG components and a single mass spectrum contained diagnostic ions for more than one TAG component. An improvement in TAG component resolution was achieved by configuring two of the HPLC columns in series (data not shown) and the acquisition of multiple mass spectral scans across each HPLC peak was used to identify individual TAG components and their fatty acid constituents.

The first group of eluting peaks (peaks 1 and 2) in Figure 3 was characterized as TAGs with two saturated and one monounsaturated fatty acid constituents associated with each TAG. Three TAG components were identified in both peaks (Table 2) and, from the relative intensities of diagnostic MAG and DAG ions, the main TAG components for peaks 1 and 2 were identified as myristoyl palmitoyl oleoyl glycerol (MPO) and myristoyl palmitoyl palmitoleoyl glycerol (MPPo), respectively (Table 2) (mass spectral data not shown). In terms of order of elution for those TAG with the same degree of unsaturation, the TAG with fatty acid constituents that possess the longer carbon chain lengths elute first. Thus, MPO eluted before MPPo. The major TAG peaks in HPLC-MS chromatograms of the TAG fraction were peaks 7 and 8 within Group 2 and peak 27 of Group 4 (Fig. 3) that consisted of one saturated and two monounsaturated constituents (Group 2), and one saturated, one monounsaturated, and one triunsaturated constituent (Group 4), respectively. The largest peak of Group 2 (peak 7) was identified as mainly palmitoyl palmitoleoyl palmitoleoyl glycerol (PPoPo). The other major Group 2 peak (peak 8) was identified as myristoyl palmitoleoyl palmitoleoyl glycerol

Fig. 3. A typical HPLC-MS chromatogram of a portion of the intact triacylglycerol fraction from five *M. rotundata* prepupae (insect equivalent = 0.067). TAG components mixtures were eluted from the column as groups of peaks according to degree of unsaturation, and within each group according to fatty acid chain length. Group num-

bers represent the total number of double bonds for the three acyl constituents of each TAG component (i.e., Group 2 consists of peaks of TAG components with a total of two double bonds: one saturated fatty acid and two mono-unsaturated fatty acid constituents).

(MPoPo). Peaks 6 and 9 in Group 2 were identified as palmitoyl palmitoleoyl oleoyl (PPoO) and myristoleoyl palmitoyl palmitoleoyl glycerol (MoPPo), respectively. The major peak 27 (Group 4) consisted of multiple TAG components, palmitoyl oleoyl linolenoyl glycerol (POLn), palmitoyl palmitoleoyl linolenoyl glycerol (PPoLn), and myristoyl palmitoleoyl linolenoyl glycerol (MPoLn). The three major peaks in Group 3 (peaks 16–18) consisted of three monounsaturated constituents and were identified as PoPoO, PoPoPo, and MoPoPo, respectively. Linoleic acid (L), as a diunsaturated fatty acid, was a common fatty acid constituent of TAG identified in peaks 21–26. The triunsaturated fatty acid linolenic acid (Ln) was either a single constituent or

multiple constituents of the TAG peaks 27–40, except for the TAG components of peaks 28 and 31 that had a palmitlinolenoyl (PLn) moiety (Table 2). Interpretation of mass spectra indicated the presence of positional isomers for the moieties of peaks 19 and 20 (MoPoMo and MoMoPo, respectively) and for two TAG components of peak 37 (LnPoLn and PoLnLn) (Table 2).

DISCUSSION

Megachile rotundata prepupae possessed copious quantities of stored lipid: 20% of the wet weight. In contrast, most insect species contain less than 10% of wet weight as lipid (Fast, 1966, 1970) and

TABLE 2. Identification of *M. rotundata* Triacylglycerols and Their Fatty Acid Constituents

Peak number ^a	Triacylglycerols and fatty acid constituents ^b	Scientific name	Peak number ^a	Triacylglycerols and fatty acid constituents ^b	Scientific name
Group 1	1 Double bond		25	PoPoL	Palmitoleoyl palmitoleoyl linoleoyl glycerol
1	MPO	Myristoyl palmitoyl oleoyl glycerol	26	MoPoL	Myristoleoyl palmitoleoyl linoleoyl glycerol
	MMO	Myristoyl myristoyl oleoyl glycerol	27	POLn	Palmitoyl oleoyl linolenoyl glycerol
	LaPO	Lauroyl palmitoyl oleoyl glycerol		PPoLn	Palmitoyl palmitoleoyl linolenoyl glycerol
2	MPPo	Myristoyl palmitoyl palmitoleoyl glycerol		MPPoLn	Myristoyl palmitoleoyl linolenoyl glycerol
	MMPo	Myristoyl myristoyl palmitoleoyl glycerol		PPoPn	Palmitoyl palmitoleoyl palmitlinolenoyl glycerol
	LaMO	Lauroyl myristoyl oleoyl glycerol			
Group 2	2 Double bonds		Group 5	5 Double bonds	
3	P00	Palmitoyl oleoyl oleoyl glycerol	28	OOLn	Oleoyl oleoyl linolenoyl glycerol
5	M00	Myristoyl oleoyl oleoyl glycerol	29	PoOLn	Palmitoleoyl oleoyl linolenoyl glycerol
6	PPoO	Palmitoyl palmitoleoyl palmitoleoyl glycerol	30	PoPoLn	Palmitoleoyl palmitoleoyl linolenoyl glycerol
7	PPoPo	Palmitoyl palmitoleoyl palmitoleoyl glycerol		MoPoLn	Myristoleoyl palmitoleoyl linolenoyl glycerol
8	MPoPo	Myristoyl palmitoleoyl palmitoleoyl glycerol	31	PoPoPn	Palmitoleoyl palmitoleoyl palmitlinolenoyl glycerol
9	MoPPo	Myristoleoyl palmitoleoyl palmitoleoyl glycerol			
10	MMoPo	Myristoyl myristoleoyl palmitoleoyl glycerol	32	PLLn	Palmitoyl linoleoyl linolenoyl glycerol
11	MoMoP	Myristoleoyl myristoleoyl palmitoyl glycerol	Group 6	6 Double bonds	
Group 3	3 Double bonds		33	OLLn	Oleoyl linoleoyl linolenoyl glycerol
14	O00	Oleoyl oleoyl oleoyl glycerol	34	PoLLn	Palmitoleoyl linoleoyl linolenoyl glycerol
15	Po00	Palmitoleoyl oleoyl oleoyl glycerol	35	MoLLn	Myristoleoyl linoleoyl linolenoyl glycerol
16	PoPoO	Palmitoleoyl palmitoleoyl oleoyl glycerol	36	PLnLn	Palmitoyl linolenoyl linolenoyl glycerol
17	PoPoPo	Palmitoleoyl palmitoleoyl palmitoleoyl glycerol		MLnLn	Myristoyl linolenoyl linolenoyl glycerol
18	MoPoPo	Myristoleoyl palmitoleoyl palmitoleoyl glycerol	Group 7	7 Double bonds	
19	MoPoMo} Isomer ^c	Myristoleoyl palmitoleoyl myristoleoyl glycerol	37	OLnLn	Oleoyl linolenoyl linolenoyl glycerol
20	MoMoPo} Isomer ^c	Myristoleoyl myristoleoyl palmitoleoyl glycerol		LnPoLn} Isomer ^c	Linolenoyl palmitoleoyl linolenoyl glycerol
21	POL	Palmitoyl oleoyl linoleoyl glycerol		PoLnLn} Isomer ^c	Palmitoleoyl linolenoyl linolenoyl glycerol
22	PPoL	Palmitoyl palmitoleoyl linoleoyl glycerol		MoLnLn	Myristoleoyl linolenoyl linolenoyl glycerol
23	MPoL	Myristoyl palmitoleoyl linoleoyl glycerol	Group 8	8 Double bonds	
	LaPoL	Lauroyl palmitoleoyl linoleoyl glycerol	39	LLnLn	Linoleoyl linolenoyl linolenoyl glycerol
Group 4	4 Double bonds		Group 9	9 Double bonds	
24	PoOL	Palmitoleoyl oleoyl linoleoyl glycerol	40	LnLnLn	Linolenoyl linolenoyl linolenoyl glycerol

^aPeaks, numbered according to their order of elution from HPLC-MS analysis of triacylglycerol fraction as indicated in Figure 3.

^bFor mixed TAGs, the position of fatty acid constituents on carbons 1, 2, and 3 of glycerol was not established. A consistent system was used for positioning and naming: shorter carbon chain lengths first, then by degree of unsaturation (e.g., MPPo).

^cThe position of fatty acid constituents for positional isomer triacylglycerols was based on interpretation and abundance of diagnostic monoglyceride and diacylglyceride mass spectral fragment ions.

for the honeybee (*Apis mellifera* L.), lipids as percentage of fresh weight range between 5.9 and 8.6% for both larval and pupal drones, workers, and queens (Robinson and Nation, 1970). Analyses of total lipid extracts from a variety of insect species have shown that the predominant lipid class is TAG (Downer, 1985) and that fat body tissue is the primary site of TAG storage (Downer, 1985; Keeley, 1985). In this study, chromatographic analyses revealed that 88% of the extractable internal lipids from *M. rotundata* were associated with the TAG fraction and ultrastructural studies revealed lipid storage within very large vacuoles (Fig. 1) located within apparent fat body cells. For the TAG fraction, the fatty acid composition and double

bond location was established by CGC-MS of the fatty acids as their TMS and pyrrolidide derivatives, respectively. We chose to analyze the fatty acids as their TMS derivatives because no clean up was required after derivatization and, losses of the more volatile polyunsaturates were minimal. A total of 15 saturated and unsaturated fatty acid structures were identified (Fig. 2 and Table 1).

The majority of the observed TAG fatty acids were unsaturated (76%) and the major fatty acid constituent (31.4%) was the monounsaturated fatty acid, palmitoleic acid (16:1, hexadec-9-enoic acid). A high ratio of unsaturated/saturated fatty acids in storage lipids has been associated to insect wintering or to species adapted to cold climates, and has

been interpreted as a mechanism to maintain the appropriate fluidity of these lipid reserves (Ohtsu et al., 1993; Joannis and Storey, 1996; Kostal and Simek, 1998). The function of increased fatty acid unsaturation, however, remains unclear, as high unsaturated/saturated fatty acid ratios are found in species that use glycogen, rather than TAGs, as their main energy reserve during winter or summer diapause (Joannis and Storey, 1996; Bennett et al., 1997; Kostal et al., 1998), as well as in species in which TAGs are the main winter energy source (Pullin, 1987; Kurthiala and Chippendale, 1989; Ohtsu et al., 1993, 1995; Joannis and Storey, 1996). Weight loss in *M. rotundata* is almost negligible throughout late summer and winter, and only increases once prepupae are exposed to warm temperatures in the spring (Kemp et al., 2004). Again, this pattern is found both in species using glycogen and in species using TAGs as their main winter energy source (Joannis and Storey, 1996).

In terms of fatty acid distribution in insects, 16:1 as the major fatty acid is not a common occurrence. Insects contain a Δ^9 desaturase that desaturates 16:0 and 18:0 fatty acids to Δ^9 monounsaturates (Stanley-Samuelson et al., 1988); however, for most insects the quantities of 16:1 are minor compared to 18:1 (Downer, 1985; Stanley-Samuelson et al., 1988). High proportions of 16:1 fatty acids have also been found in some Heteroptera (Spike et al., 1991; Bashan et al., 2002). For the total lipids from Homoptera, amounts of 16:1 were minor in aphids, ranging between 0.6–2.6% (Dillwith et al., 1993) and, for the internal lipids of nymph and adult whiteflies, 16:1 amounts were only 1–2% (Buckner and Hagen, 2003). For the Hymenoptera, the major fatty acid was 18:1 (46%) and the amount of 16:1 was only 4% (Downer, 1985).

For the TAG fatty acids from *M. rotundata* prepupae, we also detected small quantities of 16:2 and 16:3 (Fig. 2 and Table 1), and an intact TAG component was identified with a 16:3 constituent (Peak 31; Fig. 3 and Table 2). To our knowledge, this is the first reported occurrence of 16:2 and 16:3 fatty acids in insects, although a conjugated 16:2 (10,12-hexadecadienoic acid) was observed as

a precursor of pheromone biosynthesis in female silkworm moths (Ando et al., 1996). For other organisms, quantities of 16:2 have been observed in yeast cells (Ito et al., 1983; Brown et al., 1998) and marine algae (Viso and Marty, 1993), and 16:3 was present in plant leaves (Horiguchi et al., 1996) and algae (Viso and Marty, 1993).

The predominance of 16:1 (palmitoleic acid (Po)) for the TAG fatty acids was also reflected by structural identifications of intact TAGs as determined by HPLC-MS. Identification of fatty acid constituents for each TAG was made from interpretable mass spectra (Table 2), but the fatty acid position on carbon 1, 2, or 3 of the glycerol molecule was not determined. The TAG fraction from *M. rotundata* prepupae was shown to be a complex mixture of more than 40 TAG components (Fig. 3 and Table 2) with different combinations of the 15 fatty acid constituents, as shown in Figure 2 and Table 1. The major fatty acid constituent, palmitoleic acid (Po), was identified in about 75% of the identified TAG components (Table 2). As indicated by total ion current peak area in the typical HPLC-MS chromatogram of prepupae TAG (Fig. 3), the major peaks were nos. 6–9 and 27, and all identified TAG components had one or two palmitoleic acid constituents, except for the POL_N component within peak 27 (see Table 2).

Although glycerol is the most commonly encountered low-molecular weight cryoprotectant in insects (Lee and Denlinger, 1991), and has been found in *M. rotundata*, the contributions of stored lipids (TAGs) to cold hardiness in wintering insects like *M. rotundata* has not been clearly established. There is, however, an association between the synthesis and sequestration of large amounts of stored lipid in cold-hardy insects and a decrease in water content. Many insects reduce their water content before winter, at a time when cryoprotectants (glycerol, sorbitol, etc.) typically increase (Ring, 1981; Cannon and Block, 1988; Lee and Denlinger, 1991), although this reduction may only reflect increases in lipid stores (Danks, 2000). For example, in studies of the boll weevil, *Anthonomus grandis* Boh., an increase in fat correlated to a decrease in body water (Brazzel and Newsom, 1959).

For *A. grandis* from different latitudes, Keeley et al. (1977) suggested that the lower lipid content and associated higher water content in diapausing weevils from warmer climates may be necessary to survive the arid climate of southern latitudes. Larvae of the gall fly larva, *Eurosta solidaginis*, have been extensively used as a model system for studying freeze tolerance and biochemical adaptations (Storey et al., 1981; Storey and Storey, 1986, 1988). Intermediary metabolism, correlated with low temperature acclimation and winter survival studies with *E. solidaginis*, revealed that the large total glyceride reserves (an average of 312 $\mu\text{mol/g}$ wet weight) and larval weight were not altered over the course of the winter season (Storey and Storey, 1986). No correlations between lipid levels and the synthesis or degradation of polyols were observed, suggesting that *E. solidaginis* lipid reserves were not involved with cryoprotectant production.

M. rotundata prepupae overwintering in temperate regions frequently spend more than 9 months in a non-feeding state. This includes three more or less distinct periods, diapause, cold-mediated post-diapause, and springtime pupation and adult emergence (Kemp et al., 2004), during which these insects are faced with the multiple tasks of maintaining basal metabolic processes, surviving extreme winter conditions (temperatures as low as -30°C) and, perhaps equally difficult, frequent diurnal freeze thaw cycles. Although insects possess diverse patterns of energy/cryoprotectant intermediary metabolites (Lee, 1991; Table 2.1), our results for *M. rotundata* appear consistent with the argument (Downer, 1985) that TAGs, whose major repository is the fat body in insects, are frequently associated with species that undergo lengthy periods of metabolic activity (for example, diapause or migratory flights) or that have non-feeding stages of development (e.g., pupa, adult). From a bioenergetic standpoint, TAGs appear to offer several advantages over glycogen, another frequently encountered metabolic reserve (Lee, 1991; Table 2.1), including a higher caloric content per unit weight, the liberation of two times more metabolic water than is obtained from carbohydrates upon oxidation, and the capacity for storage in an

hydrous form, instead of the more bulky hydrated form as is the case for glycogen (Downer, 1985).

The water content in *M. rotundata* prepupae at the end of the wintering period was $39.1 \pm 3.5\%$ ($n = 9$) and, even though the water content has not been established for prepupae entering diapause, recent findings have indicated very low O_2 consumption and static weight for overwintering *M. rotundata* prepupae, followed by increased O_2 consumption and more rapid weight losses, during incubation, as individuals pupate and ultimately emerge as adults (Kemp et al., 2004). Thus, the large quantities of intracellular-stored lipid that we observed likely have a dual role in the overwintering physiology of *M. rotundata*. In addition to reducing intracellular water and thus increasing concentrations of cryoprotectants, lipid stores in *M. rotundata* likely have a very important bioenergetic function in support of basal metabolic functioning during diapause and subsequent development through the pupa and emerged adult. Future studies will focus on correlating lipid and water levels with metabolic activities, including the accumulation of other cryoprotectants, during pre-wintering, wintering, and post-wintering stages of *M. rotundata* development.

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